To My Daughter,

Sarah.

My Pride and Joy

To my parents for all their hard work and their belief in me. To my brothers and sisters for their words of encouragement. To the mother of my daughter for all her sacrifices. A special dedication in loving memory of my friend Peter. Thank you, Peter, for your kind words and for the coffee breaks and for taking care of the axolotls. To my cats, Dudu and Magna, who kept me company during the long hours of reading and writing

Effects of Extracts from Regenerating Organisms on Differentiation of the Human Myeloid Leukaemia HL-60 Cell Line

Sherif Suleiman

Ph.D. Dissertation

Department of Anatomy

Faculty of Medicine and Surgery

University of Malta

2020

Supervisor: Prof Jean Calleja-Agius

Co-Supervisor: Prof Pierre Schembri-Wismayer

Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

L-Università ta' Malta

Faculty of Medicine and Surgery
DECLARATION OF AUTHENTICITY FOR DOCTORAL STUDENTS

Student's Code: 130595M

Student's Name & Surname: Sherif Suleiman

Course: PMPHDA08

Title of Thesis

Effects of extracts from regenerating organisms on differentiation of the human myeloid leukaemia HL-60 cell line

(a) Authenticity of Thesis
I hereby declare that I am the legitimate author of this Thesis and that it is my original work. No portion of this work has been submitted in support of an application for another degree or qualification of this or any other university or institution of higher education.
I hold the University of Malta harmless against any third party claims with regard to copyright violation, breach of confidentiality, defamation and any other third-party right infringement.

(b) Research Code of Practice and Ethics Review Procedure
I declare that I have abided by the University's Research Ethics Review Procedures. Research Ethics & Data Protection form code

□ As a Ph.D. student, as per Regulation 49 of the Doctor of Philosophy Regulations, I accept that my thesis be made publicly available on the University of Malta Institutional Repository.

_____	SHERIF SULEIMAN	13/07/2020
Signature of Student	Name in Full (in Caps)	Date

Acknowledgements

I would like to express my gratitude to my supervisor Professor Jean Calleja Agius for her constant encouragement throughout the course of this thesis and her drive to ensure that my work is exposed to the scientific community through publications, poster and oral presentations. Also, for ensuring that my work was kept on schedule.

I am indebted to my co-supervisor Professor Pierre Schembri-Wismayer for the opportunity to join his research team and to pursue my studies. Thank you for the useful critique of this research work.

I would like to thank Dr Riccardo di Fiore, Dr Analisse Cassar, and Dr Melissa Marie Formosa for their help in setting up and planning of experiments and assistance with the use of the laboratory equipment.

A sincere thanks to Professor Neville Calleja, from the Public Health Department for the professional assistance with the statistical analysis.

A special thanks to Professor Godfrey LaFerla, Dean of the Faculty of Medicine and Surgery for the PhD scholarship which allowed me to reach to this stage in my career.

Abstract

Acute myeloid leukaemia (AML) is the most common form of acute leukaemia in adults, constituting about 80% of cases. Although remarkable progress has been made in the therapeutic scenario for patients with AML, research and development of new and effective anticancer agents to improve patient outcome and minimize toxicity are needed. In this study, the antitumour activity of crude extracts from two regenerative organisms, a fresh water planarian species-Malta (PSM) and the axolotl *Ambystoma mexicanum* (AXO) were assessed *in vitro* on the human AML HL-60 cell line. The anticancer activity was evaluated in terms of ability to influence proliferative activity, cell viability, cell cycle arrest, and differentiation. Moreover, gene expression analysis was performed to evaluate the genes involved in the regulation of these processes. The PSM extract exhibited a selective cytotoxic effect on HL-60 cells when compared to normal lymphocytes. Furthermore, cell cycle analysis and Annexin V/PI assay showed that the PSM extract induced apoptosis in HL-60 cells. AXO crude extract exhibited antiproliferative but not cytotoxic activities on HL-60 cells, with cell cycle arrest in the G0/G1 phase. Both PSM and AXO extract clearly decreased the nucleo/cytoplasmic ratio of the HL-60 cells, with an increase in nitroblue tetrazolium-positive cells. Furthermore, PSM treated cells showed an increase in CD11b- and CD14-positive cells, whilst AXO-treated HL-60 cells showed an increase in the expression of CD11b, suggesting that the extracts were able to stimulate myeloid differentiation. Finally, PSM and AXO extracts caused upregulation of *CEBPA, CEBPB, CEBPE, SPI1,* and downregulation of *c-MYC*, with PSM extract showing an increased expression of *CDKN2C* and reduction of *CDKN1A*. The data clearly show the potential anticancer activity of PSM and AXO on HL-60 cells and suggest that it could help develop promising therapeutic agents for the treatment of AML

Key words: Planarian; Axolotl; Apoptosis; Cell Proliferation; Differentiation; Leukaemia

Contents

List of Figures

List of Tables

List of Abbreviations

AEC	Apical epithelial cap
AML	Acute myeloid leukaemia
APL	Acute promyelocytic leukaemia
ATO	Arsenic trioxide
ATRA	all-trans- retinoic acid
AXO	Axolotl
BAK	BCL2 homologous antagonist/killer
BAX	BCL2 Associated X
BCL2	B Cell Lymphoma 2
BM	Bone marrow
C/EBP	CCAAT enhancer-binding protein
CBF	Core binding factor
CDKI	Cyclin-dependent kinase inhibitors
Cdk2	Cyclin-dependent kinase 2
Cdk4	Cyclin-dependent kinase 4
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
c-MYC	Avian myelocytomatosis virus oncogene
CpG	Cytosine-Guanine oligodeoxynucleotides
CR	Complete remission
CREB	cAMP response element-binding protein

CREs	cis-regulatory elements
CSC	Cancer stem cell
DMSO	Dimethyl-sulfoxide
DNMTi	DNA methyl-transferase inhibitors
ELN	European leukaemiaNet
ETS	Erythroblast transformation specific
FAB	French American British
FLT3	fms-like tyrosine kinase 3
GMP	Granulocyte/Monocyte progenitor
HDACi	Histone deacetylase inhibitors
HLA	Human leukocyte antigen
HSCs	Haematopoietic stem cells
HSCT	Haematopoietic stem cell transplantation
HTH	Helix-turn-helix
KLF4	Kruppel-like factor 4
LSC	Leukaemia stem cell
MDM2	Mouse double minute 2 homolog
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte/Erythroid progenitor
MHC	Major histocompatibility complex
MLL	Mixed lineage leukaemia
MPP	Multipotent progenitor
MSX	Msh homeobox 1
MYH11	Myosin heavy chain 11

NRAD	newt homolog of Rad
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
pRB	Retinoblastoma protein
PSM	Planaria species – Malta
RARα-PML	Retinoic acid receptor alpha – promyelocytic leukaemia
ROS	Reactive oxygen species
SOX2	(sex determining region Y)-box 2
TFs	Transcription factors
TGF*β*	Transforming growth factor beta
Th1	T helper type 1
Th2	T helper type 2
TP53	Tumour protein 53
VITD3	1,25 dihydroxy vitamin D3
WHO	World health organisation

Chapter 1

Introduction

1.1 Haematopoiesis

Haematopoiesis is the process that involves the proliferation, differentiation and maturation of the multipotent undifferentiated haematopoietic stem cells (HSCs) resulting in the production of specialized cell types with vastly different functions including B cells, T cells, macrophages, erythrocytes, platelets and granulocytes (Burda *et al*., 2010). Maintenance of HSCs depends on asymmetric cell divisions balancing self-renewal to ensure HSCs maintain their numbers, and the process of differentiation to ensure the haematopoietic cell pool remains in balance (Kanji *et al*., 2011).

Different models of haematopoietic cell differentiation have been put forward to fully understand the pathway that HSCs undertake towards differentiation into fully functional mature blood cells. The earliest model proposes a branching hierarchical differentiation of 'homogeneous' HSCs to their progenitors. With HSCs at the top of the hierarchy, the first point that branches from the tree separates cells with lymphoid potential from all other lineages (myeloid, erythroid and megakaryocytic). These then continue a series of steps until they reach terminal differentiation and lose their potency along the differentiation tree (Laurenti & Gottgens, 2018). Another hierarchical model acknowledges the heterogenous nature of HSCs and suggests that segregation occurs at a later stage with HSCs initially giving rise to a multipotent progenitor (MPP), which further differentiates into either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP). The CMP branches into megakaryocyte/erythroid progenitors (MEP) and granulocyte/macrophage progenitors (GMP) and the CLP gives rise to natural killer cells, B cells and T cells (Doulatov *et al*., 2010).

Another model reveals much less rigidity and depicts the differentiation pathways as a continuum 'cloud' (Laurenti & Gottgens, 2018). In this model, the activation of one or more transcription factors does not necessarily seal the fate of cell commitment to a particular cell lineage, but an 'oscillating' concentration of the transcription factors can shift in favour of one cell fate or a group of cell fates whilst suppressing the cell fates of another cell lineage commitment (Nerlov & Graf 1998; Ceredig *et al*., 2009).

Humans generate approximately 4.5×10^{11} haematopoietic cells every day and this feat requires tightly regulated decisions between proliferation and differentiation (Dingli *et al*., 2012; Kaushansky, 2018). This intricate regulation requires a balanced signalling and transcription network that ensures that the different cell types are formed at the right time and in right numbers (Ruijtenberg & van der Heuvel, 2016). Moreover, it needs to ensure that proliferation remains under check to avoid the development of anomalies such as cancer. For haematopoiesis to occur without obstruction of the maturation process from early progenitors to mature blood cells, an intricate balance between the extent of reduced self-renewal of early precursors and commitment to a specific lineage with particular lineage markers and identity needs to occur in an orderly and controlled fashion (Seita & Weissman, 2010). Basically, if a stem cell commits to differentiation, genes that ensure self-renewal is maintained are switched off, whereas genes that align cells along the differentiation pathway are switched on (Rosenbaeur & Tenen, 2007; Saunders *et al*., 2013; Wei & Frenette, 2018). Differentiation follows a series of lineage branching points and is directed by up- and down- regulation of particular genes, which together build a large network of factors that determine cell fate (Rosenbaeur & Tenen, 2007; Rothenberg *et al*., 2016). Once this tightly controlled system is

disrupted, it may lead to incomplete differentiation with uncontrolled proliferation of immature blood cells leading to cancer (Fouad & Aanei, 2017).

1.2 Transcription factors in myeloid differentiation

Transcription factors (TFs) are proteins which are necessary for regulation of gene transcription (Wilkinson *et al*., 2017). Regulation involves the binding of TFs to specific DNA sequences (Lambert *et al*., 2018) termed *cis*-regulatory elements (CREs) that are located either upstream, downstream or at intronic regions of target genes (Powell *et al*., 2019). TFs also exert their effect at gene regulatory regions in distant DNA sequences (Wilkinson *et al*., 2017). Binding of TFs to CREs can result either in transcription activation or transcriptional repression (Sharma & Gurudutta, 2016; Mitsis *et al*., 2020). TFs use a variety of DNA-binding structural motifs to recognize their target sequences (Inukai *et al*., 2017). The interaction between DNA and TFs involves a large number of co-operating factors including epigenetic modifications and the cooperative binding of other TFs (Mitsis *et al*., 2020).

TFs play a major role in differentiation of haematopoietic cells. They control haematopoietic development and homeostasis by causing up- or down- regulation of genes that push cells to commit to a specific lineage (Sharma & Gurudutta, 2016). Therefore, any mutations such as deletions and/or translocations that target TFs, would result in impairment of differentiation with development and progression of leukaemia (Ridinger-Saison *et al*., 2012; Niwa, 2018). This is substantiated by studies that show many known leukaemia translocations involve TF sites (Zheng, 2013; Brunet & Jasin, 2018).

1.2.1 PU.1 transcription factor

PU.1, encoded by the *Spi1* gene on chromosome 11p11.2, is an Erythroblast transformation specific (ETS) -family transcription factor that plays major and important roles in haematopoiesis (Gupta *et al*., 2009). It positively regulates many genes in haemopoietic cells including macrophages, granulocyte, dendritic- and B-cells (Rothenburg *et al*., 2019). Depending on cell fate, expression levels of PU.1 is crucial for lineage commitment. For example, GATA-1 and GATA-2 expression represses PU.1, and favour commitment to megakaryocyte/erythrocyte differentiation over myeloid lineage expression and vice versa, (Mak *et al*., 2011; Wontakal *et al*., 2012; Woolthius & Park, 2016). It has also been proposed that CCAAT enhancer-binding protein alpha (C/EBPα) and PU.1 expression levels are directly linked to neutrophil versus the monocyte fates (Avellino *et al*., 2016; Avellino & Delwel, 2017). Thus, conflicting competition among more than two TFs functions could be a vital mechanism for commitment of a bipotent cell in a progenitor stage to a single lineage program (Iwasaki *et al*., 2006).

In a bid to understand the role of PU.1 in the granulocytic versus monocytic commitment decision, studies on PU.1 (−/−) mice revealed that low levels of PU.1, direct the myeloid progenitors towards commitment to granulocytic development, whereas high levels induced cells to commit to monopoiesis (Dahl *et al*., 2003; Laslo *et al*., 2006).

Other studies have revealed that deficient PU.1 expression in mice severely impaired haematopoiesis and in some cases lead to leukaemia (Imperato *et al*., 2015; Verbeist *et al*., 2015), whilst a reduction in PU.1 expression reaching 20% of wild-type expression has been demonstrated to induce AML (Nutt *et al*., 2005; Zhou *et al*., 2015). Mutations in PU.1 have also been described in human AML (Vangala *et al*., 2003; Mueller *et al*., 2006). Furthermore, certain

oncogenic fusion proteins, such as AML-ETO and Retinoic acid receptor alpha – promyelocytic leukaemia (RARα-PML) (15;17) are also associated with graded PU.1 inhibition (Zhou *et al*., 2015).

1.2.2 CCAAT/enhancer-binding protein (C/EBP) family of transcription factors

To date, six members have been characterized, and are designated C/EBPα, C/EBP-β, C/EBP-γ, C/EBP-δ, C/EBP-ε, and C/EBP-ζ (Khanna-Gupta & Berliner, 2018). C/EBPs enhance the expression of certain genes through several interactions including with other transcription factors, protein-protein interactions and through binding to DNA promoter regions (Liu *et al*., 2009). Once bound to DNA, C/EBPs can open up the chromatin structure allowing other factors to regulate gene expression of many housekeeping and tissue-specific genes (Ren *et al*., 2014).

Within haematopoiesis, C/EBPα (Chromosome 19q13.11), C/EBPβ (Chromosome 20q13.13) and C/EBPε (Chromosome 14q11.2) are predominantly expressed in the granulocytic and monocytic lineages (Friedman, 2017). Elevated C/EBPα expression is found in the HSC, CMP and GMP, but not the CLP or MEP populations (Guo *et al*., 2014). Knock-out studies in mice further confirm the involvement of C/EBPα in haematopoiesis (Rosmarin *et al*., 2005; Haseman *et al*., 2014). C/EBPα(−/−) neonatal mice exhibit a reduction in their neutrophil and eosinophil numbers, while monocytes are still present in their peripheral blood (Friedman, 2007).

C/EBPα and C/EBPβ exhibit structural, regulatory, and functional similarities and they may functionally replace each other in certain occasions (Chen *et al*., 2000). This was demonstrated by gene replacement strategies in which C/EBPβ was expressed from the C/EBPα locus in

C/EBPα null-mice, leading to normal haematopoiesis (Jones *et al*., 2002). C/EBPα and C/EBPβ may cross-talk to ensure enough cells commit to granulocytic differentiation by orchestrating the proliferation and differentiation of granulocyte precursors (Hirai *et al*., 2015). C/EBPβ also interacts directly with PU.1 through the PU.1 DNA-binding domains, promoting gene induction (Grondin *et al*., 2007).

The transcription factor C/EBPε is expressed only in myeloid lineages and specifically in the later stages of granulopoiesis (Khanna-Gupta & Berliner, 2018). During neutrophil development, C/EBPε drives the commitment of promyelocytes to the end stage of myeloid differentiation and is also necessary for secondary and tertiary granule formation (Bartels *et al*., 2015; Goos *et al*., 2019). C/EBPε (−/−) mice develop all of the haematopoietic lineages, but they also develop myelodysplasia features, die prematurely, and their granulocytes fail to reach terminal differentiation and resemble human cases of secondary granule deficiency (Yamanaka *et al*., 1997). Cells in mice with C/EBPε (−/−) show incomplete differentiation of granulocytes with an intermediate 'paramyeloid' cell type exhibiting both monocyte and granulocyte features (Halene *et al*., 2010).

C/EBPs play an important part in cell cycle regulation (lee at al., 2019). They are mainly negative regulators of cell proliferation (Nerlov, 2007) and C/EBPα expression blocks cell cycle progression at the G1-S boundary (Johnson, 2005). C/EBPα expression in human cell lines leads to an induction of *CDKN1A* promoter activity and accumulation of p21 protein (Slomiany, 2000). It is able to stabilize p21 protein and block cell cycle progression (Johnson, 2005). Together with C/EBPβ, C/EBPα causes repression of cell cycle regulatory proteins including E2F,

retinoblastoma protein (pRB), and cyclin dependent kinases cyclin-dependent kinase 2 (Cdk2) and and cyclin-dependent kinase 4 (Cdk4) function (Al-Bitar & Gali-Muhtasib, 2019).

The repressive properties of C/EBP-α and -β raised the possibility that they could behave as tumour suppressors. Within the context of leukaemia, mutations in *CEBPA* have been described in approximately 10% of *de novo*-AML patients (Nerlov, 2007; Mannelli *et al*., 2017). This was confirmed in studies whereby several oncogenic events disrupt C/EBP-α expression or function in AML and other cancers (Roe & Vakoc, 2014; Wurm *et al*., 2017). The role of impaired transcription factors in cancer progression is supported by the ability of causing a suppression of *in vivo* tumorigenicity of AML after the re-introduction of such factors (Lourenço & Coffer, 2017; Nerlov, 2007), with targeted modulation of C/EBP activities providing a new approach to AML therapy (Truong *et al*., 2003).

1.2.3 Avian myelocytomatosis virus oncogene (c-MYC) transcription factor

c-MYC, on chromosome 8q24.21, is an oncogenic transcription factor that belongs to the helix-loop-helix (HLH) leucine zipper family (Jones, 2004). It was first discovered in retroviruses that induced aggressive myeloid neoplasms in chicken (Delgado & Leon, 2010). Through its leucine zipper domain, c-MYC dimerises with another TF, myc-associated factor x (MAX), and together they bind to specific DNA sequences in the regulatory regions of target genes (Solomon *et al*., 1993).

Thousands of c-Myc target genes have been identified by genome-wide technologies and many of these genes are involved in important cellular functions including proliferative,

glycolytic, metabolic, and differentiation pathways amongst others (Miller *et al*., 2012). In haematopoiesis, MYC family members c-MYC, n-MYC, and l-MYC are differentially expressed with early HSCs co-expressing c-MYC and n-MYC, with l-MYC moderately expressed in CLPs, megakaryocytes, and macrohpages (Delgado & Leon, 2010).

In humans, deregulation of MYC was first reported in Burkitt's lymphoma. Over half of human cancers show a dysregulated c-MYC expression with over expression as the major contributory factor in at least 75% of cancers (Carabet *et al*., 2018). Studies using several leukaemia cell line models have highlighted the importance of c-MYC deregulation particularly pertaining to cellular proliferation, apoptosis, and differentiation.

From a therapeutic point, targeting MYC could be a useful treatment approach. Several groups focused on the identification of small molecule inhibitors albeit with modest success (Brockman *et al*., 2013; Whitfield *et al*., 2017). However, being a transcription factor and not an enzyme, c-MYC lacks a binding pocket where small molecules can fit and modulate its expression (Carabet *et al*., 2018). Also, a high *MYC* copy number does not necessarily reflect a higher c-MYC expression (Paulsson et al., 2003) and hence its inactivation may not necessarily be a successful therapeutic option.

1.3 Acute Myeloid Leukaemia

Acute myeloid leukaemia (AML) is a heterogeneous group of diseases characterised by impaired differentiation and clonal expansion of myeloblasts in both the peripheral blood and bone marrow (BM) (Castelli *et al*., 2019). In the USA, the age-adjusted incidence of AML is 4.3 per 100,000 with a mean age of 68 years (Shallis *et al*., 2019). In 2020, it is estimated that over 60,000 people will be diagnosed with leukaemia in the USA alone (https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2020.htmL 2020, accessed on 16th of April, 2020). The 5-year survival rate of patients diagnosed with AML depends on the age at diagnosis and the subtype of AML, among other factors. In fact, the overall 5-year survival rate for all AML sub-types with the best available treatment for children is about 60-75%, young patients (age 15-39) is about 50-60% while in older patients, it is approximately 10% (Creutzig *et al*., 2018).

1.3.1 Incidence of leukaemia subtypes in Malta

Using the cancer statistics data collected by the Registry for Cancer in Malta, the total number of cases of all leukaemia subtypes between 2007-2018 is 496 cases (Figure 1-1.) with an average of 41.3 cases per annum. Similar to data reported in the USA (Hao et al., 2019) of the four major subtypes of leukaemia, the majority of cases in Malta are AML (44%) followed by CLL (24%) (Figure 1-3). Furthermore, similar to cancer registries in the US (Hao et al., 2019) and the European Union (https://worldcancerinitiative.economist.com/pdf/acute-myeloid-leukaemia/acute-myeloid-leukaemia-report.pdf, accessed on 13th October, 2020), leukaemia incidence increases with age (Figure 1-2). This increase could be attributed to the increase in the average life expectancy. Recent genetic analyses of large populations have revealed that

somatic mutations in hematopoietic cells leading to clonal expansion are commonly acquired during human aging (Steensma et al., 2015). Also, AML could arise secondary to treatment for other cancers.

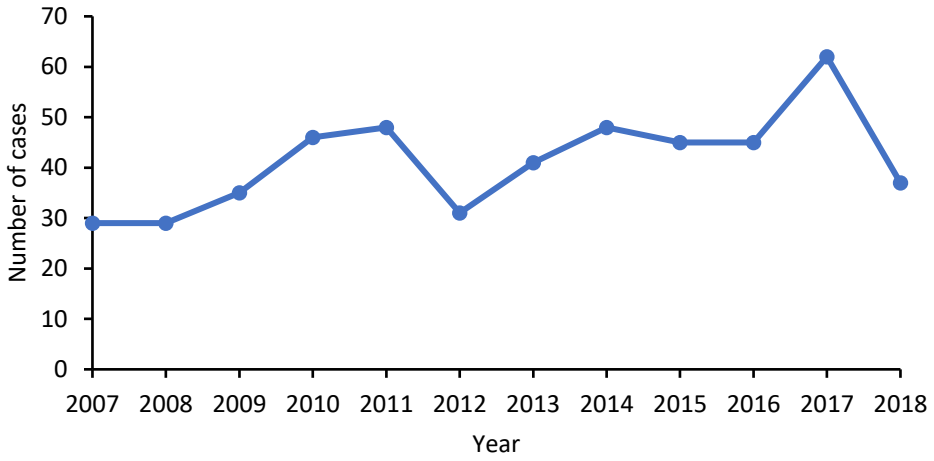

Figure 1-1: The number of cases of all leukaemia subtypes in Malta between 2007 - 2018. Data collected from the Registry of Cancer in Malta

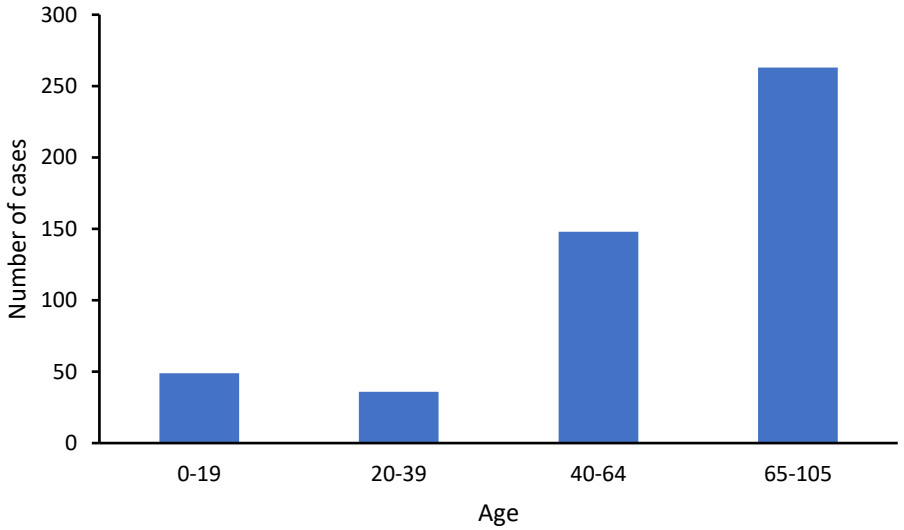

Figure 1-2: The number of cases of all leukaemia types in different age groups in Malta between 2007 – 2018. Data collected from the Registry of Cancer in Malta

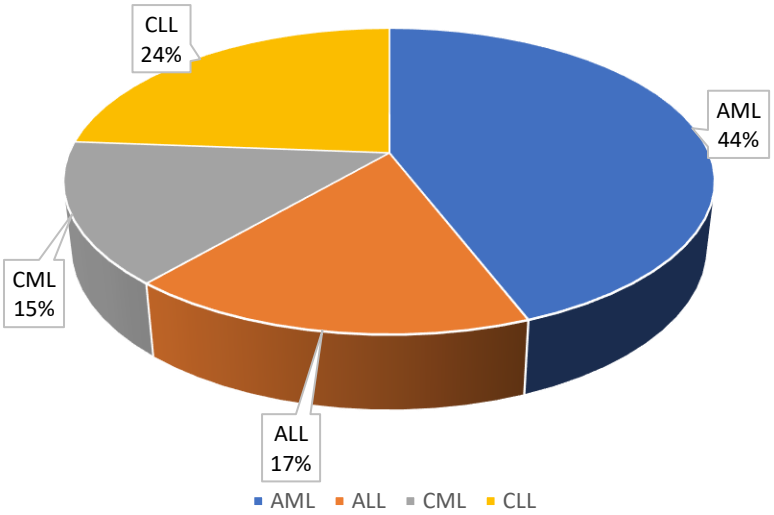

Figure 1-3: The leukaemia subtypes in Malta. The percentage number of leukaemia subtypes in Malta between 2007 and 2018. Data from the Registry of Cancer in Malta. (AML= Acute myeloid leukaemia; ALL= Acute lymphoblastic leukaemia; CML= Chronic myeloid leukaemia; CLL= Chronic lymphocytic leukaemia).

1.3.2 Classification landscape in AML

In 1976, a French-American-British (FAB) Group stratified patients into different leukaemia sub-groups (M0 to M7) based on the morphology of the abnormal presenting cells and the number of "blast cells" in bone marrow smears and the extent to which morphology recapitulates normal haematopoietic progenitors, i.e., whether the blasts are myeloblasts, monoblasts, megakaryoblasts, or erythroblasts (Table. 1-1) (Bennett *et al.*, 1976).

Table 1-1: FAB classification of AML (Adapted from Bennet *et al*., 1976). Classification is based on morphological features of the cells. The table also shows the percentage incidence of the different AML subgroups.

FAB Subtype	Morphological classification	(%) subgroup-AML cases
M0	Undifferentiated AML	5
M1	AML with minimal differentiation	15
M2	AML with maturation	25
M3	Acute promyelocytic leukaemia	10
M4	Acute myelomonocytic leukaemia (AMML)	20
M4-eos	AMML with eosinophilia	5
M5	Acute monocytic leukaemia	10
M6	Acute erythroid leukaemia	5
M7	Acute megakaryoblastic leukaemia	5

However, over the past four decades, it has become clearly evident that the FAB classification is not sufficient in predicting prognosis and/or stratifying patients into different risk groups. With the advent of cytogenetics, it became possible to examine leukaemia cells at the chromosomal level using G-band karyotyping (Mrózek *et al*., 2009). This identification of cytogenetic abnormalities at diagnosis was considered a crucial independent prognostic factor in childhood and adult patients with AML (Mrozek, 2004). However, this technique is not adequate in identifying structural and/or numerical chromosomal aberrations in almost half the patients at diagnosis, making it difficult to stratify patients into the correct risk group (Damiani & Tiribelli, 2019).

Advances in molecular genetics led to the identification of various mutations with diagnostic and prognostic impact and these have eventually been included in the revised WHO classification of haemataological neopleasms (Dohner *et al*., 2017; Arbor *et al*., 2016). The revised WHO classification incorporated the FAB morphologic classification, cytogenetic abnormalities and their prognostic significance and reflected the progress in understanding the molecular background of the disease. It was based on clinical, prognostic, morphologic, immunophenotypic, and genetic data and defined AML into six major categories (Table 1-2), AML with recurrent genetic abnormalities; AML with myelodysplasia related features; therapy related AML; AML not otherwise specified; myeloid sarcoma; and myeloid proliferation related to Down syndrome (Arber *et al*., 2016).

Table 1-2: WHO classification of AML. Adapted from Arber *et al*., 2016 and Dohner *et al*., 2017. The classification is based on genetic mutations and morphological abnormalities

AML types	**Genetic mutation/morphological abnormalities**

AML with recurrent genetic abnormalities	Genetic abnormalities
	t(8:21)(q22;q22)/RUNX1-RUNX1T1;
	inv(16)(p13.1q22);
	t(16;16)(p13.1;q22)/CBFB-MYH11; APL with PML-RARA;
	t(9;11)(p21.3;q23.3)/MLLT3-KMT2A;
	t(6;9)(p23;q34.1)/DEK-NUP214;
	inv(3)(q21.3q26.2);
	t(3;3)(q21.3;q26.2)/GATA2, MECOM;
	t(1;22)(p13.3;q13.3)/RBM15-MKL1 (megakaryoblastic); mutated NPM1;
	Provisional entity: AML with mutated RUNX1; AML with biallelic mutations in *CEBPA*
	• Provisional entities
	o Mutated *RUNX1*
	o BCR-ABL1

AML with myelodysplasia related features	• Complex karyotype • Chromosome deletions • Translocations

Therapy related AML	• Secondary mutations derived from primary cancer treatment
AML not otherwise specified	• with minimal differentiation • without maturation • with maturation • Myelomonocytic maturation • Acute monoblastic/monocytic leukaemia • Acute erythroid leukaemia • Pure erythroid leukaemia • Acute megakaryoblastic leukaemia • Acute basophilic leukaemia • Acute panmyelosis with myelofibrosis

Myeloid sarcoma	• unique clinical presentation
	  ○ de novo
	  ○ in AML relapse
	  ○ progression of MDS

Myeloid proliferation related to Down syndrome	• Transient abnormal myelopoiesis • Down syndrome associated leukaemia
Myeloid neoplasms with germ line predisposition	• Without a pre-existing disorder or organ dysfunction ○ CEBPA mutation

	○ DDX41 mutation • With pre-existing platelet disorders ○ RUNX1 mutation ○ ANKRD26 mutation ○ ETV6 mutation • With other organ dysfunction ○ GATA mutation ○ Bone marrow failure syndrome ○ Juvenile leukaemia associated with Noonan syndrome, Neurofibromatosis ○ With Noonan syndrome

The stratification of leukaemias according to genetic mutations and clinical presentation into different risk groups greatly enhanced the prediction of event free survival and overall survival. The European LeukemiaNet (ELN) proposals for diagnosis and management of AML (Figure 1-4) further complemented the WHO classification of myeloid cancers. Nowadays, these recommendations have been widely adopted in the management and diagnosis of cancer patients and within clinical trials (Dohner *et al*., 2017).

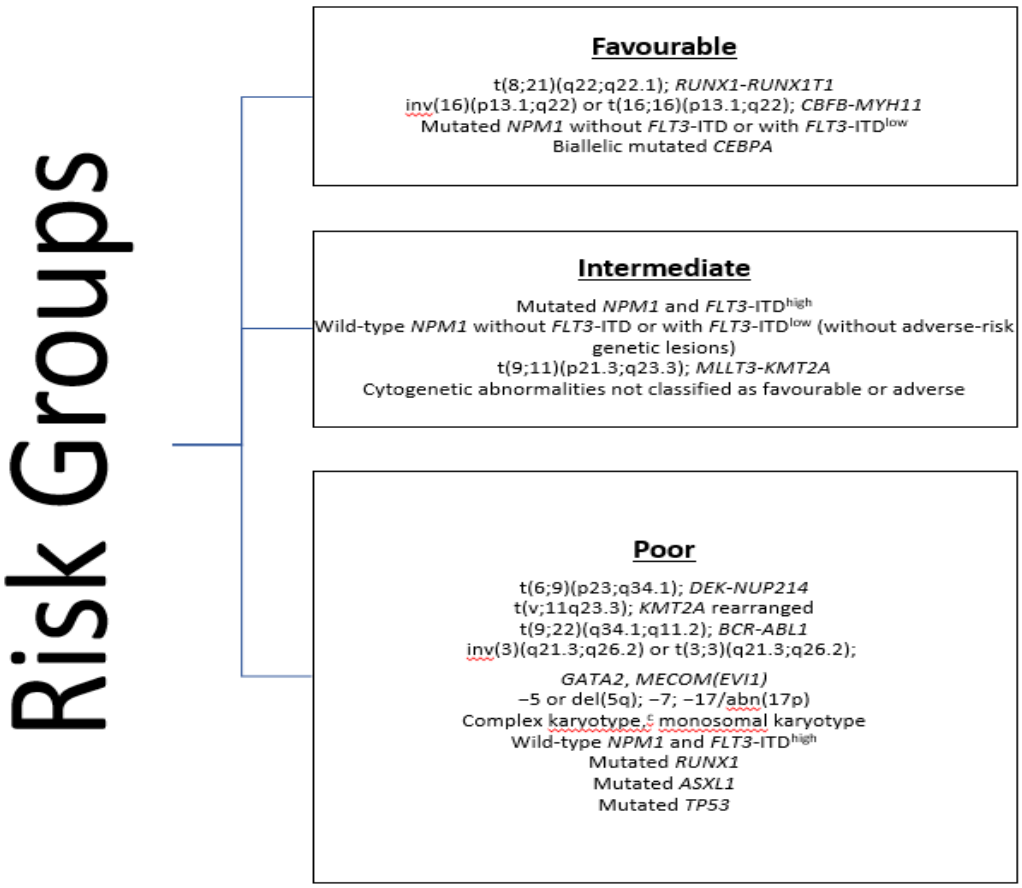

Figure 1-4: ELN risk stratification by genetics. The ELN stratification assigns patients into favourable, intermediate, and poor risk according to the genetic mutations present at diagnosis (Adapted from Dohner *et al*., 2017).

While one study found that ELN2017 effectively stratified patients into different risk groups (Harada *et al*., 2018), another study reported a discordance in risk stratification according to the proposed ELN guidelines in older patients presenting with de-novo AML (Eisfeld *et al*., 2018). A third group proposed the inclusion of genes other than those indicated by the ELN risk stratification (Papaemmanuil *et al*., 2016). The ongoing replacement of single gene assays by next generation sequencing (NGS) will facilitate further the development of prognostic and diagnostic markers. Also, exome sequencing and genome wide assays will further enhance more sophisticated prognostic systems (Estey, 2018).

1.3.3 Cytogenetic and molecular genetics of AML

The spectrum of chromosomal abnormalities associated with leukemogenesis in AML is broad and heterogeneous. Approximately 50% to 60% of newly diagnosed AML patients exhibit cytogenetic abnormalities (Kumar, 2011). Many patients present with recurring chromosomal translocations that often result in gene arrangements giving the leukaemic cells a growth advantage. Identification of cytogenetic anomalies is a significant factor in predicting remission rate, relapse, and overall survival in patients diagnosed with AML (Kumar, 2011; Rashidi & Cashen, 2015).

One of the most common translocations in AML patients (10%) is the translocation t(8;21)(q22;q22) generating the RUNX1/RUNX1T1 fusion transcript (Kamran *et al*., 2019). It can present solely or with co-operative mutations in other genes. Another common translocation with equal incidence is the t(15;17)(q22;q21) that is typical of acute promyelocytic leukaemia (APL) and involves the *PML-RARA* fusion gene (Wafa *et al*., 2016). Inversions and translocations in chromosome 16 are found in about 8 % of AML. This involves inversions between the short 'p' arm and the long 'q' arm of chromosome 16 resulting in core binding factor beta (*CBFB*) and myosin heavy chain 11 (*MYH1*) fusion transcripts (Lv *et al*., 2020). About 5 % of AML patients present with rearrangements of the mixed lineage leukaemia (MLL) (KMT2A) gene which is located on 11q23 (Meyer *et al*., 2017).

AML patients can present with deletions or duplications of part or whole chromosomal arms, such as deletions in the long arms of chromosomes (Mrozek, 2008). Two of the most common deletions are del (5q), and del (7q). They are more common in elderly patients and are

associated with a poor outcome. Chromosome monosomies in AML are also associated with an unfavourable outcome (Breems *et al*., 2008; Papaioannou *et al*. 2016; Anelli *et al*., 2017).

Mutations implicated in the pathogenesis of AML affect the overall prognosis and recurrence of AML (Yu *et al*., 2020). They often lead to activation of downstream signalling pathways resulting in uncontrolled cellular proliferation, increased resistance to apoptosis, and the blocking of differentiation. The identification of certain recurring mutations can help stratify patients into distinct prognostic sub-groups. For instance, 35% of adult patients with *de novo* AML and 45–60% of AML patients with a normal karyotype show mutations in the molecular chaperone nucleophosmin 1 (NMP1) and its presence predicts a higher likelihood of achieving CR (Walker & Marcucci, 2012; Falini *et al*., 2005). In contrast, mutations in the tumour suppressor gene [tumour protein 53 (*TP53*)] are usually associated with old age and resistance to chemotherapy (Zeichner *et al*., 2014). Patients with certain fusion proteins (*PML-RARA, RUNX1-RUNX1T1,* or *MYH11-CBFB*) are associated with a favourable risk.

Whilst approaches at classifying leukaemia into different groups has paved the way for better understanding of AML biology, however, the current classification approaches are not sufficient for accurate stratification. Thus, more work is required in elucidating the genetic and epigenetic changes in the pathogenesis of AML. This will ensure better classification and assessment of risk and better approaches to therapy (Moarii & Papaemmanuil, 2017). Table 1-3 summarises the recurring molecular genetic mutations in AML, their partners, pathogenesis and prognosis.

Table 1-3: Mutations in AML. Compiled from Ley *et al*., 2010; Winters & Bernt, 2017; Kunchala *et al*., 2018; Ball *et al*., 2019; Barbosa *et al*., 2019; Daver *et al*., 2019. The table presents the percentage of common mutations in De-novo AML mutations together with the type and biological function of the mutated gene.

Gene	% De-novo AML cases	Type of mutation	Biological function
NPM1	30-35%	Frame shift mutations common on exon 12	Molecular chaperoning Ribosome biogenesis, DNA repair Genome stability of tumour suppressor genes such as *P53*
CEBPA	5-10%	Frame shift, insertions, and deletions in the P42 and P40 isoforms	TF involved in commitment of progenitors to the myeloid lineage
FLT3	30-35%	internal tandem duplication; duplication; mis-sense mutations	Receptor tyrosine kinase
RAS	N-RAS (11-16) K-RAS (4-5)	N-RAS 1p22; K-RAS 12p12 Activating mutations	Family of oncogenes regulate signal transduction
IDH	20% IDH1 IDH2	DNA and histone hypermethylation, altered gene expression and blocked differentiation of hematopoietic progenitor cells	important in the cellular metabolism of lipid synthesis, cellular defense of oxidative damage, and oxidative respiration; histone demethylation and DNA modification
TET2	10-20%	Majority are nonsense and frameshift mutations	catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine
MLL	10%	Partial tandem duplications	epigenetic regulation of defined developmental genes
DNMT3A	15-20%	loss-of-function	DNA methylation
TP53	8%	Somatic mutations Germline mutations	Tumour suppressor gene

1.3.4 Leukaemia stem cells

The cancer stem cell (CSC) concept was first described in the nineteenth century (Capp, 2019). CSCs are a small population of immature quiescent cells, and similar to HSCs, are capable of self-renewal. Moreover, CSCs are able to maintain tumour cells by differentiation of non-stem daughter cells (Aponte & Caicedo, 2017).

AML was one of the first malignancies in which the existence of CSCs was demonstrated (Dick, 2005) with approximately 0.1–1% of cells exhibiting leukaemia stem cell (LSC) properties (Thomas & Majeti, 2017). These cells are able to initiate AML when transplanted into immunodeficient animals (Lapidot *et al*., 1994). However, not all transplanted LSCs are able to induce leukaemia in recipient animal models (Bonnet & Dick, 1997).

Clonality studies in AML patients of different age groups indicate that LSCs arise through the successive accumulation of somatic DNA mutations in HSCs (Zuna *et al*., 2009; Hong *et al*., 2008; Miyamoto *et al*., 2000). These 'early' mutations often result in enhancing self-renewal potential together with impairment of differentiation and lead to variably expanded clonal populations of pre-leukaemic HSCs in patients (Corces *et al*., 2016; Vetrie *et al*., 2020). These mutations are retained in myeloblasts of AML patients, and are often implicated as putative initiating events in leukemogenesis (Shlush *et al*., 2014). Examples of such mutations are seen in cases that precede acute leukaemia, particularly in patients with myelodysplastic syndrome (MDS) and or chronic myeloid leukaemia (CML) (Walter *et al*., 2012).

The search for genetic mutations which are common in AML within subjects with no history of haematological malignancies led to the identification of several genes implicated in AML (Jaiswal *et al*., 2014; Buscarlet *et al*., 2017). These findings were attributed to clonality and termed 'clonal haematopoiesis of indeterminate potential' (CHIP). Naturally, the incidence of CHIP increases with age (Corces *et al*., 2017) and is a known risk factor in haematological cancers. CHIP has a peak incidence of approximately 20% in otherwise healthy elderly (over 70 years) subjects (Genovese *et al*., 2014). Examples of 'early' mutational events include epigenetic regulator mutations such as DNA methyltransferase 3A (*DNMT3A*), methylcytosine dioxygenases *TET1* and *TET2*, isocitrate dehydrogenase 1 (*IDH1*) and *IDH2*, the Polycomb group protein *ASXL1*, and *TP5*3 (Vetrie *et al*., 2020). Many of these mutations are actually described in the majority of patients with AML (Lagunas-Rangel *et al*., 2017). In AML patients, these 'evolutionary ancestral' mutations can survive induction chemotherapy and remain in the bone marrow at remission thereby providing a persistent milieu of cells that ensure a poor prognostic outcome (Corces *et al*., 2017).

1.4 Therapeutic approaches in AML

1.4.1 Current standard therapy for AML

The overall therapeutic strategy in patients with AML in the last 40 years remains relatively unchanged. The standard of care for AML has been a '3+7' chemotherapy regimen, based on three days of anthracycline and seven days of cytarabine (Norsworthy *et al*., 2017). The initial approach determines whether a patient is eligible for intensive chemotherapy based on age, clinical presentation, and laboratory results. If complete remission is achieved following intensive therapy, appropriate post-remission therapy becomes essential (De Kouchkousky & Abdul-Hay., 2016; Dohner *et al*., 2015). This approach of induction and consolidation evolved from a series of large-scale clinical trials in the 1970s where patients were placed into different treatment groups and treated with chemotherapy regimens regardless of the unique presenting features of individual AML patients (Visani *et al*., 2006). Even in patients who achieved remission with chemotherapy, relapse occurred in two thirds of patients (Yilmaz *et al*., 2019) due to persistence of leukaemic cells below morphologic detection and due to chemo-resistance (Ravandi *et al*., 2018). It is thought that unsuccessful treatment and persistence of residual cancer cells in leukaemia can also be attributed to the BM microenvironment in which these cells are sequestered in niches that protect them from drugs or the body's own immune response (Shafat *et al*., 2017).

Several attempts have been made to improve induction chemotherapy by combinatorial treatment approaches, but none had shown significant improvements in outcomes without increasing toxicity (Williams *et al*., 2019). More intense combination regimens increased

complete remission (CR) rates albeit with increased toxicity and no overall improvement in survival (Anders *et al*., 2017).

Allogeneic haematopoietic stem cell transplantation (HSCT) as post-remission therapy significantly reduces leukaemia relapse but non-relapse mortality due to complications of the transplant including graft versus host disease (GvHD) and infection will counterbalance this beneficial effect (Sweeney & Vyas, 2019). The ELN group recommends allo-HSCT if the patients disease-free survival is predicted to improve by at least 10% (Sweeney & Vyas, 2019). Therefore, considering the still imperfect treatment modalities available, the need for treatment approaches tailored for individual patients becomes more pressing (Ossenkoppele *et al*., 2016).

Therapy success of AML depends on the molecular mutations at presentation and at relapse, since it has been speculated that 'pre-leukemic' HSCs survive therapy and acquire additional alterations that result in therapy failure (Corces *et al*., 2017). Since AML is a heterogenous disease, genetic subclones that persist during therapy eventually expand and re-populate the AML seen in relapse (Li *et al*., 2016). The persistence of a small number of LSCs following therapy and the eventual relapse seen in patients further strengthens the drive towards understanding the biology of AML. The aim is to improve therapeutic survival outcomes by identifying the characteristics of cells that drive disease relapse in order to improve AML therapy.

Advances in genomic sequencing and in-depth understanding of the molecular biology of AML and the bone marrow microenvironment (Ghiaur *et al*., 2015; Balderman *et al*., 2016) is paving the way for a personalised approach to AML treatment. Using novel targeted therapies offers not only effective but also reduced toxicity in AML patients. Given the molecular diversity of AML, it is unlikely that a 'single approach' therapy will provide an effective long-term solution against this disease. In the following section, different approaches to AML therapy including recent advances will be discussed including some examples of differentiation therapy, epigenetic modifiers, protein kinase inhibitors, dysregulated pathway inhibitors, and immunotherapy (Figure 1-5)

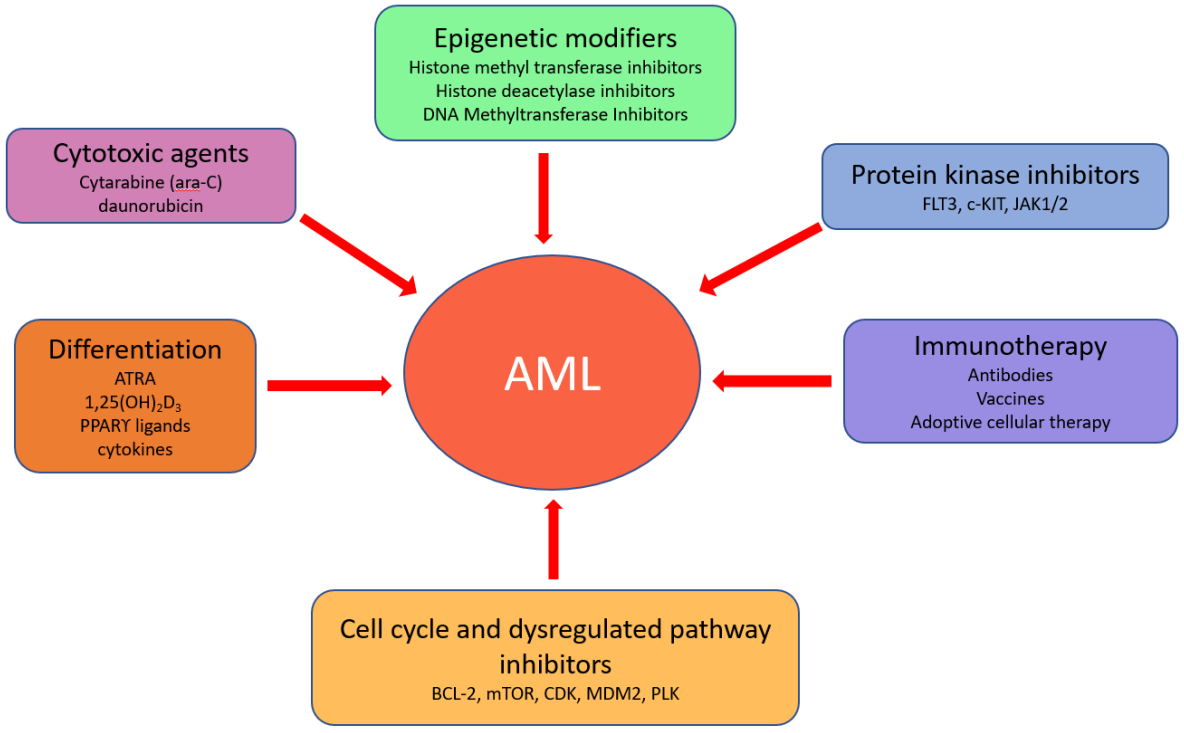

Figure 1-5: Different approaches to the treatment of AML including recent advances in treatment with some examples of cytotoxic agents, differentiation therapy, epigenetic modifiers, protein kinase inhibitors, dysregulated pathway inhibitors, and immunotherapy. Adapted from the following references (Castelli *et al*., 2016; Allis & Jenuwein, 2016; Corces *et al*., 2017; Megias-Vericat *et al*., 2019; Sillar & Enjeti, 2019; Ghosh *et al*., 2020)

1.4.2 Differentiation therapy

One of the important hallmarks of cancer is the poor differentiation of cancer cells, and differentiation therapy is key for cancer treatment (Yan & Liu, 2016). In differentiation therapy, instead of killing cells, cells are coaxed into over-riding the differentiation block and attaining a non-proliferative state. This override occurs by reactivation of endogenous differentiation programs, and in turn, cancer cells are able to resume maturation and elimination of tumour phenotypes in the process (Yan & Liu, 2016). The advantages of differentiation agents is that they tend to have less toxicity than conventional cancer treatments (de Thé, 2018; Yan & Liu, 2016).

One of the successful stories in differentiation therapy was seen in the treatment of APL. First described in 1957 by Leif Hillestad in Sweden (Degos, 2003), APL is characterised by the reciprocal translocation between chromosomes 15 and 17 and the product of this translocation is two fusion proteins (PML-RARα; RARα-PML). The accumulation of the fusion product leads to a differentiation block at the promyelocytic stage as a result of the inhibition of the retinoic acid receptor alpha (RARα) with a block in RARα-dependent transcription (Melnick & Licht, 1999; Nowak *et al*., 2009; de Thé, 2018). This inhibition is exacerbated by the formation of PML-RARα homodimers and the binding of the fusion protein to retinoic acid receptor elements (RAREs) of target genes, with subsequent epigenetic changes and recruitment of corepressors. Such corepressors include histone deactylases (HDACs) and histone methyl transferases. This results in sequestration of both retinoic acid X receptor (RXR) and wild type PML, leading to a halt in differentiation (de Stanchina *et al*., 2004; Rush *et al*., 2013; Nowak *et al*., 2009) and maintaining chromatin in a densely packed, inactive state (Noguera *et al*., 2019). PML–RARα expression also disrupts the formation of nuclear structures known as PML oncogenic domains (PODs) or PML

nuclear bodies (PML-NB). These in turn result in blunting of p53 activity/function (de Stanchina *et al*., 2004), since it is thought that p53 is channelled through PML NBs. Thus, they may represent sites for p53 post-translational modifications and ultimately determine its activation status (Garcia and Attardi, 2014). Although PML is required for efficient induction of p53 targets such as *BAX* or *CDKN1A*, *in vivo* evaluation using transcriptomic approaches have not been successful in identifying a defective p53 signature in *PML* null mice (Niwa-kawakita *et al*., 2017).

1.4.2.1 All-*trans* retinoic acid

One of the key discoveries in differentiation therapy is the vitamin A derivative, all-*trans* retinoic-acid (ATRA) (de Thé, 2018). ATRA mediates rapid differentiation of immature myeloid cells and is used as a primary therapeutic agent in the treatment of APL. Early clinical trials using ATRA for the treatment of APL reported complete haematologic remission rates of more than 90% of newly diagnosed cases with *in vitro* demonstration of differentiation of APL blast cells (Massaro *et al*., 2016). Subsequently, disease free survival and overall survival were significantly improved with combination therapy using ATRA and cytotoxic chemotherapeutic drugs including idarubicin or daunorubicin with cytarabine (Sanz *et al*., 2019). However, 14-16% of patients treated with ATRA may present with a retinoic acid-APL syndrome and experience life threatening clinical manifestations characterized by respiratory complications including interstitial pulmonary infiltrates, pleural or pericardial effusions, and fever (Patatanian & Thompson, 2008).

ATRA therapy results in the differential regulation of hundreds of genes involved in myeloid differentiation (Nowak *et al*., 2009). This includes the downregulation of *c-MYC* and upregulation of the differentiation-associated TF genes C/EBPε and PU.1, leading to

transcriptionally active chromatin. Also, pharmaco-therapeutic concentrations of ATRA result in the release of corepressors and degradation of the PML-RARα fusion protein product (Fukuoka *et al*., 2019). With ATRA therapy established as standard for induction of newly diagnosed patients with APL, further research for identifying other compounds that can tackle ATRA resistance became necessary.

Another compound that has been successfully used alone or in combination with ATRA for the treatment of APL is arsenic trioxide (ATO/As_2O_3) (Falchi *et al*., 2016). This 2000-year-old drug was reintroduced by Chinese studies for the treatment of APL and has been very effective in relapsed cases (Li *et al*., 2014). It can induce complete remission when used with or without chemotherapy or ATRA. *In vitro* studies on leukaemia cell lines have demonstrated that, at low concentrations, ATO causes partial morphologic differentiation and arrest of the APL cell line NB4 (Zhou *et al*., 2007). It also accumulates promonocytic leukaemia U937 cells at the G2/M phase of the cell cycle through SUMOylation of PML-RARα (di Masi *et al*., 2016). SUMOylated PML recruits RING finger protein 4 (RNF4), and polyubiquitylated PML-RARα can be degraded by the ubiquitin–proteasome pathway (Lallemand-Breitenbach *et al*., 2008). Theoretically, the degradation of PML/RAR*α* re-establishes the functional active sites while suppressing the effect of this translocation on the myeloid differentiation block with resultant activation of differentiation related genes and signalling pathways. These include the PML nuclear body and transforming growth factor beta (TGFβ) signalling pathway, that are crucial factors controlling the proliferation, survival and differentiation of haematopoietic cells (Liu *et al*., 2017).

Higher doses of ATO result in commitment of cells to apoptosis through downregulation of anti-apoptotic genes including the B Cell Lymphoma 2 (*BCL2*) gene (Kumar *et al*., 2014) and cell

proliferation genes such as Wilm's tumour (WT1) (Glienke *et al*., 2006). This leads to the subsequent release of pro-apoptotic factors from the mitochondria to the cytoplasm followed by activation of caspases 3 and 8 and degradation of specific substrates (Gupta *et al*., 2009). ATO elicits the formation of reactive oxygen species (ROS) by binding of arsenic atoms to cysteines of many target proteins, resulting in mitochondrial enzyme poisoning (Kumar *et al*., 2014).

1.4.2.2 Other differentiation-inducing agents

Early *in vitro* and *in vivo* studies demonstrated the ability of the physiologically active form of vitamin D, 1,25 dihydroxy vitamin D3 (VITD3) to elicit differentiation of myeloid cells (Di Rosa *et al*., 2011). Early studies showed encouraging anti-proliferative and apoptotic effects of VITD3 in cancer cells lines (Simboli-Campbell *et al*., 1996), however, it failed to induce terminal differentiation of blast cells in AML patients and was deemed not as effective as ATRA in treatment of APL (Nowak *et al*., 2009). VITD3 works by binding and subsequent activation of the vitamin D receptor (VDR). Following activation, VDR heterodimerises with RXR and in turn, binds to vitamin D responsive elements (VDREs) in the promoter region of target genes (Nurminen *et al*., 2019).

The amount of VITD3 required to induce terminal maturation of AML cells can cause lethal hypercalcemia *in vivo* (Kulling *et al*., 2017). To counter the side effects, VITD3 derived analogues were developed, and even though they are characterized by lower calcaemic side effects and stronger antineoplastic effects, clinical trials have shown overall poor results (Leyssens *et al*., 2013).

1.4.3 Epigenetic modifying agents

Epigenetic modifications comprise a class of heritable, non-genetic changes in gene expression. These modifications include DNA methylation, histone modification, and chromatin remodelling (Goldman *et al*., 2019). In healthy haematopoietic stem cells, epigenetic modifications are crucial for maintaining normal cell differentiation and haematopoiesis (Ostrander *et al*., 2020). In AML, epigenetics plays an important part in the transformation of normal haematopoietic precursors into their malignant counterpart and treatments targeting the epigenetic mechanisms in AML have been thoroughly investigated.

1.4.3.1 Histone methyltransferase inhibitors

Histone methyltransferases play an important role in gene regulation (Cheng *et al*., 2019). They can add a methyl group or an arginine residue which can result in either gene transcription or repression depending on the location in which this group or residue is added (Bannister & Kouzarides, 2011). An example of a histone methyltransferase is the Disruptor of telomeric silencing 1 (DOT1L). DOT1L targets the histone H3 lysine 79 position, or H3K79. Dysregulation of DOT1L leads to elevated H3k79 methylation and this has been implicated in the development of leukaemia with translocations of the *MLL* gene (Wood *et al*., 2018). Treatments that can selectively target and inhibit histone methyltransferases are thus of interest. Several DOT1L inhibitors have shown the ability to inhibit the histone methyltransferase, however, they lack optimal pharmacokinetic properties (Goldman *et al*., 2019).

1.4.3.2 Histone deacetylase inhibitors

Histone acetylation and deacetylation regulate genes by acetylation and deacetylation of lysine residues within the N-terminal tail protruding from the histone core of the nucleosome (Fu *et al*., 2017). Histone acetylation usually results in transcriptional activation and gene expression, while deacetylation has been linked with transcriptional deactivation and gene silencing (Zhang *et al*., 2015). Changes in histone acetylation can contribute to dysregulation of the cell cycle with an effect on cell differentiation, apoptosis, and angiogenesis, leading to cancer (Audia & Campbell, 2016). Histone deacetylase inhibitors (HDACi) can function by inhibiting G1- to S- phase transition of the cell cycle, induce mitochondria driven apoptosis, cause accumulation of DNA defective cells at M phase of the cell cycle (Bose *et al*., 2014), and induce cell differentiation (Vitkeviciene *et al*., 2019). They limit excessive deacetylation of histones and transcription factors, particularly those that regulate tumour-suppressor genes (Goldman *et al*., 2019). HDACi are useful in clinical treatments and can be used as adjuvant therapy and enhance selectivity against cancerous cells versus healthy cells (Suraweera *et al*., 2018).

1.4.3.3 DNA methyltransferase inhibitors

In AML, DNA hypermethylation of gene promoters is very common and often correlates with differentiation block. A typical DNA methylation signature of AML shows both hypermethylation and hypomethylation of Cytosine-Guanine oligodeoxynucleotides (CpG) islands in promoter regions and results in repression of tumour suppressor genes (Przybilla *et al*., 2017). The fact that this repression is reversible prompted extensive studies on using DNA methyltransferase inhibitors (DNMTi) in the treatment of AML among other cancers (Figueroa *et al*., 2010; Yang *et al*., 2010). Two of the most common inhibitors is 5-azacitidine (AZA) and 5-

aza-2'deoxycytidine (DAC). Both agents affect human AML cell lines by inducing DNMT methyltransferase degradation, DNA hypomethylation and damage (Przybilla *et al*., 2017). The disadvantage of these epigenetic drugs is that they do not act on specific hypermethylated gene promotor regions and upon withdrawal of treatment, hypermethylation of gene promoters is restored within weeks (Przybilla *et al*., 2017). Thus, patients require continuous cycles of the drugs to induce complete remission.

1.4.4 Tyrosine Kinase inhibitors

Protein kinases represent a family of enzymes that control intracellular signalling pathways (Megias-Vericat *et al*., 2019). Overexpression or gene mutations in tyrosine kinases (TK) could lead to the constitutive activation of membrane receptors or cytoplasmic TKs and in turn promote survival of cancer cells (Arshad *et al*., 2020). An example of a tyrosine kinase inhibitor target is that of the FMS-like tyrosine kinase FLT3. It is one of the most frequent somatic mutations in AML (Daver *et al*., 2019). Patients presenting with FLT3 mutations have a high relapse rate with a short remission period and reduced chances of survival after chemotherapy and transplantation (Larrosa-Garcia & Baer, 2017).

FLT3 encodes a membrane-bound receptor tyrosine kinase (RTK) that belongs to the RTK subclass III family and is typically expressed in haematopoietic progenitors and normally promotes haematopoietic development (Grafone *et al*., 2012). It plays a pivotal part in the proliferation, differentiation, and survival of multipotent stem cells. FLT3 mutations are found in approximately 20- 45% of cases of adult AML (Daver *et al*., 2019). They can be due to an internal tandem duplication (ITD), referred to as FLT3-ITD or activating missense point

mutations. FLT3-activating mutations have been shown to reduce the requirement for growth factors with increased cell proliferation, and leukaemic transformation. Over-expression of *FLT3* results in a block in differentiation and down regulation of myeloid TF expression (Kavanagh *et al*., 2017). Drugs specifically targeting FLT3 have been developed and are essential in AML cases with drug resistance (Daver *et al*., 2019). However, drug resistance remains a problem and limits the efficacy of the drug and combinations of FLT3 inhibitors together with epigenetic therapies, proteasome inhibitors, downstream kinase inhibitors, phosphatase activators and other drugs are being explored (Larrosa-Garcia & Baer, 2017). Other TK mutations include KIT and Janus kinase 2 (*JAK2*) and treatment targeting these mutations is important since their presence at diagnosis is a marker of poor outcome (Megias-Vericat *et al*., 2019).

1.4.5 Targeting transcription factors

Recently, therapeutic strategies targeting TFs have been described (Takei & Kobayashi, 2019). Small molecules that can induce the expression of TFs such as C/EBPα, commonly mutated in AML, have been described in AML cell lines and primary AML cells (Namasu *et al*., 2017). PU.1, a TF dysregulated in about 40% of AML patients, has also been targeted by small molecules (Anthony-Debre *et al*., 2017). Another TF that is up-regulated in AML is Cyclic AMP response element-binding protein (CREB). CREB inhibitors have been shown to suppress leukaemia cell proliferation in KG-1 acute myeloid leukaemia cell line (Mitton *et al*., 2016). Other therapeutic strategies include complex formation inhibitors of TFs including inhibition of *c-MYC* DNA-complex formation (Carabet *et al*., 2018), and inhibition of p53- mouse double minute 2 homolog (MDM2) complex formation (Sanz *et al*., 2019).

1.4.6 Targeting the apoptotic pathway in AML

The difficulty of targeting molecular mutations associated with AML has led to exploring ways to target pathways downstream of genetic mutations (Sillar & Enjeti, 2019). One of the most common dysregulated pathways in AML is the apoptotic pathway (Sillar & Enjeti, 2019). The BCL-2 family of proteins play a crucial role in regulating mitochondria-mediated apoptosis. They maintain the survival of cells and mitochondrial outer membrane integrity by preventing the activation of pro-apoptotic BCL2 Associated X (*BAX*) and BCL2 homologous antagonist/killer (*BAK*). Once activated, they form oligomers and result in permeabilization of the outer mitochondrial membrane, leading to a cascade of events that ultimately result in cell death (Flores-Romero *et al*., 2019).

BCL-2 is frequently overexpressed in AML in comparison to normal haematopoietic cells. Naturally, this prompted targeting *BCL-2* as a therapeutic approach for AML (Kavanagh et al., 2017). *BCL-2* inhibitors have shown activity as stand-alone agents or in combination with other drugs (Kavanagh *et al*., 2017). It is however expressed in both AML blasts and normal cells, and one would assume that such treatment would not be totally selective. However, AML has more 'primed' *BCL-2* in the mitochondrion when compared to normal cells, making *BCL-2* inhibitors more effective on AML (Konopleva & Letai., 2018).

1.4.7 Immunotherapy

Immunotherapy has become an important approach towards the treatment of cancer patients (Hu et al., 2019). This is achieved by helping the patient to boost his/her own immune system (Rothlin & Ghosh, 2020). Natural killer (NK)cells play a pivotal role in identifying cancer cells and even leukaemic stem cells. Their mode of action involves forming synapses with cancer cells and emptying their enzymatic contents, leading to cell lysis (Ben-Schmuel *et al*., 2020). It has been beneficial in increasing the clinical remission rates by eradicating minimal residual disease after chemotherapy or targeted therapy (Lin & Li., 2013). The optimal immunotherapy targets antigens that are usually exclusively expressed by leukaemia cells and are absent in normal tissues (Lin & Li, 2013).

Even though immunotherapy offers promising results, further studies are needed to overcome clinical problems including autoimmune cytotoxicity. Whilst immunotherapy is promising for treatment of leukaemia patients, it has its limitations in treatment of solid cancers. Limitations include difficulty to reach the tumour milieu in which the cancer microenvironment favours a suppressed immune surrounding (Ben-Schmuel *et al*., 2020).

1.4.8 Vaccines

The use of vaccines as targeted therapy to treat AML has been researched heavily in preclinical and clinical trials. Even though patients in clinical remission are usually immunocompromised, vaccines can mount an immune response against AML cells while circumventing normal cell death (Alatrash & Molldrem, 2011).

Peptide vaccines have been developed against leukaemia-associated self-antigens. This therapeutic approach is limited because of major histocompatibility complex (MHC) class or human leukocyte antigen (HLA) haplotype restrictions in the patient population. These vaccines are also limited in their ability to activate $CD4^+$ T helper responses and inability of maintaining long-term memory responses against antigens with DNA vaccines presenting as an alternative strategy for peptide vaccination. (Walters *et al*., 2017).

DNA vaccines combine live replicating vaccines that induce broad cellular and humoral immune responses with the safety and ease of manufacturing a non-live, non-spreading platform (Weiner & Nabel., 2018). Stimulation of innate immunity is triggered by the bacterial DNA backbone and is the primary step in the induction of immunity against weak tumour antigens. However, effective immune responses against leukaemic cells is impeded by the weak immunogenicity of cancer cells. Ongoing clinical trials are targeting ways to increase leukaemia cell immunogenicity and enhance delivery systems (Riley *et al*., 2019).

Recently in the USA, a solid vaccine was tested for its efficacy in eliminating leukaemia cells in mice. In principle, the vaccine contains biomolecules that are embedded in a scaffold. These molecules attract the body's dendritic cells and activate them, along with antigens that exhibit specificity to AML cells. This results in the activated dendritic cells taking up the antigens from the vaccine site and presenting them to T cells. These in turn are triggered to mount a response against the AML cells. Interestingly, vaccines with no AML antigen in them were just as effective at providing protection as vaccines containing either AML cell contents or WT-1 peptide (The Harvard Gazette, 2020).

1.4.9 Antibodies

Monoclonal antibodies (mAbs) have enhanced the therapeutic approach for numerous diseases including AML. Antibody therapeutic approaches in AML treatment include antibodies that facilitate the body's own immune cell response. Natural Killer (NK) cell antibody-dependent cell-mediated cytotoxicity (ADCC) engages either NK cells or T-cells to redirect cytotoxicity against AML targets in a highly efficient manner; and toxin conjugated antibodies that combine a cytotoxic drug or a radioactive isotope to a targeted antibody (Williams *et al*., 2019). Antibody therapy targeting in AML includes targeting surface marker antigen expression, checkpoint inhibitors and microenvironment targeting. These include antibodies against surface antigens such as CD33 (e.g. lintuzumab), antibodies conjugated to toxins or radioactive particles in various anti-CD33, anti-CD123, and anti- CD45 formulations (Williams et al., 2019; Assi et al., 2018). Even though significant benefits in clinical outcomes were reported, treatment-related toxicities have provided a setback in their progress, with most of the adverse clinical manifestations occurring as a result of the dysregulation in the immune system balance as a result (Pardoll, 2012). Also, challenges remain because of the biology of cancers. AML exhibits a dysfunctional and suppressive immune compartment (Epperly *et al*., 2020) and the expression of somatic mutations or mutation burden that can be recognized as non-self, is relatively low (Lawrence *et al.*, 2013).

1.4.10 T-cell therapies

Adoptive immunotherapy is based on *ex vivo* engineering and expansion of immunologically active T cells. Once isolated, the cells are infused into the patient with the aim of inducing direct cytotoxicity against cancer cells. T cells with engineered chimeric antigen receptors (CAR-T cells) are a novel immunotherapeutic strategy in treatment of AML (Hofmann *et al*., 2019). However,

only a few clinical trials have been investigated for AML treatment (https://clinicaltrials.gov/ct2/results?cond=amL&term=car&cntry=&state=&city=&dist=)(Acessed on 23rd March, 2020) .

Despite the success of CD19-CAR-T cells in B-cell malignancies, studies of antibody targeted CAR-T cells in AML have been less prominent, primarily owing to the AML microenvironment that reduces the antitumor activity of CAR T cells (Epperly *et al*., 2020). Thus, different strategies are being sought with alternative CAR-T cell targets whereby a combinatorial effect of CAR-T cells with antibodies such as check-point inhibitors or other pharmacological products may result in improved therapeutic results (Bachmann, 2019).

1.5 Natural products and cancer therapeutics

Natural products from plants, animals, and micro-organisms have always played a pivotal role in the treatment of human diseases, including cancer (Calixto, 2019). Nowadays, technological advances paved the way for a more intense search towards natural product discovery. This led to the development of open access applications and analysis systems to identify interesting compounds based on their chemical structures. A few examples are ChemGPS-NP and TCMAnalyzer (Saldívar-González *et al*., 2018).

With chemotherapy as the standard treatment for cancer and approximately 80% to 90% of cancer deaths directly or indirectly attributed to drug resistance, the search for new drugs has become even more important (Yuan *et al*., 2017). It is broadly accepted that overcoming resistance to drugs will never be achieved through the use of single drug or a combination of drugs that target a single gene or pathway and that natural products can be a very good source of compounds in the fight against cancer (Chamberlin *et al*., 2019). Most biologically active natural products are secondary metabolites, and the fact that these metabolites evolve in response to challenges, to protect the species from the hostile environment, makes them attractive candidates as cancer therapeutic drugs (Matulja *et al*., 2020).

1.5.1 Plant derivatives

The plant kingdom is comprised of thousands of plant species and only around 10% have been studied and tested for treating different diseases. Even though only a small percentage has been studied, more than 60% of clinically approved anticancer drugs are derived from

plants (Choudhari *et al*., 2020). Plant products such as alkaloids and other compounds isolated from plants, play significant roles in cancer therapy with anti-proliferative and apoptotic properties (Kowalczyk *et al*., 2016; Petruczynik *et al*., 2019).

Examples of plant-derived anticancer drugs include Inhibitors of topoisomerase I such as camptothecins (isolated from the bark of a tree native to China), and of topoisomerase II such as epopodophyllotoxins (isolated from the roots of the mayapple plant). These drugs are active in several solid tumours and haematological malignancies (Jain *et al*., 2017). Polyphenols such as gossypol (isolated from cottonseeds and roots) have been found to induce apoptosis through generation of reactive oxygen species (ROS) (Cao *et al*., 2018).

Microtubule formation-inhibiting drugs represent some of the most effective anticancer drugs. They exert their effect by binding to tubulin, and disrupting the spindle apparatus (Stanton *et al*., 2011). They are classified as microtubule stabilizers or destabilizers (Bates & Eastman, 2017). The most common microtubule inhibiting drugs are the vinca alkaloids and the taxanes (van Vuuren *et al*., 2015). Vinca alkaloids were primarily isolated from the pink periwinkle plant (*Catharantus roseus*) (Moudi *et al*., 2013). Even though vinca alkaloids are structurally close, their effects depend on the cancer cell type (Amin *et al*., 2009). One important alkaloid is vincristine. It is a chemotherapeutic drug that is very effective in treating leukaemias, lymphomas and sarcomas (Martino *et al*., 2018). A structurally related drug, vinblastine, is effective in treating solid tumours (Lee *et al*., 2015). Extracts containing polyphenols, flavonoids and anthraquinones have also been described in the treatment of cancer with some also acting as epigenetic modifying agents (Saldívar-González *et al*., 2018).

1.5.2 Micro-organisms

Actinomycin D, a polypeptide antibiotic isolated from the genus *Streptomyces*, was the first microbial agent tested for its anti-tumour efficacy (Farber *et al*., 1960). Actinomycin D intercalates into DNA, preventing the progression of RNA polymerases and it is toxic at high concentrations (Liu *et al*., 2016).

A very interesting approach to therapy is by live cancer-targeting bacteria. Once virulence has been minimised (Kim *et al*., 2015), many facultative or obligate anaerobic bacteria exhibit fundamental tumour-targeting and tumour-killing activities (Duong *et al*., 2019). Live tumour-targeting bacteria ideally would selectively colonize tumours, inhibit tumour growth, and enhance the effect of chemotherapeutic drugs while reducing any harm to the host cells (Duong *et al*., 2019).

Even though a well-known attenuated *Salmonella Typhimurium* strain (VNP20009) showed strong inhibitory effects on tumour proliferation in mouse models (Wang *et al*., 2016), however, early clinical trials in aggressive cancers showed modest results in cancer regression (Toso *et al*., 2002). It has also been associated with increased morbidity associated with liver disease in mouse models (Coutermarsh-Ott *et al*., 2017). Attempts to maximize bacterial tumour colonization and anticancer effects continue to be investigated (Duong *et al*., 2019).

Anthracyclins are microbial derivatives and they have a prominent role in cancer treatment (McGowan *et al*, 2017). The four most common anthracyclines are doxorubicin, daunorubicin, epirubicin and idarubicin and they been used to treat haematological malignancies, carcinomas,

and soft tissue sarcomas. Anthracyclines cause inhibition of topoisomerase II, nuclear DNA damage, and induction of ROS, leading to cell death (Marinello *et al*., 2018). However, anthracycline treatment can lead to cardiac and renal toxicity (Neuendorff *et al*., 2020). Novel anthracycline derivatives are continuously being tested for their anticancer efficacy and increased selectivity (Marinello *et al*., 2018).

1.5.3 Marine organisms

Marine organisms represent a large biodiversity of multicellular organisms with more than two million species formally described. Marine natural products are one of the most important sources of bioactive substances including those from marine bacteria, fungi, sponges, cniderians, molluscs, tunicates, and echinoderms amongst others (Blunt *et al*., 2018). Despite challenges to ensure enough supply, marine-derived active compounds and secondary metabolites are being explored as sources for anticancer, antimicrobial, antiviral and anti-inflammatory drugs (Barzkar *et al*., 2019). To date, thousands of new compounds have been isolated from marine species and many of these are bioactive secondary metabolites with valuable therapeutic potential (Khalifa *et al*., 2019). What makes marine organisms interesting as a source of anticancer drugs is the fact they live in hostile environmental conditions including extreme temperature, salinity, pH changes. They also need to protect themselves from predators which encourages them to produce bioactive secondary metabolites as a form of defense mechanism (Raimundo *et al*., 2018).

Extracts from several marine organisms show anti-proliferative and cytotoxic effects on different cancer cell lines (Isbilen *et al*., 2018). Marine sponges and their microbial associates produce a vast number of pharmacologically and chemically diverse compounds such as peptides, alkaloids, steroids, terpenoids and sesquiterpenes (Anjum *et al*., 2016). These

compounds exhibit various biological activities including antioxidant, antidiabetic, anticancer, antihypertensive, antiviral, anti-obesity, and anti-proliferative properties. (Yun *et al*., 2019).

The first successful marine-drug was isolated from a Caribbean sponge named *Cryptotethyacrypta.* The isolated nucleoside was spongothymidine and its derivative, Ara-C (also known as 1-beta-d-Arabinofuranosylcytosine or cytarabine) was approved by the United States Food and Drug Administration (FDA) and is currently in use for the treatment of leukaemia and other cancers (Ercolano *et al*., 2019).

Many marine-derived compounds have promising pharmaceutical potential and have been included in clinical trial phases. Ziconotide, a venom from cone snails was one of the first FDA-approved intrathecal infusion formulations for the treatment of severe, chronic pain (Smith & Deer, 2009). Kahalalide from green algae is a peptide that has been tested for its anticancer properties (Wang *et al*., 2015). Synthetic drugs isolated from sponges have shown potent anti-leukaemic activity in *ex vivo* and *in vitro* experiments against AML (Ercolano *et al*., 2019). Eribulin mesylate is a synthetic product isolated from the Poriferan Halichondria okadai. The drug is used to treat breast cancer (Dybdal-Hargreaves *et al*., 2015). Synthetic marine-derived alkylating agents extracted from tunicates are currently being tested in randomized phase III clinical trials in many countries (Yun *et al*., 2019). Steroidal extracts from sea urchins have been shown cytotoxic effects when tested against several human cancer cell lines including HL-60 cells (Thao *et al*., 2015).

To date, clinical trial studies with isolated compounds are not sufficient. This could be due to bureaucracy with obtaining the necessary technical and ethical approvals, recruitment of volunteers, or obtaining sufficient quantities of the metabolite of interest. In the future, genetic technology would allow easier isolation and biosynthesis of marine natural products.

1.6 What is regeneration?

Regeneration is best described as a homeostatic process of renewal that involves a well-balanced and coordinated process of restoration of lost or damaged cells, tissues and organs. Once regeneration is successful, the organism regains full structural and functional integrity of the lost or damaged part. It is characterised by tightly controlled and orderly overlapping processes of inflammation, tissue reconstruction and remodelling (Charni *et al*., 2017). This process does not just orchestrate the restoration of lost tissue but must also integrate the newly regenerated tissues with the pre-existing one. Moreover, regeneration must be able to have an orderly control to initiate and maintain signals that are capable of controlling the cell cycle and correct or eliminate possible mutations. This also involves spatio-temporal recognition of the missing parts that need to be replaced. Once regeneration is complete, the organism needs to ensure that proliferative signals are switched off because failure to do so would lead to abnormal tissue repair, fibrosis, and uncontrolled cell growth leading to cancer (Oveido & Beane 2009).

In humans, the ability to replace cells within a specific tissue is limited. With the exception of finger tips and the liver, humans are unable to regenerate complex structures that combine the structure and function of multiple tissues (Shieh & Chang. 2015). In humans, limited repair to tissues such as bone, peripheral nerves, and skin cells can occur following trauma, however, these mechanisms are not as efficient and error free as the regenerative abilities in certain animals. Interestingly, even though these organisms are far superior in their regenerative capacities when compared to humans, the signalling pathways that regulate regeneration,

wound healing, and cancer are highly conserved in both humans and organisms with regenerative capabilities (Makanae *et al*., 2014).

It is well known that species such as hydra, planarians, echinoderms, annelids and amphibians, can completely regenerate several tissues, organs, or even whole-body parts (Tanaka & reddien, 2011). Different organisms exhibit either morphallactic or epimorphic regeneration or a combination of both (Londono *et al*., 2018). In morphallactic regeneration, such as that seen in hydra, the remaining part of the amputated organism is remodelled to regenerate the missing parts of the body (Agatha *et al*., 2007). In contrast, in epimorphic regeneration such as limb regeneration in axolotls, the amputated structure forms a stump which in turn provides stem/progenitor cells that will form a bud called the blastema. The interaction of the blastema with underlying cells initiates a program of de- and re-differentiation of cells at the wound site with missing tissue structurally and functionally restored (Bely & Nyberg 2010). Regeneration in flatworms such as planaria displays both epimorphic regeneration with the formation of a blastema, and morphallactic regeneration with remodelling of the remaining tissue to achieve full regeneration following amputation (Ivankovic *et al*., 2019). Extensive research has already elucidated the mechanisms behind the ability of these organisms to regenerate body parts (Pinsino *et al*., 2007).

1.6.1 Regeneration and Cancer

In 1935, Waddington postulated the existence of 'individuation fields', agents which control the growth of different parts of an organism in a flawless way so that a normal individual is formed (Waddington, 1935). Extending this knowledge to the behaviour of cancer cells, these individuation fields of the 'epigenetic landscape', were thought to maintain their control over

normal cells, and the escape of these cells from its influence may lead to cancer. In turn, if the cancer cells were exposed to these individuation fields, they can be brought back under control. This theory might be supported by the observation that spontaneous tumours are rarely observed in animals which are capable of regeneration (Oviedo & Beane, 2009). However, it cannot be true for all regenerating organisms since the reactions of planarians and axolotls to carcinogen exposure are different. Whereas axolotls resisted cancer formation, exposure of planarians to carcinogens induced tumour formation (Van Roten *et al*., 2018). This could be explained by the fact that planarians have a single *P53* gene (Pearson & Alvarado, 2010). Furthermore, the tumour microenvironment plays a pivotal role in carcinogenesis, whereby there is direct interaction between the tumour and the microenvironment in which it resides (Foster. 1963; Ingram. 1971; Quail & Joyce, 2013).

Dvorak (2015) best described cancers in relation to wound healing as 'tumours being 'wounds that never heal'. Indeed, growing evidence strongly suggests that tissue regeneration, wound healing and carcinogenesis share common signalling pathways that involve increased proliferative capacity and survival as well as inflammatory and angiogenic processes. However, in contrast to wound healing and regeneration, cancer is not self-limiting with uncontrolled cell proliferation, invasion, and metastasis being hallmarks of cancer cells (Charni *et al*., 2017).

1.7 Planarians

There are several hundred planarian species worldwide. There are marine, fresh water and terrestrial planarians and their regenerative abilities vary greatly (Sluys and Riutort, 2018; Vila-Farré and Rink, 2018). Planarians including *Schmidtea mediterranea* or *Dugesia japonica* are able to regenerate the whole body as opposed to planarians with decreased regenerative abilities such as *Dendrocoelum lacteum*, or the planarian *Bdelloura candida* with limited regenerative capabilities (Vila-Farré and Rink, 2018). Planarians belong to the taxonomic class Turbellaria (or flatworms) which belongs to the phylum Platyhelminthes.

Planarians possess three tissue layers, bilateral symmetry, and a bulk of sensory organs concentrated at the head end. Their brain is attached to nerve cords and they possess two eye spots (Umesono and Agata, 2009). They also possess a simple intestinal system that is highly branched, an excretory system and a reproductive system consisting mainly of a pair of ovaries, testes, and yolk glands with the copulatory organs located at the tail end (Sluys and Riutort, 2018). They can produce sexually and/or asexually (Vila-Farré and Rink, 2018). They feed via a muscular pharynx which is the sole opening that also functions as the anus of the animal. They lack both circulatory and respiratory systems and vary in size from a few millimetres to more than one metre, depending on the species (Sluys and Riutort, 2018).

Planarians have long been investigated in the fields of developmental and regenerative biology (Elliot & Sanchez-Alvarado, 2013). They have also triggered a huge interest in neuroscience and the study of neurodegenerative disorders due to their ability to regenerate

new neurons (Ross *et al*., 2017). They are highly resilient organisms that are able to regenerate completely even from the smallest of fragments (Elliot & Sanchez-Alvarado, 2013). This regenerative capability is attributed to a specialised group of adult stem cells called neoblasts that are spread throughout the bodies of these worms, save the tip of the head region and the centrally located pharynx (Rink, 2013). These proliferative stem cells are essential for physiological tissue homeostasis and post-injury regeneration in planarians (Lei *et al*., 2016). Neoblasts exhibit large nuclei and little cytoplasm and are the only identified somatic cells with mitotic abilities in the adult planarian, comprising approximately a third of the cells in an adult worm. They often possess filopodia-like extensions with prominent RNA/protein granules (chromatoid bodies) that share similar morphological and molecular characteristics to the RNA/protein granules found in the germ cells of many animals (Ivankovic *et al*., 2019).

Planaria regeneration is a typical example of the overlapping of 'epimorphosis' in the form of *de novo* tissue remodelling, and 'morphallaxis' by remodelling of existing tissue (Agata et al., 2007; Ivankovic *et al*., 2019). Following amputation of a planarian, a blastema is formed at the injury site and this gives rise to the lost body parts (Reddien & Sánchez Alvarado, 2004). Unlike the axolotls, the blastema does not have cells that undergo de-differentiation. In planarians, the de-differentiation stage is absent and mitotically active immature neoblasts, peaking early on after wounding, rapidly migrate to the wound epithelium. If the wound requires the replacement of missing tissue, a second surge of neoblast proliferation occurs (Scimone *et al*., 2014). Cells called rhabdites migrate to the injury site and release their mucoid contents producing a protective mucosal covering. The released contents possibly have immunological functions that aid in wound healing and regeneration (Hayes, 2017). The fragment containing the head will continue to move, whilst the trunk end remains stationary during regeneration. A

thin layer of epithelium covers the wound early on following amputation and the process does not involve proliferation of cells but rather by cell spreading. The spreading involves both dorsal and ventral epithelial cells, which in turn lose their characteristic morphology to enable them to cover the wound. Unlike in humans, scarring does not seem to occur in planarians. This ensures that the epithelium is in direct contact with tissues at the site of amputation, which is important for successful regeneration (Karami *et al*., 2015).

Regenerative tissues are exceptionally resilient to carcinogenic compounds, making the understanding of the process of regeneration and the regenerative niche a useful research tool for the development of potential oncology drug targets (Oviedo and Beane, 2009). Experimental near-lethal irradiation and eradication of planarian neoblasts results in failure of regeneration with the injection of healthy donor neoblasts successfully enriching the neoblast population and re-establishing regenerative capacity (Lei *et al*., 2016).

Several toxic compounds including microplastics and their effects on neoblasts and planaria regeneration have been studied (Gambino *et al*., 2020; Hagstrom *et al.*, 2018). Results from early studies reveal that when exposed to carcinogenic agents, neoblasts respond by forming benign and malignant growths (Foster, 1963). It was observed that when planarians are subjected to long term carcinogen exposure, the DNA repair mechanisms responses in regenerating planaria are better than non-regenerating planarian control groups (Oviedo & Beane, 2009). It was suggested that planarians are able to evade tumorigenesis, and the increase in proliferation of planarian stem cells is more a controlled repair mechanism as opposed to uncontrolled proliferation as seen in cancer (Van Roten., 2018).

Data from knockdown experiments using planaria as a study model to understand cancer biology and characterisation of neoblasts reveals a myriad of genes and protein products that are implicated in regeneration (Onal *et al*., 2012). Many of these are highly conserved mechanisms and signalling pathways. Several genes that maintain pluripotency in mammalian embryonic stem cells (ESCs) and neoblasts from planarians, together with expression of epigenetic regulators and post-transcriptional regulatory genes characteristic of ESCs and neoblasts, are conserved (Onal *et al*., 2012). Also, mass spectrophotometric studies revealed several interesting pro hormones and peptides that are implicated in regeneration (Ong *et al*., 2016).

Gene knockdown studies and gene expression analysis aided in decoding the regenerative capabilities of planaria (Roberts-Galbraith *et al*., 2016). Also, atlases from single-cell sequence analysis have revealed previously uncharacterised cell types expressing genes with no apparent homologs in other phyla (Plass *et al*., 2018). Protein databases have also been constructed from sequenced planarian transcripts (Castillo-Lara & Abril, 2018).

Genome assembly studies reveal a highly polymorphic and repetitive genome of planarians *Schmidtea meditteranea* (61.7%) when compared to humans (46%). Using stringent basic local alignment search tool (BLAST) protein alignments, 11584 planarian transcripts (52%) with human homologues were identified (Onal et al., 2012). With the genome of planarians including *Schmidtea mediterranea* already sequenced and containing more than 20,000 genes with protein products (Adamidi *et al*., 2011) and the identification of major cell types and pathways (Swapna *et al*., 2018), together with the development of web applications such as PlanNET that predicts important protein-protein interactions (Lara & Abril., 2018), the

planarians offer an interesting model not only for the understanding the pathways linking cancer and regeneration but the identification of interesting molecules that can be isolated and tested for their effect on cancer cells. In fact, metabolomic studies in planarians revealed the presences of several bioactive molecules that could be of interest including amines, thiols, and nucleotides (Natarajan et al., 2015).

1.8 Salamanders and regeneration: The story of the Axolotls

Salamanders are one of the oldest laboratory animal models used for research experiments. In 1768, Lazzaro Spallanzani first described limb regeneration in axolotls (Nowoshilow *et al*., 2018). The axolotl belongs to the class *Amphibia* family: *Ambystomatidae*. Described originally as larvae, they were later classified as adults when it was observed they were able to reproduce (Suleiman *et al*., 2019). The axolotl became a very important model in the field of embryology and developmental biology (Reiß et al., 2015), as well as research on thyroxine. Nowadays, the axolotl plays an important role in investigating and understanding stem cell biology (Zielens *et al*., 2016), cancer (Boilly *et al*., 2017), scarless wound healing, and aging (McCusker *et al*., 2011).

The axolotl is one of the few tetrapods that is capable of regenerating entire structures such as limbs, tail, heart, eye lens, and central nervous system including the brain and spinal cord (Godwin *et al*., 2017). Earlier work on the effect of carcinogens on axolotls has shown that they are resistant to tumour formation upon exposure to carcinogens (Ingram *et al*., 1971). In other experiments, tumour cells show regression or are incorporated into the normal tissues of the organism when implanted (Oviedo & Beane, 2009). The axolotl limb regeneration model is amongst the most studied regeneration models and the fact that many genes and signalling pathways are conserved between humans and axolotls makes this organism an ideal model in therapeutic research. In fact, even though the axolotl genome is 10 times the size of the human genome at 32 gigabases (GB), it roughly encodes a similar number of proteins as humans (Nowoshilow *et al*., 2018). There is extensive conservation of synteny (blocks of order within sets of chromosomes that are being compared with each other) between humans and axolotls

with similarities in the length of conserved segments and genome size (Voss *et al*., 2011). Several other reasons make the axolotls a very attractive model for research, including their high reproductive rate and low maintenance requirements in the laboratory. In comparison to the newt (Notophtalmus viridescence), axolotls are much easier to breed. The fact that axolotls are sexually mature and yet maintain a larval state could prove to be pivotal to cancer research (Suleiman *et al*., 2019).

Following limb injury in axolotls, regeneration is initiated and the basal membrane keratinocytes travel to the amputation site and cover the wound. They proliferate and form the outer wound epidermis and within days the nerves are formed. The structure is referred to as the apical epithelium cap (AEC). The contact between the AEC and the cells beneath it, that are part of the remaining limb, help determine which parts of the limb need replacement and also the correct orientation of the newly formed structures. The AEC provides signalling molecules that promote regeneration. This includes growth factors and proteins which are known to be essential for limb outgrowth during development and adult salamander regeneration (Purushothaman *et al*., 2019). The interaction between the AEC and the underlying tissue also results in the formation of the blastema, which resembles an embryonic limb bud both structurally and functionally (Stocum, 2017). During the early stages of regeneration, the AEC supports blastema formation by enhancing the de-differentiation of cells. This consists of cell cycle re-entry of post-mitotic differentiated cells (Dall'Agnese & Puri, 2017). The blastema is likely to be composed of different types of dedifferentiated cells derived from muscle cells, dermal fibroblasts, as well as activated stem/progenitor cells. Together with dedifferentiation, protease-induced histolysis of tissue and the release of cells from the tissue structure occurs. Proteomic studies have identified several genes that are associated with dedifferentiation, including msh-homeobox 1 (*MSX*), newt homolog of Rad (*NRAD*), *RFRNG* and *NOTCH* (Stocum *et*

al., 2017). Blastema cells do not de-differentiate to reach a pluripotent cell mass, nevertheless, they still express three of the four TF genes used to reprogram adult somatic cells to pluripotency, namely, Kruppel-like factor 4 (*KLF4),* sex determining region Y-box 2 (*SOX 2*)*,* and *c-MYC* (Campbell *et al*., 2011). Changes in chromatin associated proteins were detected, suggesting transcriptional changes, chromatin modification, and upregulation of tumour suppressors, which is required for neuronal and skeletal differentiation (Campbell *et al*., 2011). Several proteins have been associated in blastema formation, most notably, the axolotl orthologs cold-inducible RNA-binding protein (cirbp) and kazal-type serine peptidase inhibitor domain 1 (kazald1) (Bryant *et al*., 2016). Other proteins have also been identified including proteins implicated in synthesis and maintenance of the extracellular matrix structure, regulation of epithelial to mesenchymal transition (EMT), regulation of cell maturation, differentiation, and proliferation (Campbell *et al*., 2011).

Successful regeneration will only proceed if none of the above-steps are impaired or obstructed. Also, for successful signalling and induction of regeneration, sufficient nerve damage is necessary, for both initial blastema formation and growth and development of the blastema during the early and mid-stages of regeneration. If there is insufficient nerve damage at the site, regeneration will not commence (Farkas & Monaghan, 2017). Also, studies on limb models where denervation or diversion of the nerve at different stages of blastema formation occurred, this resulted in regeneration failure (Suzuki et al., 2005). Moreover, the reduction in the number of macrophages leads to impaired communication between the epidermis and underlying cells and results in regeneration failure (Godwin *et al*., 2013). Similar crosstalk between cancer cells and neuronal cells has been observed, whereby a reciprocal interaction results in cancer cells stimulating neuronal outgrowth with neuronal cells inducing cancer metastasis (Deborde *et al*., 2017). Involvement of nerves in tumour growth and metastasis has been described for several cancers, including basal cell carcinoma, gastric, and prostate cancers

(Saloman *et al*., 2016; Boilly *et al*., 2017). It appears that neurotransmitters released by nerves and proteins such as BMP2 and FGFs have a direct effect on both regeneration and cancer microenvironments (Boilly *et al*., 2017).

1.9 The potential of regenerative organisms in cancer treatment research

From a cancer perspective, the ability of axolotls (*Ambystoma mexicanum*) and planarians to faithfully replicate regeneration following injury including dedifferentiation of cells (in the case of axolotls), gain of proliferative capabilities, and the subsequent differentiation of cells in the blastema without producing abnormalities, is of great relevance (Suleiman *et al*., 2019). The axolotl limb regeneration model and the planaria model provide an important research tool to dissect the pathways that enable regeneration capable animals to evade cancer formation. To aid in identifying any active molecules, factors or proteins that can possibly cause differentiation of cancer cells, it is important to focus on secretary factors including proteins secreted within the extracellular matrix that can have a direct effect on the tumour microenvironment (Suleiman *et al*., 2019). It is equally important to dissect in detail the process of regeneration and characterize the different factors involved in regeneration within the context of cancer treatment. The ability to identify the factors that cause re-differentiation will be helpful in cancer treatment. Such factors are important because, during regeneration, they act on early progenitors (blastema) and result in differentiation into the more complex structures. Molecular characterization of regeneration will help identify and isolate the factors involved in re-differentiation. This is important since many factors in de-differentiation result in re-programming cells to pluripotency (Friedmann-Morvinski & Verma, 2014) and such factors are implicated in CSCs. Success of such therapeutic approaches depends on whether the molecules or factors can actually be taken up by ligands or cell surface receptors and whether they have a differentiation, apoptotic, and/or cytotoxic effect on cancer and normal cells (Suleiman *et al*., 2019).

With the advancement in molecular biology and development of molecular techniques, the focus is on the identification of genes, TFs and signalling pathways that modulate the cellular events governing regeneration and cancer. In addition, the potential benefit of understanding the fields of cancer and regeneration could ultimately lead to the development of novel medical treatments of great importance. Also, new technologies such as CRISPR, RNA-seq, together with other molecular techniques and *in vitro* modelling have been very successful in identifying new protein interactions that govern signalling pathways, further allowing the understanding of pathway regulation (Franco & Hess, 2014). Although much has been elucidated about the mechanisms of regeneration in the axolotls and planarians, the fine lines between controlled and uncontrolled cellular proliferation as seen in regeneration and cancer are not fully understood. From this perspective, studying the overlapping stages of regeneration and focusing on the factors and/or molecules that cause re-differentiation becomes very important.

There are huge challenges in designing drugs that not only target the cancer cells but can also modulate the microenvironment to one that does not favour the cancer cell phenotype. By understanding the processes governing cellular proliferation, de-differentiation, cell cycle arrest, and re-differentiation using animal models like the axolotls and planarians, further light may be shed on the pathways linking regeneration and cancer with the ultimate aim of discovering novel therapeutic approaches in the treatment of cancer.

1.10 Cell line used in this study

1.10.1 The HL-60 cell line

The Hl-60 cell line has been described as a cell line that closely resembles patient derived cells (Harrison *et al*., 2016). Some of the properties of HL-60 cells including its bilineage differentiation potential have made this cell line an attractive model for studies involving potential therapeutic drug discoveries. Despite promyelocytic features, since they lack the t(15;17) typical of APL, HL-60 cells have been classified as AML-with maturation (AML-M2) according to the FAB classification. The HL-60 cell line used in this study has a deleted *TP53* gene and amplified *c-MYC.* From a therapeutic point of view, having drugs that show an increased activity against cells with inactivated p53, would theoretically result in an increased therapeutic index. In this scenario, normal cells with intact *TP53* that are often targets of toxicity (i.e. bone marrow cells) would be more protected (Damia & Broggini, 2004).

HL-60 cells represent a convenient model to study biochemical factors regulating myeloid differentiation, as these cells can be induced to differentiate along either monocytic or granulocytic lineages. Treatment of HL-60 cells with dimethyl sulfoxide (DMSO) or retinoic acid (RA) leads to granulocytic differentiation, while monocytic differentiation can be induced by agents such as phorbol 12-myristate 13- acetate (PMA), 1,25-dihydroxy vitamin D3 (VITD3), or sodium butyrate.

The HL-60 cells lack specific markers for lymphoid cells, but express surface receptors for Fc fragment and complement (C3), which have been associated with differentiated granulocytes.

They exhibit phagocytic activity and responsiveness to a chemotactic stimulus commensurate with the proportion of mature cells (Gallagher et al., 1979).

1.11 Rationale

Acute myeloid leukemia (AML) is a blood cancer characterized by the proliferation of clonal precursor myeloid cells with differentiation arrest. AML is the most common form of acute leukaemia in adults constituting about 80% of cases. Its incidence is about 1.3 per 100,000 for those under 65 years and about 12.2 cases per 100,000 for those over 65.

At present, the standard therapeutic approach for AML consists of chemotherapy, and secondarily, stem cell transplantation. However, in many cases, AML cells are not eradicated, with persistent cells reappearing after a period of remission. Also, the conventional therapies have often shown cytotoxic effects to normal tissues and cells. Thus, seeking new treatment strategies for AML has become a pressing and urgent matter.

The use of differentiation inducing agents that overcome the block in differentiation and allow cells to mature and die has been shown to be successful in *in vitro* and *in vivo* treatment of several types of cancers, most effectively in APL.

In recent years, significant emphasis has been placed on the identification of novel agents from natural sources that could be used in the fight against cancer. Natural products that are isolated from living organism sources may contain bioactive compounds that have the potential for prevention or treatment of major diseases. Many of these have already demonstrated diverse biological actions, including anti-cancer activities (Hwang et al., 2019). Therefore, the screening of natural products against cancer is needed.

The mechanisms governing regeneration and cancer formation are closely linked whereby dysregulation of the well-balanced and coordinated process of regeneration leads to cancer. Thus, the identification of biologically active molecules from regenerative organisms could lead to major benefits, with directions on how to develop therapeutic approaches for cancer treatment in humans.

Many different animal model at different developmental stages, and several *in vitro* systems have been used to study cancer and regeneration independently. However, the urodele amphibian *Ambystoma Mexicanum* (axolotls) and planarians (flatworms), are two of the earliest models exploring malignant transformation and adult regeneration (Ingrid, 1971; Foster, 1963). From this perspective, this research project is focused on using extracts from both organisms that possess high regenerative capabilities. Extracts from planarians and axolotls were tested for their ability to exert an effect on the acute myeloid leukaemia cell line HL-60, which exhibits bilineage differentiation potential and more importantly has been described as a cell line that closely mimics patient derived leukaemia cells (Harrison *et al.*, 2016). What makes these organisms of interest is the fact that following injury, they are able to regenerate a fully functional structure and are resistant to cancer induction.

Since most cancers are caused by a block in differentiation, addition of either extract could lead to over-riding of the differentiation block and activation of differentiation signalling pathways thereby correcting the initial defect that initiated this block.

The fact that both organisms can temporarily allow their stem cells to hyper-proliferate after injury and manage to control the mechanisms responsible for replacing the missing body parts

and ultimately revert to normal cellular physiological levels, indicates the existence of molecular mechanisms that aid in wound healing and recovery from tissue loss without resulting in abnormal cellular proliferation that is synonymous with cancer.

The aim of this project is to determine the effects of planaria species – Malta (PSM) and AXO extracts on the leukaemia cell line HL-60. The objectives are as follows:

- Preparation of crude extracts from regenerative organisms (planarians and axolotls);
- Determination of the effects of these extracts on the leukaemia HL-60 cell line;
- Elucidation regarding whether the extracts can cause differentiation, apoptosis, and cytotoxic effects;
- Evaluation of the changes in gene expression of several TFs implicated in myeloid differentiation together with cell cycle regulators and apoptotic markers.

Chapter 2

Materials and Methods

2.1 Materials

The list of materials, consumables, and cell lines used are shown in Tables 2-1, 2-2, and 2-3 respectively.

Table 2-1: List of reagents used

Reagents	Brand	City	Country of origin
RPMI-1640 medium	Biowest	Meda	Italy
Foetal Bovine Serum	Biowest	Meda	Italy
Protease Inhibitors	Sigma-Aldrich	Darmstadt	Germany
Bovine serum albumin	Sigma-Aldrich	Darmstadt	Germany
Dry pellet feed	JBl	Neuhofen	Germany
DMSO	Sigma-Aldrich	Milan	Italy
Tricane	Sigma-Aldrich	Darmstadt	Germany
Sulfamerazine	Sigma-Aldrich	Darmstadt	Germany
Penicillin/streptomycin	Sigma-Aldrich	Darmstadt	Germany
Histopaque 1077	Sigma-Aldrich	Darmstadt	Germany
phytohaemagglutinin	Sigma-Aldrich	Darmstadt	Germany
Absolute ethanol	Sigma-Aldrich	Darmstadt	Germany
phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	Milan	Italy

3-(4,5-Dimethylthiazol-2yl)-2,5-Diphenyltetrazolium Bromide (MTT)	Sigma-Aldrich	Milan	Italy
Hoechst 33342	Sigma-Aldrich	Milan	Italy
PE-labelled mouse anti-human CD14	BD Sciences	California	USA
Alexafluor-labelled mouse anti-human CD14	BD Sciences	California	USA
AnnexinV/PI Kit	BD Sciences	California	USA
DC protein assay kit II	Bio-Rad	Milan	Italy
Trypan Blue	Thermofisher scientific	Waltham	USA
Propidium Iodide Solution	Sigma-Aldrich	Milan	Italy
Ribonuclease A	Sigma-Aldrich	Milan	Italy
RNaseZAP	Qiagen	California	USA
RNeasy mini kit	Qiagen	California	USA
RNase free water	Thermofisher scientific	Waltham	USA
Reverse transcriptase kit	Qiagen	California	USA
QIAzol® Lysis reagent	Qiagen	California	USA

Table 2-2: List of Consumables

Consumables	brand	City	Country of origin
50 mL tubes	Labbox labware	Barcelona	Spain
2 mL centrifuge tubes	Labbox labware	Barcelona	Spain
0.22µm filters	Sigma-Aldrich	Darmstadt	Germany
T75 tissue culture flasks	Starlab	Hamburg	Germany
5 mL polystyrene tubes	BD Sciences	California	USA
96 well plates	Orange Scientific	Athens	Greece
12 well plates	Orange Scientific	Athens	Greece

Table 2-3: Cell Lines used in this study

Cell line	Brand	City	Country of origin
HL-60 cell line (ATCC-CCL-240)	ATCC	Manassas, Virginia	USA
KG1a cell line (ATCC-CCL246.1)	ATCC	Manassas, Virginia	USA

2.2 Equipment

The list of equipment used is shown in Table 2-4

Table 2-4: List of Equipment

Equipment	brand	City	Country of origin
Laminar flow hood	SafeFast Elite	Ferrara	Italy
Stuart Homogeniser	Cole-Parmer	Stafforshire	UK
Tecan Spark microplate reader	Biotek Instruments	Milano	Italy
Countess II FL Automated Cell Counter	Thermofisher scientific	Waltham	USA
Sonicator	VWR	Leicestershire	England
BD Facs Calibur	BD Sciences	California	USA
NanoDrop™ 2000 spectrophotometer	Thermofisher scientific	Waltham	USA
Bio-Rad CFx96 real-time PCR system	Bio-RAD	California	USA
EVOS FL Auto 2 Cell Imaging System	Thermofisher scientific	Waltham	USA
BD FACS Calibur flow cytometer	BD Sciences	California	USA

2.3 Planaria

2.3.1 Planaria maintenance

Live planarians were collected from Buskett forest, limits of Rabat, Malta (Figure 1-6). The planaria species collected for this study was designated as planaria species-Malta or PSM since the species was not identified. Planarians are identified based on gross morphological appearance and histological studies. These are then compared to traits that are already described in literature. Furthermore, molecular characterization can aid in the identification of the species that was collected from the forest in Buskett. In this study, I attempted to collect planarians with a similar morphological appearance to reduce the possibility of using different species.

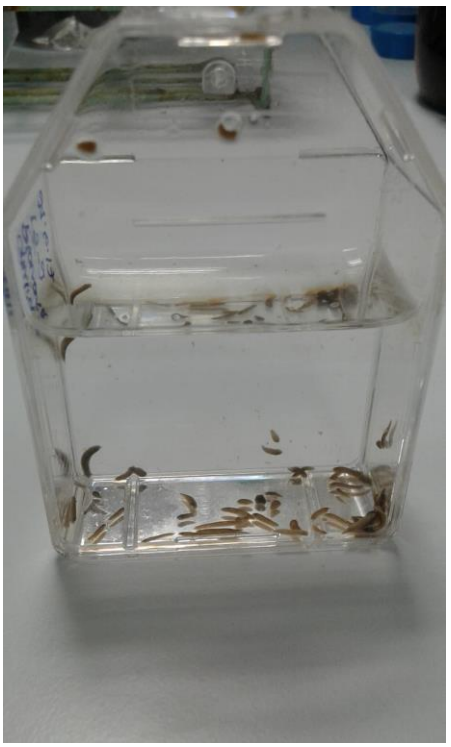

Figure 2-1: Planaria species-Malta (PSM). PSM in a T-75 flask after washing in artificial pond water

To capture the planaria, small pieces of approximately 2 cm x 2 cm of fresh bovine liver were left in the freshwater for 2 hours in container traps. Planarians feeding on liver were collected in Tupperware® plastic containers. In the laboratory, the organisms were transferred to a clean Tupperware® container and washed thoroughly with artificial pond water that was previously prepared (Table 2-5). The organisms were kept in the dark in artificial pond water at room temperature (RT) between 21-25 °C at all times until processing. Prior to preparation of the crude extract from the planarians, the organisms were starved for 7 days to avoid contamination from gut contents.

Table 2-5 Composition of the artificial pond water

Stock solution x 10	**Volume**
NaCL	8.77g
KCL	0.45g
$CaCL_2$	11.1g
H_2O	1 Litre

2.3.2 Preparation of the wet regenerative and non-regenerative crude extract from PSM

The procedure was adapted from McGann *et al*., 2001. PSM were maintained in the laboratory as already described in section 2.3.1. On day 0 of the extract preparation, starved planarians (n=40) were placed in a plastic container containing artificial pond water. The planarians were cut with a sharp sterile surgical blade medially. On day 7, the anterior and

posterior regions of the regenerating planarians were transferred to a sterile 50 mL centrifuge tube. The time point was selected to coincide with the differentiation and patterning process. Non-regenerating planarians were also collected in separate sterile 50 mL tubes and all manipulations were performed on ice and away from direct sunlight (Figure 2-2). The tissue collected was suspended in 10 mL of ice-cold Roswell Park Memorial Institute 1640 (RPMI-1640) medium with inactivated FBS and a cocktail of three protease inhibitors [2 µg/mL leupeptin/2 µg/mL A-protinin/1 mM phenylmethylsulfonyl fluoride (PMSF)]. Samples were ground with a hand-held electronic tissue homogenizer for 30 s, hand homogenized in a sterile glass homogeniser for 3 min, and sonicated for 30 s. The crude mix was transferred to sterile 2 mL centrifuge tubes and cell debris was removed following centrifugation. Initially, the crude mix was spun at 2000 g for 25 min at 4° C. The supernatant was collected and spun again at 100,000 g for 60 min at 4° C. The final supernatant was filter sterilised using a 0.22µm filter and the wet extract collected in a separate sterile 2 mL micro-centrifuge tube. The protein content was assayed using the DC protein assay kit II, and the tubes were labelled as PSM-R for the regenerative extract and PSM-NR for the non-regenerative extract. All extracts were stored in 1 mL aliquots in a −80°C freezer.

2.4 Axolotl

2.4.1 Animals and Tissue Collection

The axolotls were purchased from a local pet shop in Malta. They were of similar age and size (4-6 months old and approximately 5-7 cm in length).

The procedure was adapted from McGann *et al*., 2001. Axolotls (*Ambystoma mexicanum*) were maintained in the laboratory at 21°C in separate plastic containers filled with tap water and fed twice a week with dry pellets specifically formulated with proteins and fats from aquatic animals (Figure 2-1). Water changes were performed after each feed. They were also kept at an appropriate cycle of light (9 am – 6 pm) and dark (6 pm – 9 am) and air was continuously pumped in using an air pump.

Figure 2-2: The axolotl *Ambystoma mexicanum*. Each axolotl was kept in a separate plastic container

Limb amputations were performed on animals anesthetized transdermally with 0.1% tricaine in a water container for 15 min and observed by the naked eye to ensure there was no movement of the animal (n=20). Amputation of the limb was performed in a class II laminar flow hood cabinet and all materials used during the procedure were sterilised. Regenerating limb tissue was collected as follows. On Day 0, a hind limb was amputated by cutting just proximal to the elbow and the amputation site was trimmed to create a flat surface. After amputation, the axolotls were placed on ice for 1 hour, transferred to a 0.5% sulfamerazine solution overnight, and then back into normal water environment. The limb collected on day 0 was treated as the non-regenerating control limb and was designated the initials AXO-*NR*. Early regenerating tissue (Day 7 post-amputation), designated AXO-R, were collected (using the same methods) by re-amputating the limb 0.5–1.0 mm proximal to the wound epithelium and removing any residual bone. The time points were selected so that re-differentiation and patterning were represented, as this starts occurring at day 7. Tissue was extracted 2–3 mm proximal to the forelimb elbow and all bones were removed. Immediately after collection, all tissues were processed immediately for preparation of the wet extract

2.4.2 Preparation of wet extracts from AXO-R and AXO-NR

Tissues collected from the procedure in section *2.4.1* were kept on ice during the preparation of wet extracts. The non-regenerating tissue (Day 0) and regenerating tissues (Day 7) were transferred to 50 mL tubes containing a sterile solution of ice-cold RPMI-1640 medium with inactivated FBS. The tissues were placed in 10 mLs of media containing three protease inhibitors (2 µg/mL leupeptin/2 µg/mL A-protinin/1 mM PMSF). The tissues were ground with an electronic tissue homogenizer for 1–2 min, hand homogenized in a sterile glass homogeniser for 10–15 min, and sonicated for 30 s. The wet preparation was transferred to 2mL microcentrifuge tubes and the cell debris was removed in two centrifugation steps. The homogenate was first spun at 2000 g for 25 min at 4 °C. The supernatant was collected and spun again at 100,000 g for 60 min at 4 °C. The final supernatant was filter sterilised using a 0.22 µm filter and the wet extract collected in a separate sterile 2 mL micro-centrifuge tube. The protein content was assayed using the DC protein assay kit II, and the tubes were stored in 1 mL aliquots in a −80 °C freezer (Figure 2-2).

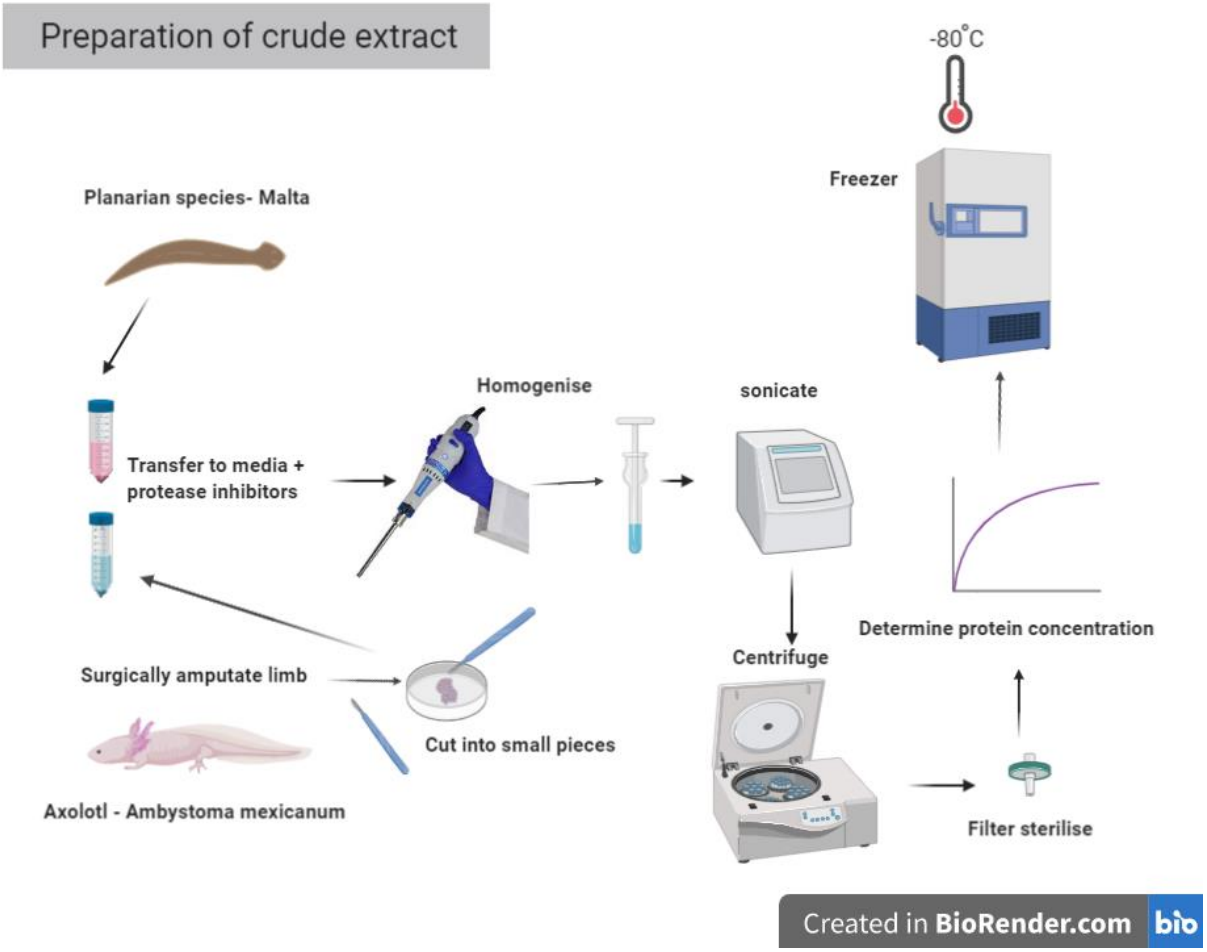

Figure 2-3: The procedure followed for the preparation of wet crude extracts from planarians and axolotls. For planarians R- and NR- extracts, 40 organisms were used. For the preparation of the AXO R- and NR- extracts, a total of 20 axolotls were used in the study.

2.5 Protein determination from wet extract preparations

The DC Protein Assay is a colorimetric assay for protein quantitation based on the Lowry assay. In this procedure, 5 µL of reagent S were added to 250 µL of reagent A. The amount prepared depended on the number of samples in each run. This solution is referred to as working reagent A. To prepare a standard curve, 100 mg of bovine serum albumin (BSA) was weighed using an analytical balance and dissolved in phosphate buffered saline (PBS). Five dilutions were prepared at 0.2 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, and 1.5 mg/mL. A standard curve was prepared each time the assay was performed. Next, 5 µL of standards and samples were pipetted in triplicate in a 96 well plate and 25 µL of working reagent A were pipetted in each well, followed by 200 µL of Reagent B. The contents of the plate were mixed on a shaker for 10 s and after 15 min of incubation at RT, the absorbance was read at 750 nm using the Tecan Spark spectrophotometer. The mean of the 3 wells for each concentration was established and a standard curve for the BSA was plotted and the concentration of the unknown samples (AXO- and PSM- extracts) was determined.

2.6 Ethical approval

All procedures performed in this study involving animals and collection of lymphocytes were covered by ethical approval from the University of Malta Research Ethics Committee, Ref. No.: FRECMDS_1819_002.

2.7 Cell culture

2.7.1 Media preparation

RPMI medium in powder form was prepared by transferring the contents of the container to a measuring cylinder containing a magnetic stirrer. 2 g of sodium hydrogen carbonate was added to ensure the pH of the media is kept stable. While stirring, the pH of the medium was adjusted to 0.1-0.3 pH units below the desired pH since the pH may rise during vacuum filtration. Adjusting to the desired pH was done using 1M HCL or 1M NaOH. The medium was brought to the final volume with tissue culture grade water. The following steps were performed inside a class II safety cabinet. The medium was placed in a stainless-steel kettle and a vacuum pump used to filter sterilise the medium by passing through a 0.22 μm Sterivex filter before collection in sterile T75 flasks. The flasks were stored at 4 °C until use. When required for growing cells, inactivated FBS at 10% final concentration, and antibiotics, penicillin/streptomycin at 1% concentration were added.

2.7.2 HL-60 cell line

Human acute myelocytic HL-60 cell line (ATCC-CCL-240) was supplied by the America Type Culture Collection (ATCC) and grown in RPMI 1640 medium supplemented with 10% inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2.

2.8 Lymphocyte cytotoxicity assay

Human peripheral blood lymphocytes of healthy donors were isolated from buffy coats using density gradient centrifugation on Histopaque-1077. All procedures were carried in sterile conditions in a class II laminar flow cabinet. The buffy coat was collected from the national blood transfusion center (NBTC) after the appropriate approval from the NBTC director. Samples were processed immediately upon arrival in the tissue culture laboratory.

The buffy coat containing blood was transferred to sterile 50 mL tubes and 1 part of buffy coat was diluted with 3 parts PBS. 35 mL of the diluted blood was layered carefully on 15 mLs Histopaque-1077. The tubes were centrifuged at 500 g and 20 °C for 25 min and the mononuclear cell layer was carefully collected and washed twice in PBS. The cell pellet was resuspended and cultured in RPMI 1640 medium supplemented with 10% inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Microscopic analysis confirmed the presence of lymphocytes. The lymphocytes were also stimulated to proliferate through addition of 1 % v/v phytohaemagglutinin (PHA) and left for 2 days to ensure lymphocytes are activated prior to treatment with extracts.

2.9 Cell viability assay

2.9.1 3-(4,5-Dimethylthiazol-2yl)-2,5-Diphenyltetrazolium Bromide (MTT)

The MTT assay is based on the ability of mitochondrial dehydrogenases in living cells to reduce MTT tetrazolium salt to formazan. MTT is used to test the metabolic activity of the cell. Cells (2 x 10^4/well) were seeded in 96-well plates and treated with different concentrations of PSM extract and AXO extract for 24-72 h. MTT was added to cells after treatment in at a final concentration of 0.5mg/mL, and the plates were incubated for 4 hours at 37°C. The assay was used to determine cell viability following the treatment of the HL60 line with extracts from both the axolotls and planaria. Metabolically active cells were able to cleave the yellow tetrazolium salt MTT to purple formazan crystals. The cells, containing the insoluble formazan product were then precipitated by centrifugation at 500 g for 10 min. The supernatant was removed, and the formazan crystals were dissolved in 120 µL dimethyl sulfoxide (DMSO) with gentle shaking at RT. Absorbance at 570 nm and reference wavelength at 405 nm was measured using the spark spectrophotometer.

2.9.2 Trypan Blue

The Trypan Blue azo dye exclusion test is used to determine the number of viable cells present in a cell suspension with live cells excluding the dye, whereas dead cells allow entry of the dye and stain blue/grey. Following treatment, 20 µL of cell suspension was mixed with an equal amount of the stain in a microcentrifuge tube and the contents were incubated for 5 min at RT. Total cell number and viability were evaluated by Trypan blue exclusion assay using Countess II FL Automated Cell Counter. The technique was used in conjunction with the MTT assay whereby low MTT results on the spectrophotometer were validated by a reduction in the

number of cell count and/or an increase in the number of cells staining with trypan blue due to death following treatment.

Additionally, IC50 values, which represent the concentration at which a substance exerts half of its maximal inhibitory effect (Sudomova *et al*., 2019), were calculated for both HL-60 cells and lymphocytes following extract treatment and staining with trypan blue, using the online tool Quest Graph™ IC50 Calculator (AAT Bioquest, Inc., Sunnyvale, CA, USA) (https://www.aatbio.com/tools/ic50calculator) (Accessed on 5th April, 2018). The IC_{50} values of the extract were used to elicit the selectivity index (SI), which describes the selectivity of a compound toward the cancer cells. The calculation is based on the ratio of IC_{50} of proliferating lymphocytes and the HL-60 cells (Demir *et al*., 2018).

To ensure proper activation of lymphocytes, PHA activated lymphocyte numbers were higher when compared to untreated controls thereby confirming that the lymphocytes successfully proliferated.

2.10 Morphological assessment of differentiation potential

2.10.1 Nitroblue Tetrazolium Assay (NBT)

The NBT assay was used to assess the ability of extracts from axolotls and planaria to induce differentiation of the HL-60 cell line as previously described (Collins *et al*., 1979). The assay was based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. NBT was prepared at a final concentration 0.2% w/v NBT in PBS (0.02 g in 10 mL PBS). Following treatment with PSM-R, PSM-NR, AXO-R, AXO-NR extracts, 20 µL of cell suspension was added to 20 µL of PMA activated NBT reagent and the mixture was incubated at 37 °C for 1 hour in the dark and the percentage number of NBT positive cells showing blue/black formazan deposits was scored using a light microscope. Cells were counted in five consecutive non-overlapping microscopic fields at a magnification of 400x.

2.10.2 Morphological examination for apoptosis and differentiation

HL-60 cells (2 x 10^4/well) were seeded in 96-well plates and treated with PSM and AXO extracts, DMSO 1.6% to induce differentiation, 10% DMSO to induce cell death and PMA (10 nM). All morphological analyses, described below, were performed using the EVOS FL Auto 2 Cell Imaging System.

Apoptotic morphology was studied in cells stained with Hoechst 33342. In particular, cells were stained with Hoechst 33342 (5 µg/mL medium) for 15 min at 37°C and visualized using an appropriate filter for DAPI. Cells were evaluated on the basis of their nuclear morphology, noting the presence of homogeneous chromatin, condensed chromatin, and fragmented nuclei.

In addition, cytospin preparations on microscopic slides were stained with Leishman's stain. Leishman’s stain was added to the slides (15 min) and PBS at pH6.8 was added to the slides at a ratio of 1-part Leishman’s stain and 3 parts PBS. Following staining, slides were washed in tap water and left to dry before analysis under the microscope. Cells were Judged for their nucleo-cytoplasmic ratio, cytoplasmic staining intensity, nuclear indentation, and chromatin staining by two reviewers.

2.11 Flow cytometry analysis

All flow cytometry analyses were performed by FACSCalibur flow cytometer (Becton Dickenson, Oxford, UK) and the data were analysed utilizing Cell Quest software. At least 1 x 10^4 events were acquired for each sample.

2.11.1 Detection of differentiation potential

During differentiation, cells express different surface antigens/markers that reflect the cell at different stages of maturation. Antibodies against these antigens were used to further elucidate the differentiation lineages the HL-60 cell line follows. PE-labelled mouse anti-human CD14 and Alex Fluor-labelled mouse anti-human CD11b antibodies were used to assess for differentiation following treatment with PSM and AXO extracts.

2.11.1.1 Staining Procedure

Following treatment, control and test cells were collected in 5mL tubes, cell counts determined using a haemocytometer, and cell numbers adjusted as recommended by the manufacturer (BD Sciences, California, USA) for optimal staining of cells (1 x 10^6 cells in 100 µL). The cells were centrifuged at 500 g for 5 min and the media was decanted. Cells were washed twice in 1mL PBS. After decanting the supernatant, cells were split in two test tubes and marked as antibody and isotype. 500 µL of blocking agent (PBS + 2mM EDTA + 10% FBS) was added to reduce nonspecific binding of the antibody. Cells were incubated in the blocking buffer at RT for 10 min. The contents of the tube were split into equal volumes in two separate 5 mL tubes and tubes were labelled as antibody and isotype. The tubes were centrifuged at 500 g for 5 min and supernatant was decanted. In the meantime, a master mix was prepared for the

antibody and the isotype depending on the number of conditions used in the experimental set up. The master mix was prepared in the dark to avoid photo-bleaching of the fluorescent labelled antibody. The master mix was made up of staining buffer (50 µL/test) (Table 2-6) and antibody or isotype (5 µL/test) (according to manufacturer's recommendation). 50 µL of master mix were added to the test tubes containing cells and the tubes were incubated on ice on a rotating platform for 30 min. Following incubation, 500 µL of 0.1% tween 20 wash buffer (Table 2-6) was added to each test tube. The tubes were incubated for 10 min on ice on a rotating platform. Tubes were centrifuged, and the supernatant was decanted. The tween wash step was repeated twice and the cell pellet was finally resuspended in 500 µL of staining buffer. The tubes were kept on ice until analysis was performed. The percentage expression of cell markers was determined by comparison with isotype control by constructing cell population gates provided in the analysis software

Table 2-6 Solution preparation for CD marker staining

	1 litre solution
Blocking Agent	PBS, 2mM EDTA, 10% FBS
Wash Buffer	PBS, 0.1% Tween 20
Staining Buffer	0.05M TBS @ pH7.4, 1% Human Serum, 2% FBS, 0.1% Azide

2.11.2 Cell cycle analysis

In this experiment, 500 µL of cells in different treatment conditions were transferred to a 2mL microcentrifuge tube. Cells were washed two times in 1 mL of 1 x PBS. After centrifugation at 800 g for 5 min, the supernatant was decanted and 1 mL of 70% ethanol was added drop-wise to the pellet while vortexing to ensure all cells were fixed and more importantly to minimize clumping. Cells were fixed to allow entry of the dye PI which is otherwise actively pumped out by living cells. The cells were left in 70% ethanol for 30 min at 4°C in the dark. After incubation, the fixed cells were centrifuged at 800 g for 5 min. The cells were then washed two times in 1 x PBS and 200 µL of Ribonuclease A. Then 200 µL of a 50 µg/mL PI was added. The mix was transferred to a 5 mL polystyrene tube and samples were then placed in the dark at 37 °C for an hour. After incubation, the tubes were transferred on ice until analysis by flow cytometry.

2.11.3 Detection of Apoptosis

The protocol was performed as recommended by the manufacturer of the kit (BD Sciences, California, USA). A positive control was included to ensure successful staining. To obtain a positive control, cells were incubated with camptothecin (5µM) for 5 hours at 37°C. Following treatments, cells were washed twice with cold PBS and resuspended in 1x binding buffer at a concentration of 1 x 10^6 cells/mL. Then 100 µL of the solution (1 x 10^5 cells) were transferred to a 5 mL test tube. Next, 5 µL of FITC Annexin V and 5 µL PI were added to the tube containing cells and the contents were gently mixed using a vortex. The tubes were incubated for 15 min at RT (25°C) in the dark. After incubation, 400 µL of 1X binding buffer was added to each tube and the cells were put on ice and analysed by flow cytometry within 1 hour.

Viable cells were negative for both annexin-V and PI (annexin V^-/PI^-; lower left quadrant), early apoptotic cells were positive for annexin-V staining (annexin V^+/PI^-; lower right quadrant), late apoptotic/necrotic cells were positive for both annexin-V and PI staining (annexin V^+/PI^+; upper right quadrant) and necrotic cells were positive for PI staining (annexin V^-/PI^+; upper left quadrant).

2.12 RNA extraction and real-time RT-PCR

Following treatment, HL-60 cells were washed x 2 in PBS and suspended in 350 µL of QIAzol® Lysis reagent to ensure maximal recovery of RNA prior to extraction. The pellet was thoroughly homogenised by repeated pipetting and the suspension was frozen at -80°C until extraction. Treated and untreated cells were collected in triplicate.

Prior to extracting RNA from the samples, the work surfaces were wiped with RNaseZAP to ensure that surfaces are free from RNase. The RNeasy® Mini Kit was used to extract RNA from the treated and untreated cells following the manufacturer's instructions. Cell lysates were removed from the -80°C freezer and allowed to thaw on ice. Chloroform (140 µL) was added to the cell lysates and following centrifugation, the upper phase of the lysate was collected for the subsequent RNA isolation steps.

Absolute ethanol (350 µL/sample) was then added to the lysates, creating conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. The samples (700 µL/sample) were then applied to the RNeasy mini columns and the columns placed in the supplied 2 mL tubes. Total RNA bound to the membrane and contaminants were washed away (collected in the 2mL tube) by centrifugation for 15 s at 8000 x g. Buffer RW1 (700 µL/sample) was added to the RNeasy columns and the tubes were centrifuged for 15 s at 8000 x g to wash the columns. At this point, the flow-through and collection tubes were discarded. The RNeasy columns were transferred into 2 mL collection tubes and 500 µL Buffer RPE/ sample was pipetted onto the RNeasy columns. The tubes were centrifuged for 15 s at 8000 x g to wash the columns and the flow-through was discarded. The previous step was repeated twice to reduce

the carryover of the RPE buffer to the next step and the columns were placed in new 2 mL tubes and centrifuged at high speed for 1 min.

RNA was then eluted in RNase-free water. To elute the RNA, the RNeasy columns were transferred to new 1.5 mL collection tubes and 30 µL RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. The tubes were centrifuged for 1 min at 8000 x g to elute the RNA and the RNA concentration was determined using the NanoDrop^{TM} 2000 spectrophotometer.

The purity of total RNA was assessed spectrophotometrically at 260 nm and 280 nm, with an A260:A280 of 1.80-2.00 considered acceptable. RNase free water was used as a blank. The samples were store at -80^{o}C until analysis.

2.12.1 cDNA synthesis

RNA samples were thawed on ice and the following steps were performed in a 96 well plate. About 300 ng of RNA of each test sample was transferred to a 96 well plate and RNase free water was added to a total volume of 12 µL. The volume of RNA and H_2O added depended on the concentration of RNA in each test sample (Table 2-7). Genomic DNA (gDNA) wipeout buffer (2 µL) was added to each well to remove any contaminating genomic DNA and the plate was incubated at 42 ^{o}C for 2 min.

Table 2-7. Genomic DNA elimination reaction components

Component	Volume/reaction µL	Final concentration
gDNA Wipeout Buffer, 7x	2 µL	1x
Template RNA	Variable (300 ng)	
RNase-free water	Variable	
Total Volume	14	-

After incubation, the plate was placed on ice. In the meantime, the reverse-transcription master mix (Quantiscript Reverse Transcriptase, Quantiscript RT buffer, and RT primer mix) were prepared on ice.

The master mix (6 µL) was added to the 14 µL of the gDNA wipe-out buffer and RNA template (Table 2-8). The plate was incubated at 95 ˚C for 3 min to inactivate Quantiscript Reverse Transcriptase and the plate was stored at -20 ˚C until the quantitative PCR was carried out.

Table 2-8. Reverse-Transcriptase reaction components

Component	Volume/reaction µL	Final concentration
Reverse Transcriptase master mix		
Quantiscript Reverse Transcriptase	1	
Quantiscript RT Buffer 5x	4	1x
RT Primer Mix	1	
Template RNA		
Entire genomic DNA elimination reaction	14	
Total Volume	20	

2.12.2 Real-Time PCR

The Bio-Rad CFx96 real-time PCR system was used for studying the gene expression in the HL-60 cell line after treatment with AXO and PSM. The expression of several TFs, cell cycle regulators, and genes encoding apoptosis regulating proteins were tested (Table 2-10). The thermocycler cycles consisted of 10 min at 95 °C, 40 cycles at 95 °C for 15 secs and 60 °C for 1 min. Prior to running the reaction on the thermocycler, a master mix containing polymerase enzyme mix, RNAse free water, and the fluorescent labelled reporter probe of interest were prepared on ice (Table 2-9).

Table 2-9. Master Mix preparation for RT-PCR

Component	**Volume µL**	**Final concentration**
Master Mix 2x (Taq DNA polymerase, dNTPs)	5	1x
TaqMan probe 20x	0.5	1x
Water	3.5	
cDNA	1	
Total Volume	10	-

These were dispensed in 96 well plates. For each probe, two housekeeping genes (HPRT1 and YWHAZ) were used. cDNA was added and the 96 well plate was covered and allowed to run on the Bio-Rad thermocycler.

Table 2-10. Taqman® Gene Expression Assays. Several genes implicated in maturation, cell cycle regulation, and apoptosis

GENE	ASSAY ID	CATALOGUE NUMBER
BCL2	Hs00608023_m1	4331182
BAX	Hs00180269_m1	4331182
CDKN1A	Hs00355782_m1	4331182
CDKN2C	Hs00176227_m1	4331182
c-MYC	Hs00153408_m1	4331182
SPI1	Hs02786771_m1	4331182
CEBPA	Hs00269972_s1	4331182
CEBPB	Hs00270923_s1	4331182
CEBPE	Hs00357657_m1	4331182
HPRT1	Hs02800695	4331182
YWHAZ	Hs01122445_g1	4331182

The relative expressions of mRNAs were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schimttgen, 2001) and normalized against the geometric average expression *HPRT1* and *YWHAZ* reference genes (Vandesompele *et al*., 2002).

2.13 Statistical analysis

All experiments were performed in triplicate and repeated at least twice. Excel was used to present the median and the inter-quartile range (IQR) data. The SPSS statistics software [(version 20 (IBM, Armonk, New York, USA)] was used for statistical analysis. Normal distribution was assessed by Kolmogorov-Smirnov test. Since data were not normally distribution they were analysed using the non-parametric Kruskal-Wallis test, followed by Dunn-Bonferroni's post hoc analysis. Significance was accepted at a $p < 0.05$. Statistical analysis was carried out on the results to accept or reject the Null Hypothesis (H0). Statistically significant results will mean that H0 is rejected and that treatment had an effect on cells

2.13.1 Representation of Statistics on Results

All graph statistics are labelled as follows with the prefix indicating the paired statistical comparisons:

* indicates a statistical difference of $p < 0.050$

** indicates a statistical difference of $p < 0.001$

Chapter 3

Planaria Extract Results

3.1 The effects of the Planarian Species – Malta (PSM) extract on metabolic activity of HL-60 cells

The effect of the PSM crude extract on the metabolic activity of HL-60 and KG1-a cells was assessed using the MTT assay (Figure 3-1). The MTT assay is a colorimetric reductive assay used to determine cell viability and proliferation based on the ability of metabolically active cells to enzymatically convert the yellow tetrazolium compound MTT to purple water insoluble formazan by dehydrogenases occurring in the mitochondria of living cells and other reducing agents and enzymes located in other organelles such as the endoplasmic reticulum (van Tonder *et al*., 2015).

As seen in Figure 3-1, The effect of the regenerative (R-)and non-regenerative (NR-) PSM crude extracts were tested for their effect on cell viability of HL-60 cells following 24, 48, and 72 h treatment. Overall, there is a significant effect across all treatment concentrations when compared to untreated cells on all days ($p<0.001$). After 24, 48, and 72 h treatment, there was a marked and significant reduction in cell viability with the higher R- and NR- PSM doses (0.5-2mg) as compared to the lower dose (0.031-0.250mg) group ($p<0.05$). After 48 h, there was a quasi-dose dependent effect of PSM (R- and NR-) observed with increasing concentrations. However, the PSM extract reduced KG-1a metabolic cell activity at 48 h at a concentration of 1.0 and 2.0 mg/ mL only. In fact, after 48 h treatment, metabolic activity was reduced by 14% when compared to the untreated cells (results not shown). Since preliminary data showed that the PSM extract is particularly effective on HL-60 cells, further investigations using only the HL-60 cell line were carried out.

The MTT assay is a relative test in which the mitochondrial activity of treated cells is compared to that of untreated cells and the results arbitrarily scaled at 100% for the untreated cells. When there is a significant reduction in MTT results, showing reduced viable cell numbers, this could be due to cytotoxic effects (Mathieu *et al*., 2009) or anti-proliferative/cytostatic effects (Alley *et al*., 1988). Therefore, further preliminary tests to confirm the cytotoxic and/or anti-proliferative effect of PSM extract on HL-60 cells were performed.

Since both R- and NR- showed similar effects, only the NR- PSM was used in the subsequent experiments.

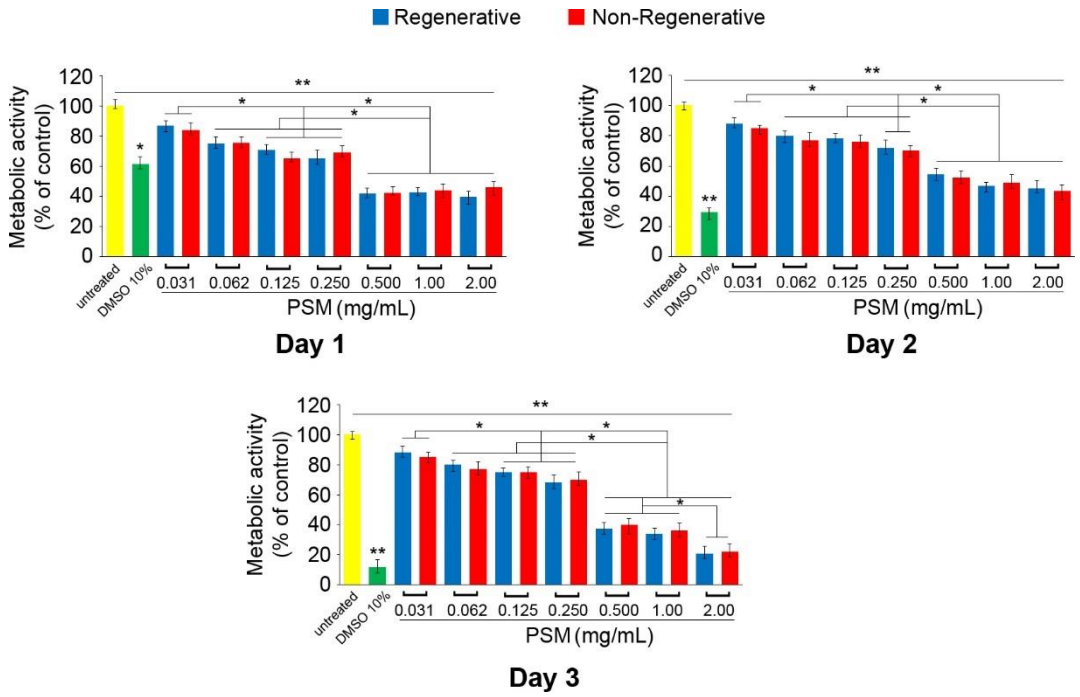

Figure 3-1: The effects of the Planarian species- Malta (PSM) R- and NR- extracts on cell viability of HL-60 cells. Data are presented as median and inter-quartile range (error bars) (n = 3 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

3.2 Planaria extract reduces the growth rate and viability of HL-60 cells

The potential cytotoxic effects of PSM crude extract on HL-60 cells and activated lymphocytes was evaluated by the trypan blue exclusion assay. The test was used to determine the number of viable cells present following 24, 48, and 72h treatment. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes whereas dead cells do not (Strober, 2015). HL-60 cells were treated for various times (24-72 h) with various doses of PSM extract (1 - 1000 µg/mL).

As shown in Figure 3-2(A), PSM extracts caused a significant reduction in cell number of HL-60 cells in a quasi-dose dependent manner over 72h when compared to untreated cells on the same day. At 24 h treatment, a significant decrease (p<0.05) in cell numbers was observed in treatment concentrations between 62.5-1000 µg/mL when compared to untreated cells. This could be due to cytostatic effects caused by bioactive molecules in the extract as cell numbers did not change between Day 0 and 24 h at 62.5 µg PSM, or cytotoxic effects due to a reduction in cell number at concentrations 125-1000 µg. At 48 h treatment, a significant reduction in the proliferation curve was observed between 31.25-1000µg when compared to untreated cells on the same day. After 72h, a significant decrease in cell numbers was observed in treatment concentrations between 125-1000 µg (p<0.05) when compared to untreated cells on the same day. However, there was an increase in the proliferation curve at 62.5 µg concentration after 72 h and this could be due to the loss of activity of any bioactive molecules in the extract and possibly the presence of resistant cells and probably an interplay of both factors.

Interestingly, trypan blue staining confirmed that the reduction in cell numbers is due to both a suppressive mechanism exerted by the PSM treatment and a cytotoxic one at higher concentrations [Figure 3-2(C)]. In fact, following 24 h treatment, PSM treatment shows a significant reduction in viability of 20-55% between 62.5-1000 μg PSM concentrations respectively.

Moreover, there is a significant percentage growth inhibition of HL-60 cells when compared to untreated cells (p<0.05), but only at 48 h, a quasi- dose dependent effect was observed with growth inhibition of 18, 51, 64, 71, 73 and 84% for 31.2, 62.5, 125, 250, 500 and 1000 μg/mL respectively when compared to untreated cells. Notably, 500 and 1000 mg/mL of PSM extract almost totally suppressed the proliferation of HL-60 cells within 72 h [Figure 3-2(B)]. Taken together with trypan blue staining [Figure 3-2(C)], it is clear that at higher concentrations, PSM is cytotoxic.

Overall, determination of cell numbers using trypan blue staining revealed that growth inhibition caused by PSM extract treatment is, only partly, due to reduction in cell viability [Figure 3-2(C)].

Based on the preliminary results of MTT and trypan blue, PSM at 62.5 μg/mL for 48 h was used in the subsequent experiments. After 48 h treatment of HL-60 cells with PSM, there is a 20% reduction in MTT when compared to untreated cells [Figure 3-1(B), 48 h], 20% reduction in cell viability after trypan blue staining [Figure 3-2(C), 48 h], and 51% growth inhibition after 48 h when compared to untreated as seen in Figure 3-2(B). To be able to investigate further the possible anti-proliferative, cytotoxic, and differentiation potential of the PSM extract, all the subsequent experiments were performed at the PSM 62.5μg/mL for 48 h.

To demonstrate the selectivity of PSM, HL-60 cells and activated lymphocytes were incubated with increasing concentrations of PSM at 48 h treatment only. PSM extract exhibited significant growth inhibitory effects on HL-60 cells when compared to activated Lymphocytes [Figure 3-2(D)]. The calculated half-maximal inhibitory concentration (IC_{50}) value in HL-60 cells treated with PSM was 61.037 µg/mL, while the IC_{50} value of PSM treated activated Lymphocytes was calculated as 162.53 µg/mL (Table 3-1). The table (3-1) also shows that the selectivity index (SI) based on the ratio of IC50 of activated lymphocytes and HL-60 cells is 2.7, suggesting that the extract has selectivity towards the cancer cells and not normal cells.

Table 3-1: Antiproliferative effects and selectivity of PSM toward HL-60 cells at 48 h.

Compound	HL-60 IC_{50} (µg/mL)	Activated lymphocytes IC_{50} (µg/mL)	SI
Planaria Species-Malta extract	61.037	162.593	2.7[a]

a. Selectivity Index, calculated based on the ratio of IC_{50} activated lymphocytes and the HL-60 cells.

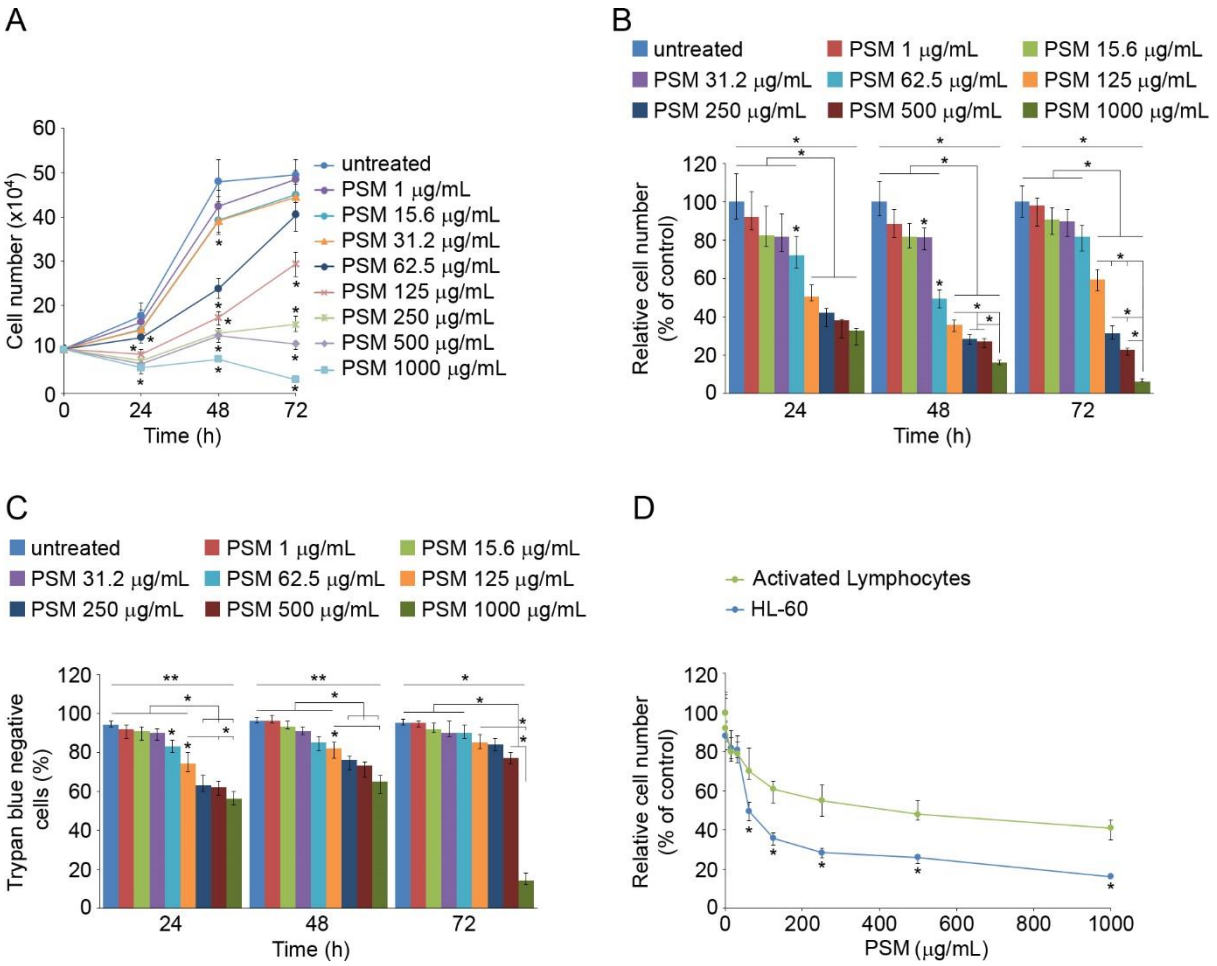

Figure 3-2: Effects of PSM extract on cell number (A), relative cell growth (percentage cell number of PSM treated cells relative to untreated cells) (B) and cell viability (C) of HL-60 cells. Cells were treated with PSM extract as indicated for 72 h. (D) Effects of PSM extract on lymphocytes and HL-60 cells after 48 h treatment. The number of viable cells was determined by trypan blue exclusion. Data are presented as median and inter-quartile range (error bars) (n = 3 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

3.3 Planaria extract induces differentiation and apoptotic morphological changes of HL-60 cells

To further confirm the effect of PSM extract, morphological analyses was performed on the HL-60 cells. In this experiment, HL-60 cells were incubated with a lethal dose of DMSO (10%) as a positive control for morphologically apoptotic cells. DMSO (1.6%) and PMA (10 nM) were used as positive controls for granulocytic and monocytic differentiation respectively. Morphological assessment of HL-60 cells was also performed following treatment with PSM at 62.5µg.

Phase-contrast microscopy [Figure 3-3(A)], show untreated control cells as well-rounded cells. Nuclear staining using Hoechst 33342 [Figure 3-3(B)] reveals a well-rounded and smooth nucleus. Morphology typical of immature cells is further confirmed after staining with Leishman's stain [Figure 3-3(C)] with cells showing fine chromatin and a scant amount of basophilic cytoplasm and the typical increased N/C ratio with large nuclei and scant cytoplasm.

Treatment of cells with 10% DMSO reveals typical features of apoptosis as seen in all three images: phase contrast microscopy, Hoechst 33342 and Leishman's stain. Examination under phase contrast microscopy [Figure 3-3(A)] reveals loss of cell volume. Increased nuclear staining intensity typical of condensed nuclei is evident after staining the cells with Hoechst 33342 [Figure 3-3(B)] and Leishman's stain reveals the typical loss of cell volume, condensed nucleus and cytoplasm [Figure 3-3(C)]. There is no evidence of cytoplasmic swelling that is typical of

necrotic cells. Also, in necrotic cells the DNA is not condensed and the edges of the nucleus are less clearly defined.

Incubation with 1.6% DMSO typically induces myeloid differentiation. Also, apoptotic cells are also present. Phase contrast microscopy reveals well rounded cells [Figure 3-3(A)] with smooth edged cytoplasmic membrane and fragmented cells typical of apoptotic fragmentation. Hoechst 33342 staining [Figure 3-3(B)] reveals smooth and evenly stained nuclei, though smaller in size when compared to untreated cells. Morphological examination following Leishman's stain [Figure 3-3(C)] reveals a mixed population of cells with a high N/C ratio and a basophilic cytoplasm, and cells with a reduced N/C ratio and greyish blue cytoplasm indicative of cells with a differentiation potential.

Induction of differentiation by PMA usually results in HL-60 cells differentiation exhibiting monocytic/macrophage morphology with adhesion and elongation of the cells as observed in both the unstained and Leishman stained HL-60 cells.

HL-60 cells treated with PSM extract increased the N/C ratio in HL-60 cells as seen in both the unstained and stained images. Also, apoptotic cells were observed in all three images. Although differentiation of the cell line did not recapitulate the lobed morphology of primarily seen in more differentiated cells, it resulted in a decreased nuclear size which is clearly evident following Hoechst 33342 and Leishman staining.

In summary, preliminary results using MTT, trypan blue, and cell morphology suggest that PSM extract exhibits anti-proliferative effects on HL-60 cells, together with induction of cell differentiation and apoptosis and thus merited further investigation.

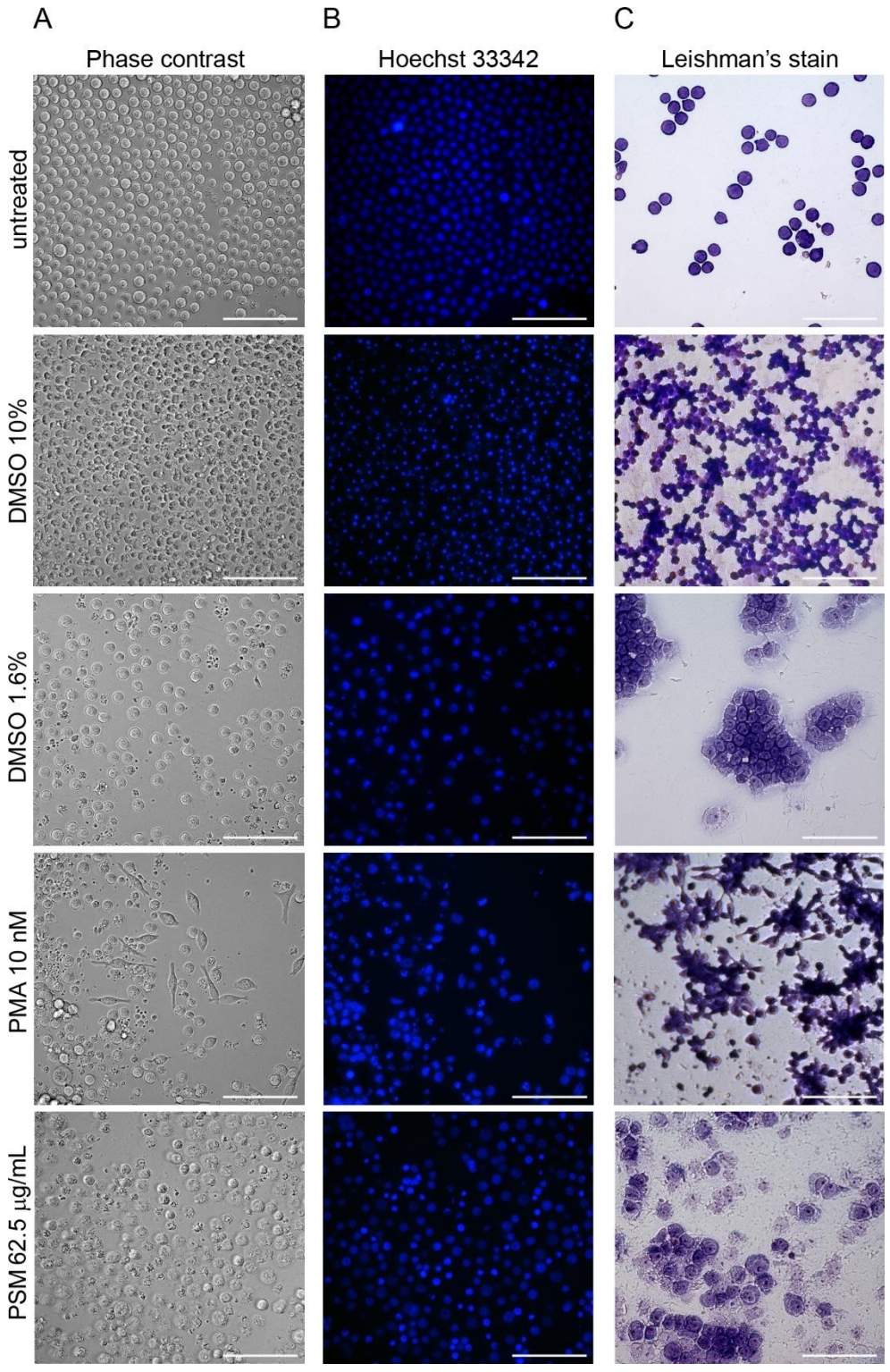

Figure 3-3: Morphological changes of HL-60 cells treated with 62.5 µg/mL of PSM extract after 48 h. (A) Phase contrast microscopy images, B) fluorescence microscopy images after staining with Hoechst 33342 and (C) Leishman staining assay. HL-60 cells incubated with DMSO (10%), DMSO (1.6%) and PMA (10 nM) were used as positive controls for apoptosis, granulocytic and monocytic differentiation, respectively. The scale bar is 100 µm. The images were a representative of three independent experiments.

3.4 Planaria extract induces apoptosis and increases the level of sub-G0/G1 of HL-60 cells

To further characterise the effect of PSM extract on HL-60 cell cycle distribution and cell death, flow cytometry analysis of propidium iodide (PI-) stained nuclei and Annexin V/PI staining were performed respectively.

For cell cycle analysis, HL-60 cells were incubated with DMSO (1.6%) and PMA (10 nM) as positive controls for differentiation (granulocytic and monocytic respectively), DMSO (10%) as a positive control for apoptosis. Untreated and PSM treated cells were also stained with PI for 48 h as shown in Figure 3-4. In untreated cells, [Figure 3-4(A)] DNA content histograms indicated that 46.2% was distributed in G0/G1 phase, 38.1% accumulated in S phase, 13.1% was in G2/M phase and only 2.6% in the sub-G0/G1. Cells treated with 10% DMSO exhibited an increase (35.8%) in the sub-G0/G1 phase of the cell cycle. These cells are usually identified as apoptotic cells with fractional DNA content. Treatment with 1.6% DMSO and PMA results in a block or cell cycle arrest at the G1 phase of the cell cycle of 74.4% and 92.2% respectively. Also, treatment with 1.6% DMSO resulted in an increase in the sub-G0/G1 phase (20.4%) when compared to the untreated cells (2.6%). When HL-60 cells were incubated with PSM extract (62.5 µg/mL) for 48 h, a significant increase in the sub-G0/G1 (16.1%) peak and a significant reduction in the S- or synthesis phase of the cell cycle peak (29.2%) is seen when compared to untreated cells [Figure 3-4(B)].

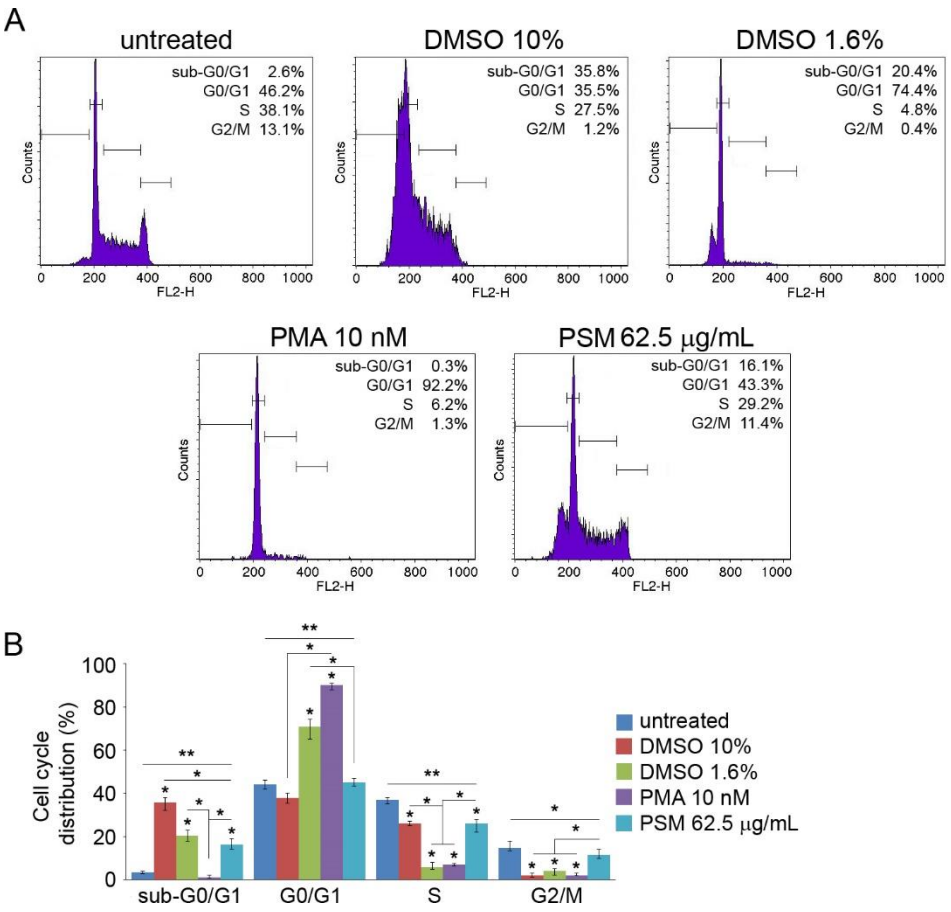

Figure 3-4: Effects of PSM extract (62.5 µg/mL) treatment on cell cycle distribution in HL-60 cells after 48 h. (A) Histogram plots of flow cytometry analysis performed in HL-60 cells. (B) Graph summarizing the cell cycle distribution. HL-60 cells incubated with DMSO (10%), DMSO (1.6%) and PMA (10 nM) were used as positive controls for apoptosis, granulocytic and monocytic differentiation, respectively. Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's post hoc analysis (* p < 0.05; ** p < 0.001).

Following treatment of HL-60 cells, the percentage number of apoptotic cells was determined by staining with annexin V/PI. Annexin V/PI double staining allows the identification of viable cells (Annexin V negative, PI negative), cells in early apoptosis (Annexin V positive, PI negative), late apoptosis or necrotic cells (Annexin V positive, PI positive), and dead cells (Annexin V negative, PI positive).

As seen in the dot plots [Figure 3-5(A)], the majority (93.76%) of untreated cells are present in the lower left (LL) quadrant. These cells are composed of viable cells that are Annexin V/ PI negative. In comparison, treatment with 10% DMSO results in a significant increase in early apoptotic (43.07%) and late apoptotic (54.08%) cells, clearly seen in the lower right (LR) and upper right (UR) populations on the dot plot respectively. A significant increase is also seen in both early and late apoptotic cells (LR and UR) following treatment with 1.6% DMSO and 10 nM PMA [Figure 3-5(A)]. Following PSM extract treatment for 48 hrs, 22.38% apoptotic cells (early plus late apoptotic cells) were found in HL-60 cells, which were significantly higher than that of untreated cells (6.24%) [Figure 3-5(A)].

Since the analysis represents a single observation (48 h), the movement of cells through the different stages (Annexin V^-/ PI^-, Annexin V^+/ PI^-, Annexin V^+/ PI^+, Annexin V^-/ PI^+) cannot be observed. Taken alone, the PI/ Annexin V results cannot rule out necrotic cell death, however, together with morphological examination following Hoechst 33342 and Leishman staining and cell cycle analysis (Sub-G0/G1) results, it is likely that cell death is due to apoptosis.

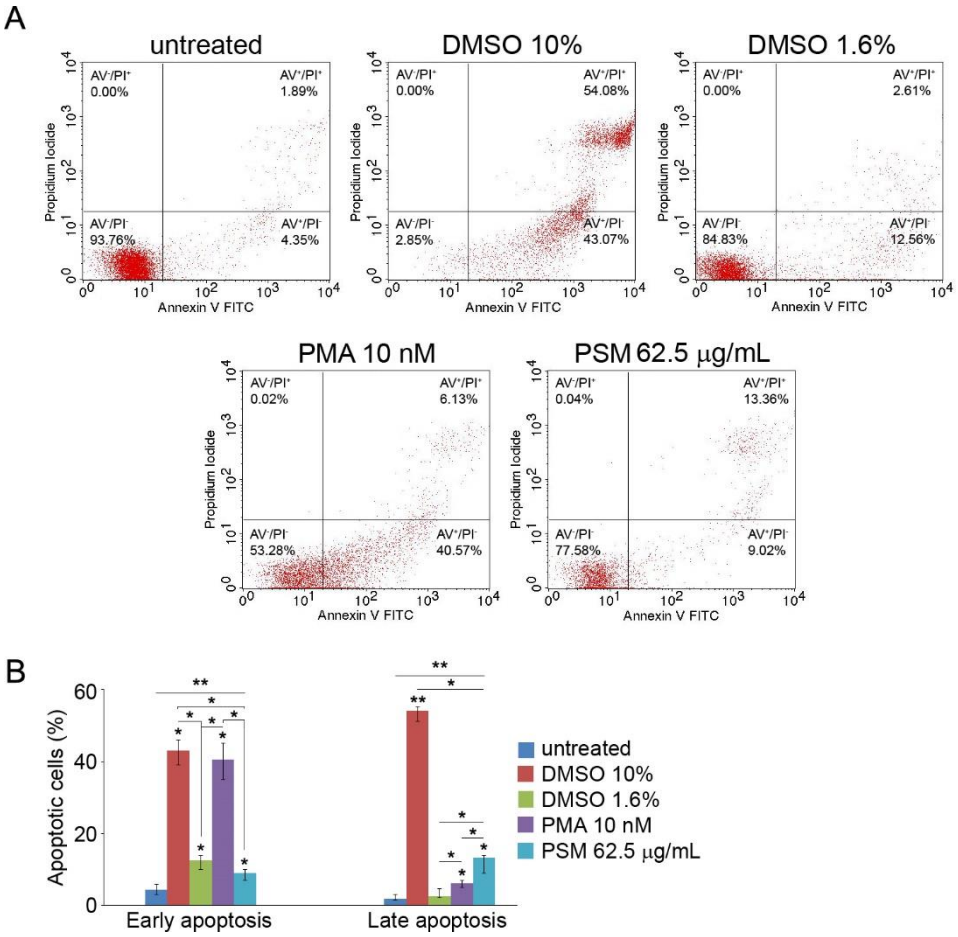

Figure 3-5: Apoptotic activity of HL-60 cells in response to treatment with DMSO (10%), DMSO (1.6%), PMA (10 nM) and PSM extract (62.5 µg/mL) for 48 h. Apoptotic cells were quantified by flow cytometry after staining with PI and FITC-Annexin V. Four fractions (PI-/Annexin V-, PI-/Annexin V+, PI+/Annexin V+, PI+/Annexin V-) were analysed. (A) Representative flow cytometric dot plots. (B) The summary of populations stained with only Annexin V (early apoptosis) and with both Annexin V and PI (late apoptosis). Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's post hoc analysis (* $p < 0.05$; ** $p < 0.001$).

3.5 Induction of differentiation in HL-60 cells after treatment with PSM extract

To investigate the possibility that PSM induces myeloid differentiation in HL-60 cells, the NBT assay was used to measure generation of ROS, which increases with HL-60 cell differentiation (Batista *et al*., 2005). 1.6% DMSO was used as a positive granulocytic differentiation marker and untreated cells as NBT negative control.

As shown in Figure 3-6(A), following treatment of HL-60 cells with the PSM extract (62.5 µg/mL), there was a significant increase (31.5%) in cells containing blue NBT formazan deposits. Incubation of cells with 1.6% DMSO also caused an increase in ROS production (15.5%) while untreated cells lacked NBT formazan deposits.

Further confirmation of the ability of PSM to cause differentiation of HL-60 cells was performed by flow cytometric investigation using CD11b and CD14 differentiation markers. During the course of differentiation of early human myeloid cells toward monocytes/macrophages and granulocytes, cell surface expression of CD14 (monocytic) and CD11b (granulocytic) antigens increases dramatically (Rosmarin *et al*., 1989; Goyert *et al*., 1988). In HL-60 differentiation studies, the expression of CD11b is usually considered a granulocytic/monocytic differentiation marker, whereas the expression of CD14 is limited mainly to monocytic and macrophage expression.

In these experiments, HL-60 cells incubated with DMSO (1.6%) and PMA (10 nM) were used as positive controls for granulocytic and monocytic differentiation, respectively.

After 48 h treatment, there was a significant increase (p<0.001) in the expression of CD11b in PSM extract (62.5 µg/mL) treated cells (48.62%) when compared to the untreated control group (8.09%) [Figure 3-6(B)]. Treatment with 1.6% DMSO and 10 nM PMA resulted in expression of CD11b in 20.3% and 40.97% of HL-60 treated cells respectively.

PSM treatment resulted in a significant increase (p<0.05) in the expression of the surface antigen CD14 (22.06%) when compared to untreated cells (3.34%).

From the NBT and surface antigen expression (CD11b and CD14), it is clearly apparent that PSM induces ROS formation and the expression of myelomonocytic differentiation markers.

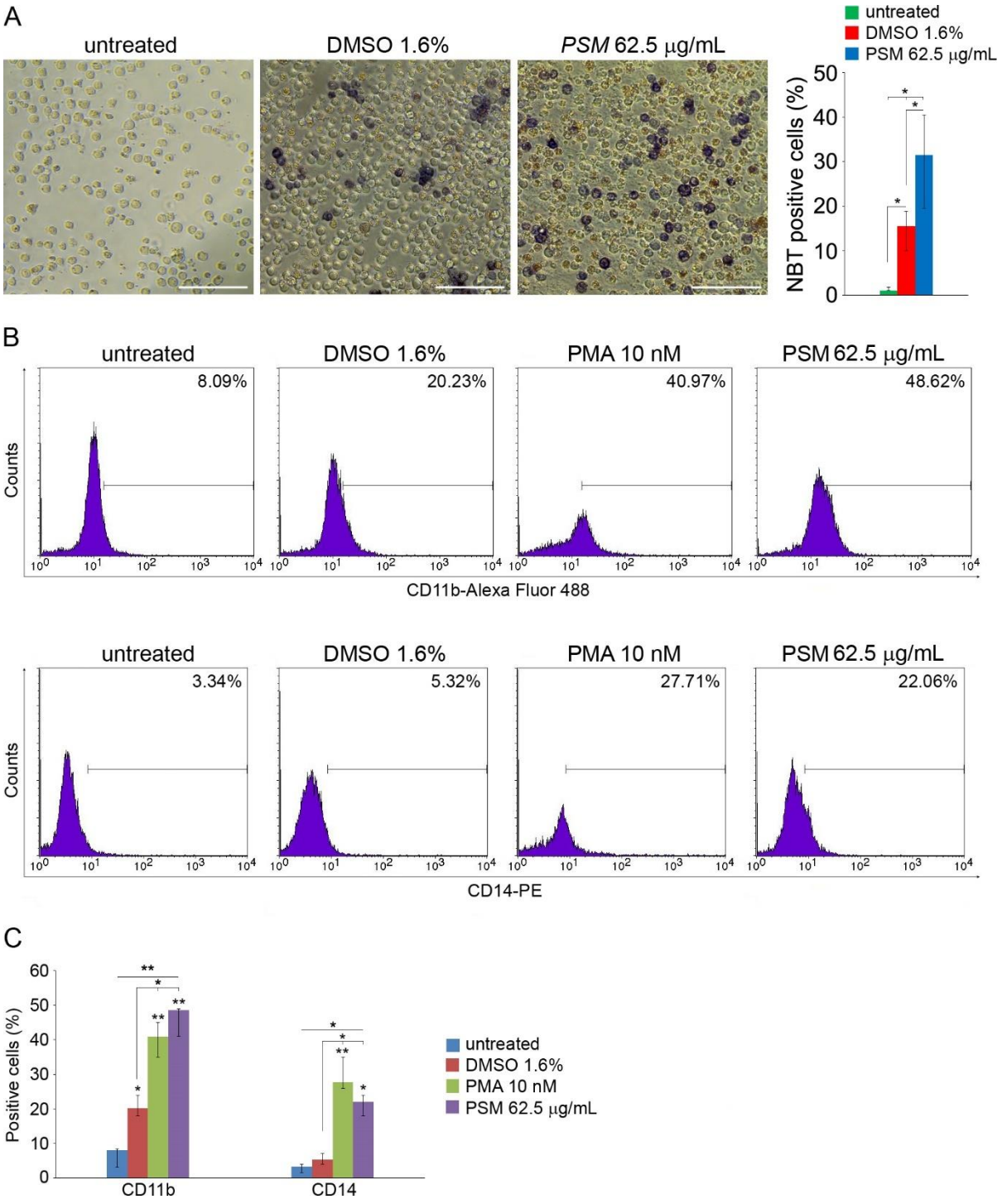

Figure 3-6: PSM extract-induced cell differentiation in HL-60 cells. (A) Nitroblue tetrazolium (NBT) reduction assay. Cells were treated with PSM extract (62.5 µg/mL) for 48 h. Microscopy images display cells containing intracellular blue-black formazan deposits (scale bar = 100 µm). Graph summarizing the percentages of NBT positive cells. HL-60 cells incubated with DMSO (1.6%) were used as positive controls for granulocytic differentiation. (B) Cytometric analyses showing cell surface expression of CD11b (top panels) and CD14 (bottom panels) in HL-60 cells treated with DMSO (1.6%), PMA (10 nM) and PSM extract (62.5 µg/mL) for 48 h. (C) Graph summarizing both CD11b and CD14 reactivity. Data are presented as median and inter-quartile range (error bars) (n = 3 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's post hoc analysis (* $p < 0.05$; ** $p < 0.001$).

3.6 Effects of PSM extract treatment on gene expression in HL-60 cells

To further investigate the anti-proliferative, cytotoxic and differentiation induction of PSM treated cells at the molecular level, gene expression studies using RT-PCR were performed. In this study, the expression levels of genes involved in the regulation of differentiation (*SP1, CEBPA, CEBPB*, *CEBPE,* and *c-MYC*), cell cycle regulation (*CDKN1A* and *CDKN2C*), and apoptosis (*BCL2* and *BAX*) were investigated at 24h and 48h following treatment with PSM. In these experiments, DMSO (1.6%) and PMA (10 nM) were used as positive controls.

3.6.1 Effects of PSM extract treatment on differentiation regulating transcription factors

Within haematopoiesis, C/EBPα, C/EBPβ and C/EBPε are predominantly expressed in the granulocyte and monocytic lineages (Scott *et al*., 1992; Muller *et al*., 1995; Radomska *et al*., 1998). C/EBPε has been identified as a critical regulator of terminal granulopoiesis. Also, PU.1 is a TF with multiple roles in haematopoiesis.

Two time points (24 h and 48 h) were chosen since gene expression follows a dynamic and temporal expression with certain genes expressed earlier and in turn regulate the expression of other genes necessary for lineage commitment. Gene expression of SPI1 might either precede the expression of 'late' acting genes such as C/EBPε or is expressed simultaneously with other TFs (such as C/EBPα and C/EBPβ) to decide cell fate. Also, several studies reported that the major gene expression changes throughout differentiation occurred in the first 48 hours (Rincon *et al*., 2018).

At 24 h treatment [Figure 3-7(A)] the data obtained by RT-PCR indicated down-regulation of *c-MYC* mRNA expression in response to PSM, DMSO, and PMA treatment. The decrease parallels the appearance of differentiation markers in this study [Figure 3-7(A)] and other studies (Filmus & Buick, 1985; Ely *et al*., 1987). The resultant down-regulation of *c-MYC* following treatment with PSM is statistically significant (log2 fold change of -0.75, $p<0.05$) when compared to untreated cells. It has been reported that terminal differentiation and cell growth arrest are essentially induced by *c-MYC* down-regulation (Nguyen *et al*., 1995).

After 48 h treatment, the reduction of *c-MYC* expression remains significantly down-regulated in DMSO (log2 fold change of -0.25) and PMA (log2 fold change of -0.35) treated cells albeit the reduction is less pronounced when compared to 24 h time point. In PSM treated levels, *c-MYC* returns to baseline levels (log2 fold change of 0.20). It has been reported that *c-MYC* expression was drastically reduced within a few hours during differentiation of DMSO-treated HL-60 cells and that expression increased again, and that the expression level remained low afterwards (Siebenlist *et al*., 1988).

The reduction in *c-MYC* RNA transcripts after 24 h treatment with PSM coincides with a significant increase in all TFs [*CEBPA* (log2 fold change of 1.33, $p<0.001$), *CEBPB* (log2 fold change of 3.15, $p<0.001$), *CEBPE* (log2 fold change of 0.5, $p<0.05$), *SPI1*(log2 fold change of 0.74, $p<0.05$)] that play a major role in differentiation of haematopoietic cells. mRNA expression of early acting TFs (*CEBPA*, *CEBPB)* are significantly greater ($p<0.001$) than *SPI1* ($p<0.05$) and the late acting TF *CEBPE* ($p<0.05$) when compared to untreated cells.

At 48 h incubation in PSM, *SPI1* (log2 fold change of -0.15) levels understandably were reduced since lineage committed cells do not require further stimulation by overexpression of *SPI1* and interestingly enough, *CEBPE* is significantly increased (log2 fold change of 1.93, $p<0.001$) after 48 h treatment. The strong and sustained expression of *CEBPB*, when accompanied by transient expression of *CEBPA* leads to the differentiation towards monocytes, while, when accompanied by the sustained expression of *CEBPE*, it leads the differentiation process to granulocytes (Marchwika & Marcinkowska, 2018).

3.6.2 Effects of PSM extract treatment on apoptotic pathways in HL-60 cells

It is clear from the previous results that PSM induces apoptosis as seen in the Annexin V/PI, cell cycle analysis (sub-G0/G1) and morphology results. To further investigate apoptosis at the molecular level, mRNA expression of the *BCL2* and *BAX* gene was carried out. The BCL-2 anti-apoptotic proteins that enhance survival of cells and their complex interaction with pro-apoptotic proteins such as Bax leads to crucial decisions in cell survival and death (Sillar & Enjeti, 2019).

Following 24 h treatment of HL-60 cells with PSM, DMSO, and PMA, a significant reduction of *BCL2* in all treatments is seen when compared to untreated cells [PSM (log2 fold change of -1.28, $p<0.05$; DMSO (log2 fold change of -0.5, $p<0.05$); PMA (log2 fold change of -1.75, $p<0.001$)] [Figure 3-7(B)]. PMA exhibited a more pronounced *BCL2* repression when compared to untreated cells alone. At 48 h treatment, there was a marked and significant reduction in all three treatment conditions when compared to untreated cells [PSM (log2 fold change of -2.29, $p<0.001$; DMSO (log2 fold change of -3.29, $p<0.001$); PMA (log2 fold change of -4.0, $p<0.001$)]. Interestingly, the significant down-regulation of *BCL2* is accompanied by an up-regulation in the pro-apoptotic *BAX*

[Figure 3-7(B)]. After 24 h treatment, there is a significant increase in expression of *BAX* in PSM, DMSO, and PMA treated cells when compared to untreated cells [PSM (log2 fold change of 1.67, $p<0.05$; DMSO (log2 fold change of 1.69, $p<0.05$); PMA (log2 fold change of 2.5, $p<0.001$)]. Again, PMA at 48 h induces a more pronounced and significant increase in *BAX* expression. *BAX* expression following 48 h is more significant among all treatment conditions when compared to untreated cells [PSM (log2 fold change of 3.42, $p<0.001$; DMSO (log2 fold change of 4.38, $p<0.001$); PMA (log2 fold change of 5.0, $p<0.001$)]. When compared to both controls, PSM significance is $p<0.05$ at both 24 and 48 h treatment.

3.6.3 Effects of PSM extract treatment on cell cycle regulators

The expression of Cyclin-dependent kinase inhibitors (CDKI) *CDKN1A* and *CDKN2C* was determined following treatment of HL-60 cells with PSM. Following 24 h treatment, *CDKN1A* is significantly increased in PSM, DMSO, and PMA treated cells [PSM (log2 fold change of 1.64, $p<0.05$; DMSO (log2 fold change of 2.0, $p<0.001$); PMA (log2 fold change of 2.5, $p<0.001$)] when compared to untreated cells [Figure 3-7(C)]. When compared to untreated alone, *CDKN1A* significance is more pronounced following PMA and DMSO treatment ($p<0.001$) compared to PSM treatment ($p<0.05$). PMA and DMSO are potent inhibitors of cell cycle progression and this can also be seen in the cell cycle results with accumulation of 92.2% and 74.4% of PMA and DMSO treated cells in the G0/G1 phase respectively [Figure 3-4(A)]. After 48 h treatment, there is a significant repression of *CDKN1A* in PSM treated cells [PSM (log2 fold change of -4.28, $p<0.001$) when compared to both DMSO, PMA and untreated cells [Figure 3-7(C)]. *CDKN1A* is also downregulated following 48 h in both DMSO and PMA [DMSO (log2 fold change of -0.36; PMA (log2 fold change of 0.5)] treated cells, however, the results are not significant when compared to untreated cells. The down regulation of *CDKN1A* could be explained by the fact

that increased *CDKN1A* expression at 24 h is sufficient to cause cell cycle arrest and induce differentiation. After 48 h, p21, which can act as a tumour suppressor and an oncogene (Kreis *et al*., 2019), is not required and eventually returns back to baseline levels.

At 24 and 48 h treatment, *CDKN2C* mRNA expression is significantly expressed in PSM, DMSO and PMA [(PSM 24 h, log2 fold change of 1.35, $p<0.05$; PSM 48 h, log2 fold change of 0.51, $p<0.05$); (DMSO 24 h, log2 fold change of 1.66, $p<0.001$; DMSO 48 h, log2 fold change of 1.98, $p<0.001$); (PMA 24 h, log2 fold change of 1.0, $p<0.05$; PMA 48 h, log2 fold change of 0.35, $p<0.05$)] treated HL-60 cells when compared to untreated cells [Figure 3-7(C)]. Interestingly, unlike p21, which is a universal cell cycle inhibitor (El-Deiry, 2016), p18 (the protein product of *CDKN2C*) is implicated specifically in granulocytic cell differentiation (Lolascon *et al*., 1998).

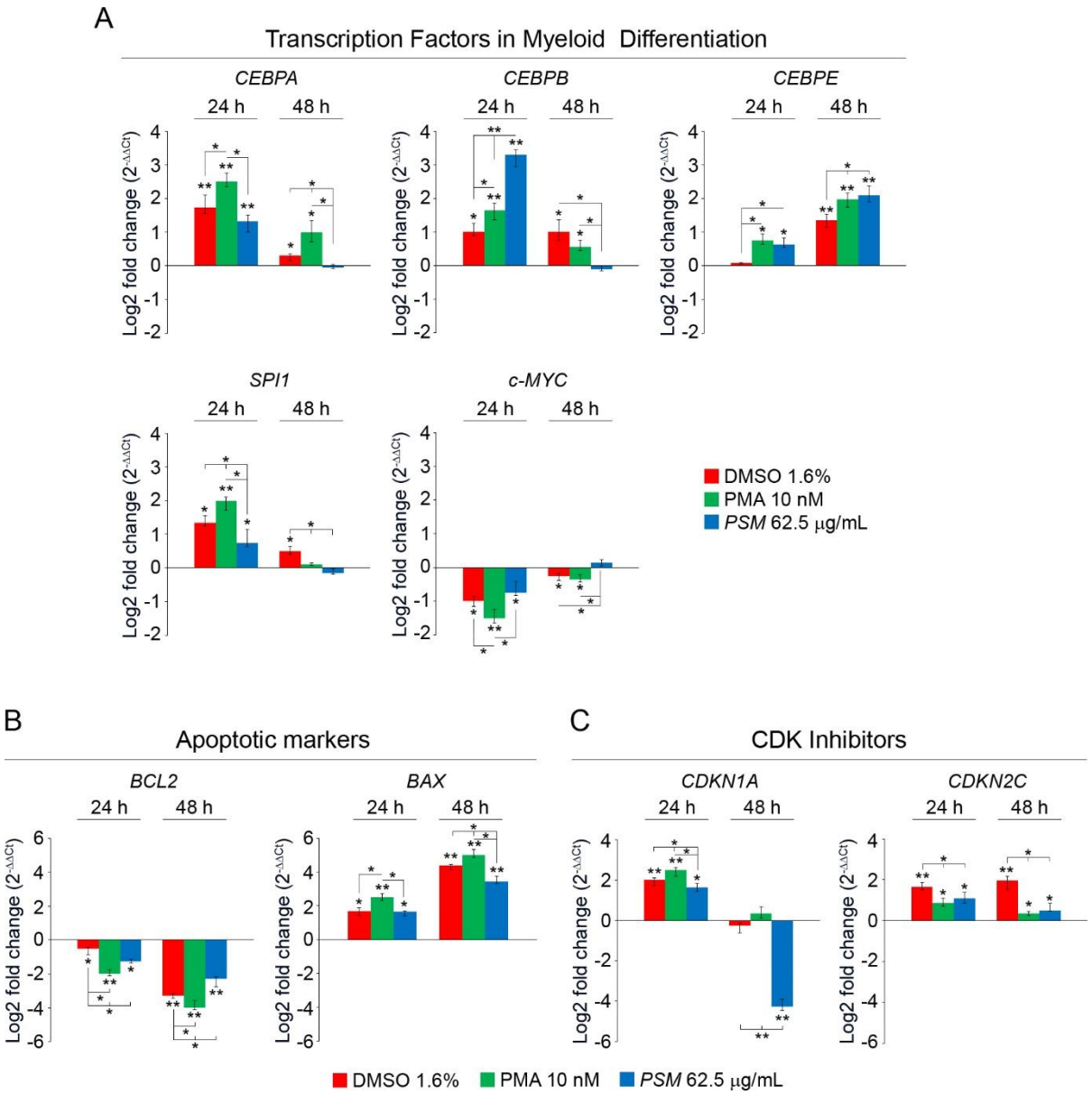

Figure 3-7: Differentially expressed genes in response to PSM extract treatment in HL-60 cells. Cells were treated with PSM extract (62.5 µg/mL) for indicated times. mRNA expression of genes involved in cell differentiation (A), apoptosis (B) and cell cycle (C) regulation was determined by Real-time RT-PCR. HL-60 cells incubated with DMSO (1.6%) and PMA (10 nM) were also used as positive controls. Relative transcript levels were determined using the $2^{\Delta\Delta}$Ct method and normalized to *HPRT1* and *YWHAZ* reference genes. Expression levels in untreated cells were treated as calibrators. Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's post hoc analysis (* $p < 0.05$; ** $p < 0.001$).

Chapter 4

Axolotl Extract Results

4.1 Effects of the axolotl *Ambystoma mexicanum* extract on metabolic activity and cell growth of HL-60 cells

First, the effects of axolotl crude extract (AXO) (regenerative and non-regenerative) was tested on the metabolic activity of HL-60 and KG1a cells by the MTT assay. Both cell lines were treated for various times (24-72 h) with various doses of AXO extract (0.250 – 2.0 mg/mL). 10% DMSO was used as a positive cytotoxic control.

As shown in Figure 4-1(A), both R- and NR- AXO extracts significantly reduced HL-60 metabolic cell activity from 24 to 72 h but only at concentrations of 1.0 and 2.0 mg/mL, compared to untreated cells (p<0.001). In fact, after 72 h treatment, metabolic activity was reduced between 40-50% in both R- and NR- AXO extracts with no significant difference observed between both extracts. Thus, the non-regenerative extract was used for the rest of this study since it is easier to collect and does not require repeated invasive interventions. However, AXO extract slightly reduced KG-1a metabolic cell activity at 72 h at a concentration of 2.0 mg/ mL. In fact, after 72 h treatment, metabolic activity was reduced less than 20% (results not shown). Since preliminary data showed that AXO extract is particularly effective on HL-60 cells, further investigations using only the HL-60 cell line were carried out.

Next, the antiproliferative effects of AXO extract on HL-60 cells was examined by the trypan blue assay. In these experiments, as shown in Figure 4-1(B) and 4-1(C), even though the cell counts increased when compared to Day 0, however, there was a significant reduction in cell numbers after 24 h treatment with 2mg (p<0.05) AXO when compared to untreated cells on the same day. Following 48 h treatment, concentrations between 0.5-1mg (p<0.05) and 2mg

(p<0.001) showed a significant reduction in cell number when compared to untreated cells on the same day. At 72 h, even the lowest concentration (0.25mg) was significant (p<0.05) when compared to untreated cells. In fact, at 72 h a dose-dependent effect was observed with growth inhibition of ~ 15, 24, 32 and 50% for 0.25, 0.50, 1.00 and 2.00 mg/mL, respectively, compared to untreated cells on the same day. Importantly, growth inhibition caused by AXO extract treatment was not due to a decreased viability [Figure 4-1(D)] but rather a suppression in cell numbers.

Based on the obtained data, to investigate the possible anticancer effects of the *A. mexicanum* extract, all the subsequent experiments were carried out by incubating HL-60 cells with the 2.0 mg/mL concentration for 72 h.

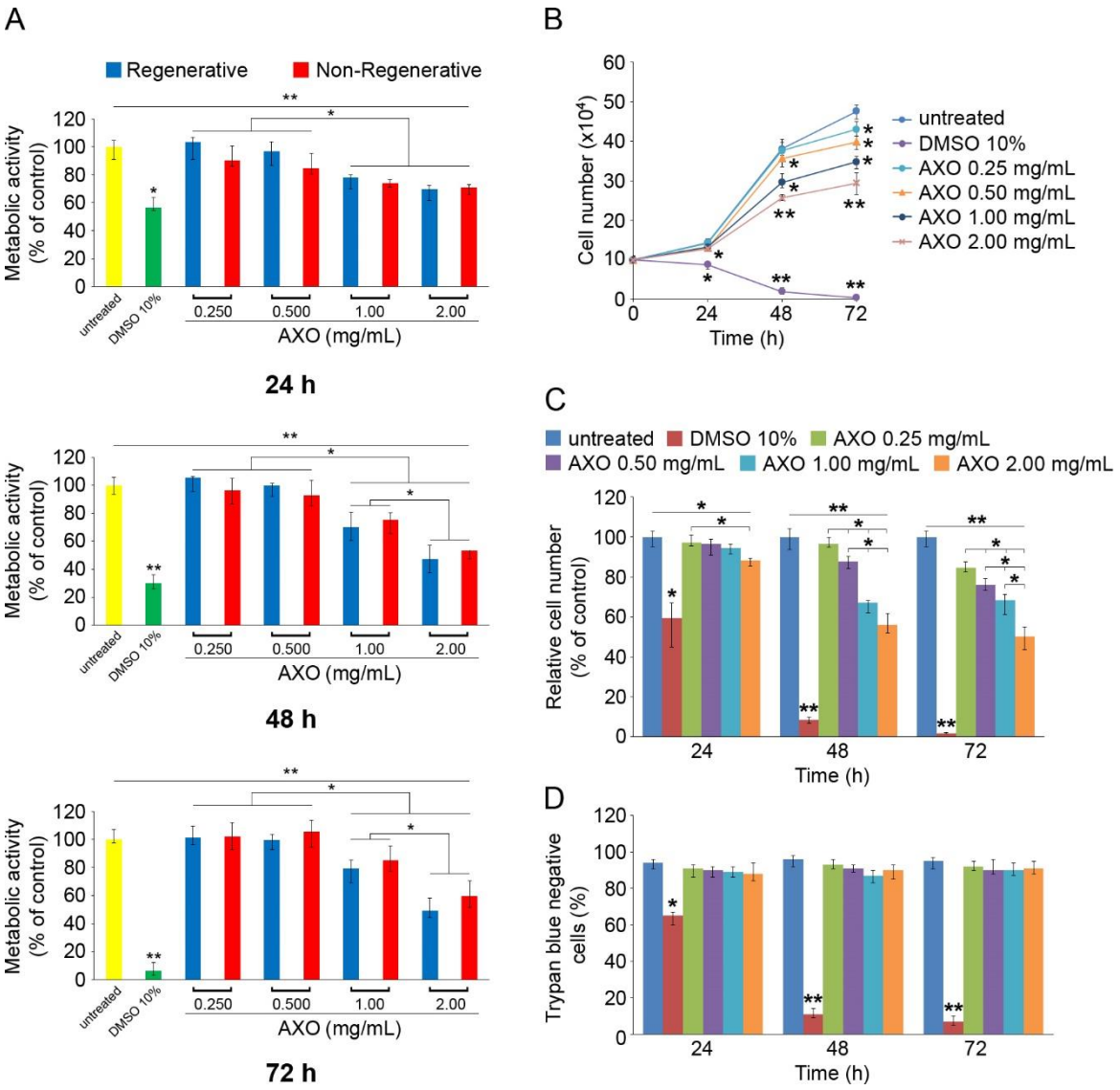

Figure 4-1: Effects of the axolotl (AXO) crude extract on metabolic activity (A), cell proliferation (B), relative cell growth (C), and cell viability (D) of HL-60 cells. Cells were treated with AXO or DMSO extract as indicated for 72 h. The metabolic activity was determined by MTT. In graphs A and C, treatment was expressed as a percentage of the control (untreated cells) on the same day. The number of viable cells was determined by trypan blue exclusion. Data are presented as median and inter-quartile range (error bars) (n = 3 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's post hoc analysis (* $p < 0.05$; ** $p < 0.001$).

4.2 Cell cycle analysis of HL-60 cells after treatment with axolotl *Ambystoma mexicanum* extract

To further explore the effect of AXO extract on growth of HL-60 cells, flow cytometry analysis of PI-stained nuclei was performed. In these experiments, HL-60 cells incubated with DMSO (1.6%) and PMA (10 nM) were used as positive controls for differentiation. As shown in Figure 4-2(A) and 4-2(B), DNA content from flow cytometry analysis indicated that among the untreated cells, 58.2% were distributed in G0/G1 phase, 26.4% were accumulated in S phase, 13.1% were in G2/M phase and only 2.3% were in sub-G0/G1. When HL-60 cells were incubated with AXO extract (2.0 mg/mL) for 72 h, a significant increase (p<0.001) in the number of cells accumulated at the G0/G1 phase (75.1%), with concomitant loss of S- (16.8%) and G2/M- (7%) phases when compared to untreated cells. Also, treatment with 1.6% DMSO and PMA resulted in a block or cell cycle arrest at the G1 phase of the cell cycle of 85.5% and 93.4%, respectively. Taken together, the lack of a sub-G0/G1 phase and accumulation of cells at the G0/G1 phase following treatment with AXO further confirms that the extract does not exhibit a cytotoxic effect and inhibition of cell proliferation of HL-60 cells is induced via a G0/G1 phase arrest.

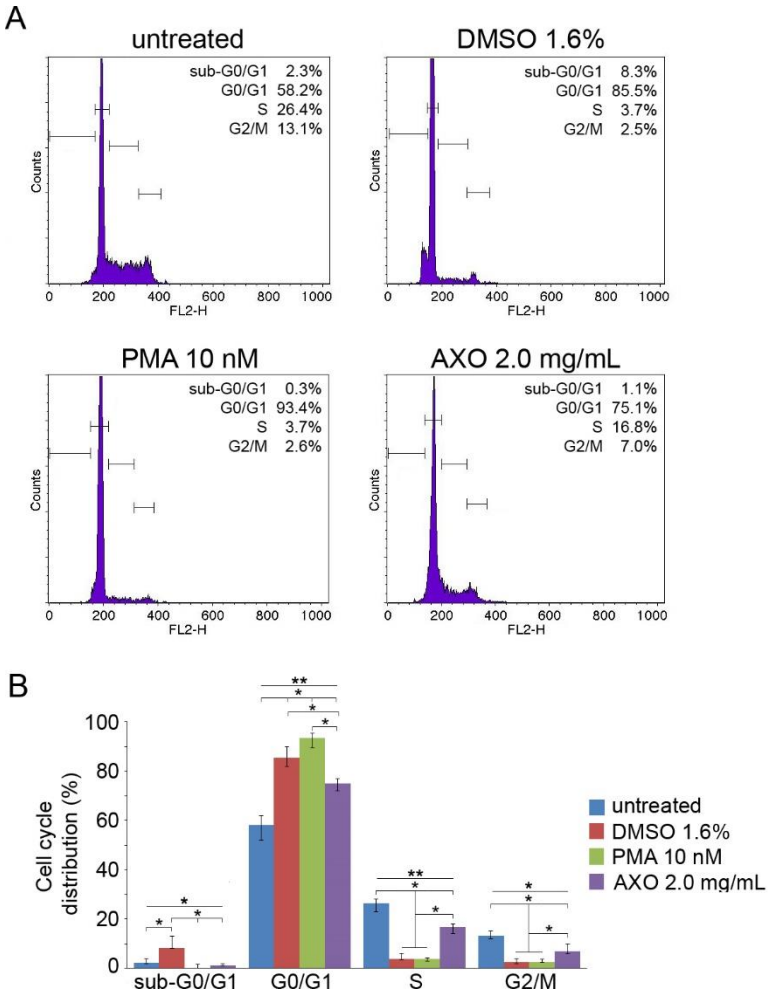

Figure 4-2: Effects of AXO extract (2.0 mg/mL) treatment on cell cycle distribution in HL-60 cells after 72 h. (A) Histogram plots of flow cytometry analysis performed in HL-60 cells. (B) Graph summarizing the cell cycle distribution. DMSO (1.6%) and PMA (10 nM) were used as positive control for granulocytic and monocytic differentiation, respectively. Data are presented as median ± inter-quartile range (n = 2 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis test. Differences between groups were subsequently determined by Dunn (* $p < 0.05$; ** $p < 0.001$).

4.3 Induction of differentiation in HL-60 cells after treatment with *Ambystoma mexicanum* extract

Since proliferation inhibition is tightly correlated to a terminal differentiation program (Ruijtenberg & van den Heuvel, 2016), the effect of AXO extract on the differentiation of HL-60 cells was also investigated. Analysis of morphological changes of HL-60 cells treated with AXO extract was investigated following Leishman staining. In these experiments, HL-60 cells were incubated with DMSO (1.6%) and PMA (10 nM), used as positive controls for granulocytic and monocytic differentiation, respectively. As evidenced by microscopy [Figure 4-3(A)], untreated cells showed typical immature leukaemia blast morphology with cells exhibiting a high N/C ratio and small rim of basophilic cytoplasm, while AXO extract (2.0 mg/mL) reduced the N/C ratio in HL-60 cells at 72 h.

The NBT assay was performed to detect the expression of oxidative burst enzymes during cell differentiation [Figure 4-3(B)] with an increase in NBT positive cells (15%) after treatment by AXO extract (2.0 mg/mL) for 72 h.

To further characterize the differentiation mediated by AXO extract, flow cytometry analysis for myelomonocytic markers CD11b and CD14 was performed. In these experiments, HL-60 cells incubated with DMSO (1.6%) and PMA (10 nM) were used as positive controls for granulocytic and monocytic differentiation, respectively. A significant increase (55.41%) in the presence of CD11b-positive cells was observed in HL-60 cells treated with AXO extract (2.0 mg/mL) for 72 h; whereas the proportion of CD14-positive cells did not change [Figure 4-4(A

and B)]. Overall, these findings suggest that AXO extract is capable of inducing granulocytic differentiation in HL-60 cells *in vitro*.

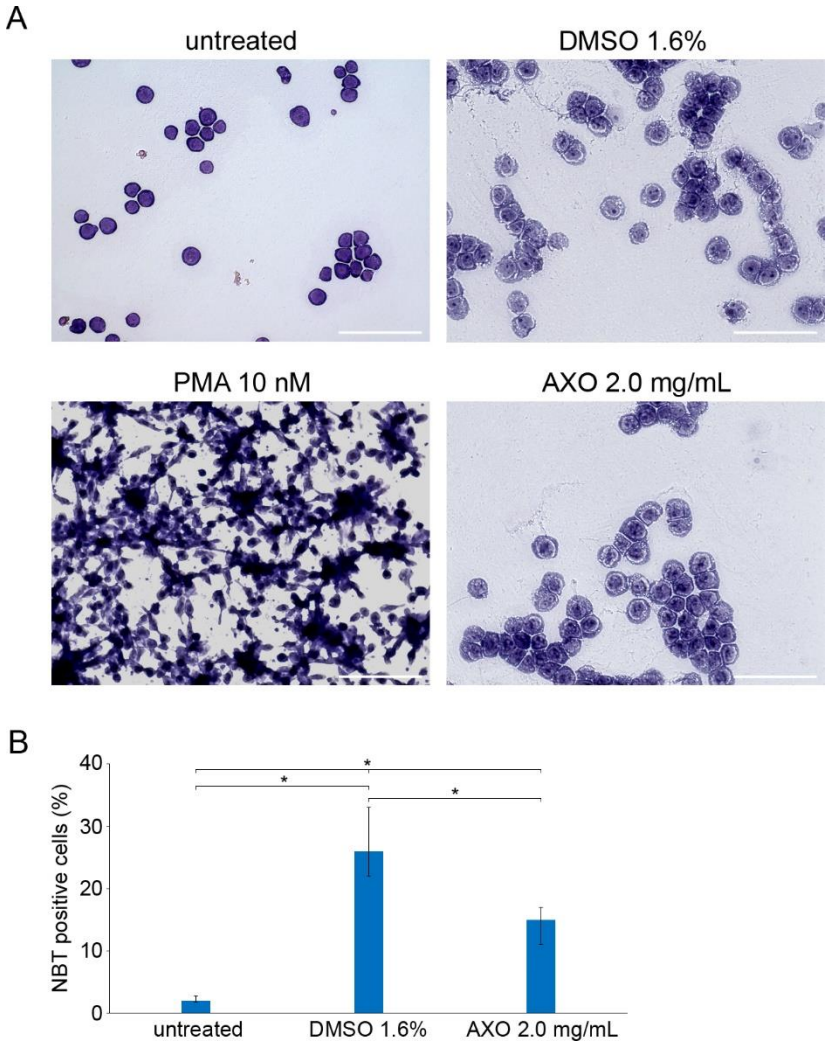

Figure 4-3: AXO extract changes the morphology of HL-60 cells. (A) Leishman staining. The scale bar is 100 µm. The images were a representative of three independent experiments. DMSO (1.6%) and PMA (10 nM) were used as positive controls for granulocytic and monocytic differentiation, respectively. (B) Nitroblue tetrazolium (NBT) reduction assay. Cells were treated with AXO extract (2.0 mg/mL) for 72 h. HL-60 cells incubated with DMSO (1.6%) were used as positive controls for granulocytic differentiation. Graph summarises the percentages of NBT positive cells. Data are presented as median and inter-quartile range (error bars) (n = 3 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

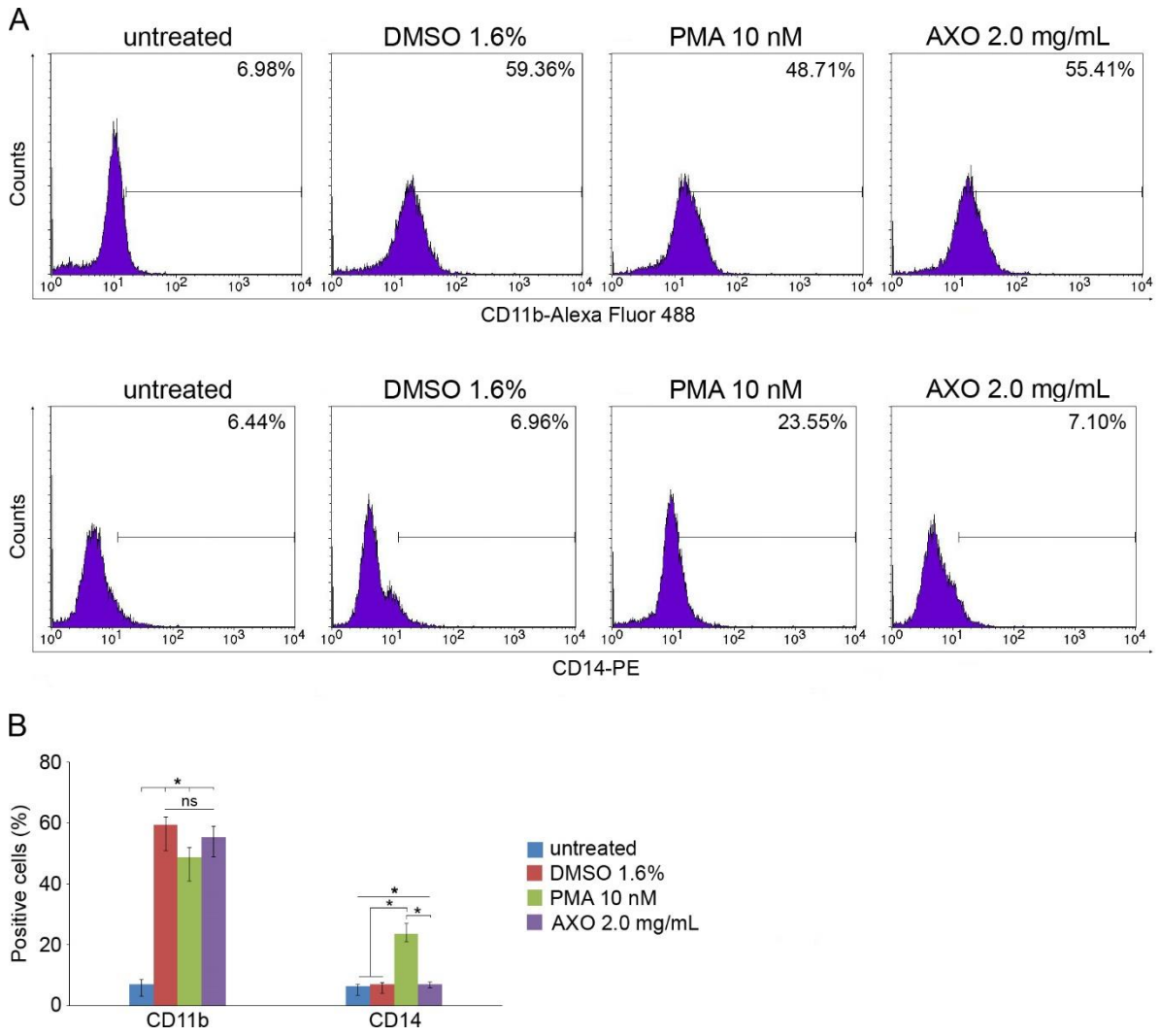

Figure 4-4: AXO extract-induced cell differentiation in HL-60 cells. (A) Cytometric analyses showing cell surface expression of CD11b (left panels) and CD14 (right panels) in HL-60 cells treated with DMSO (1.6%), PMA (10 nM) and AXO extract (2.0 mg/mL) for 72 h. (B) Graph summarizing both CD11b and CD14 reactivity. Data are presented as median and inter-quartile range (error bars) (n = 3 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ns, not significant).

4.4 Effects of Ambystoma mexicanum extract treatment on gene expression in HL-60 cells

To investigate the molecular mechanisms by which AXO extract may alter the cell cycle progression and the differentiation state of HL-60 cells, the expression of genes involved in the regulation of differentiation (*CEBPA*, *CEBPB*, *CEBPE*, *SPI1 and c-MYC*) and cell cycle (*CDKN1A* and *CDKN2C*), was quantified by RT-PCR real-time analysis. In these experiments, HL-60 cells incubated with DMSO (1.6%) were used as a positive control for granulocytic differentiation.

As shown in [Figure 4-5(A and B)], the treatment with AXO extract (2.0 mg/mL), compared to untreated counterpart, significantly increased the expressions of *CEBPA* (log2 fold change of 0.15, $p<0.05$), *CEBPB* (log2 fold change of 1.15, $p<0.05$), *CEBPE* (log2 fold change of 0.35, $p<0.05$), *SPI1* (log2 fold change of 0.85, $p<0.05$), *CDKN1A* (log2 fold change of 1.44, $p<0.001$) and *CDKN2C* (log2 fold change of 0.85, $p<0.05$); whereas strongly reduced the expression of *c-MYC* (log2 fold change of -0.75, $p<0.05$) after 48 h treatment. The results also show that, after 72 h, the treatment with AXO extract further increased the expressions of *CEBPE* (log2 fold change of 1.35, $p<0.001$) and *CDKN2C* (log2 fold change of 2.0, $p<0.001$); whereas the other genes showed an expression trend similar to that obtained in cells treated with AXO extract for 48 h.

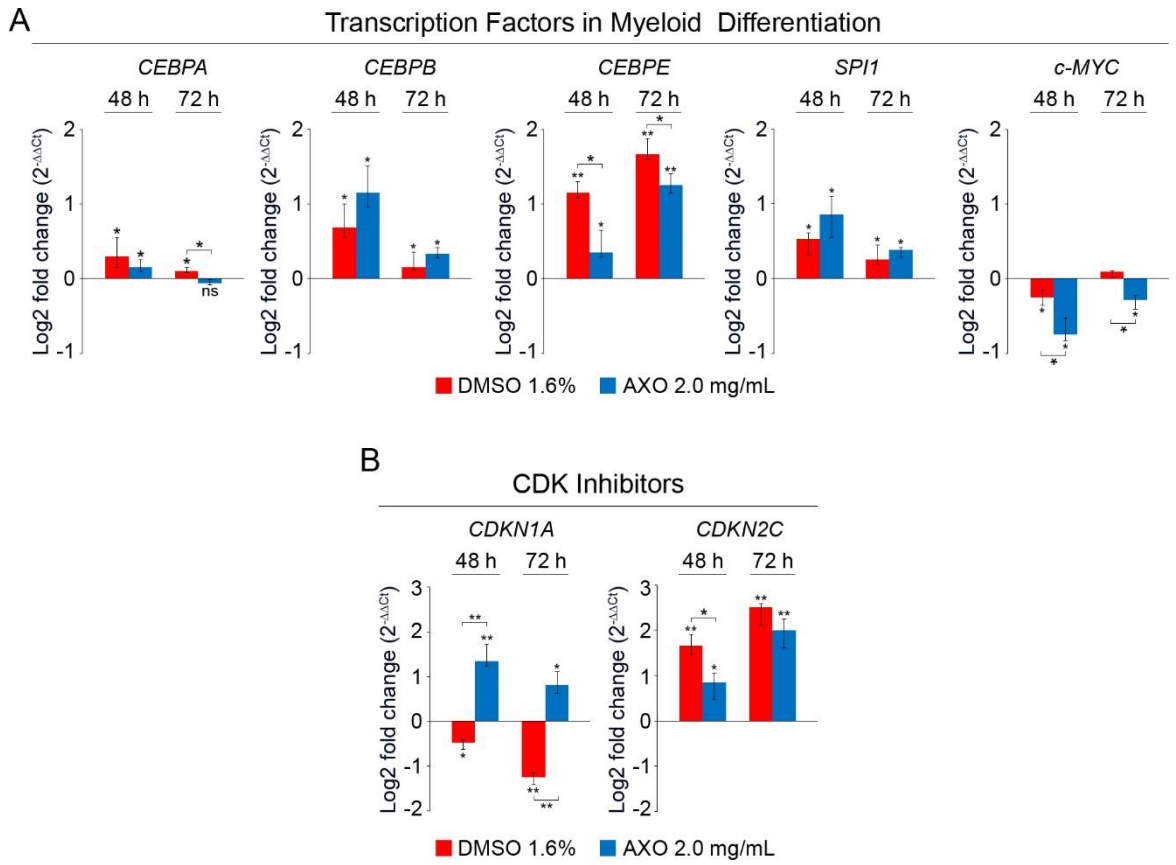

Figure 4-5: Differentially expressed genes in response to AXO extract treatment in HL-60 cells. Cells were treated with AXO extract (2.0 mg/mL) for indicated times. mRNA expression of genes involved in cell differentiation (a) and cell cycle (b) regulation was determined by Real-time RT-PCR. HL-60 cells incubated with DMSO (1.6%) were also used as positive controls for granulocytic differentiation. Relative transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to *HPRT1* and *YWHAZ* housekeeping genes. Expressions in untreated cells were treated as calibrators. Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analysed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

Chapter 5

Discussion

This is the first study that tested extracts from two regenerative organisms, a fresh water planarian species Malta (PSM) and the axolotl *Ambystoma mexicanum* (AXO) on their anti-tumour activity in terms of modulating proliferative activity, cell viability, cell cycle arrest, and differentiation. Both PSM and AXO showed interesting results with the PSM extract exhibiting a selective cytotoxic effect on HL-60 cells when compared to normal lymphocytes. PSM cytotoxicity was confirmed by morphology, cell cycle analysis and Annexin V/PI assay which revealed that the PSM extract induced apoptosis in HL-60 cells. AXO crude extract exhibited antiproliferative but not cytotoxic activities on HL-60 cells, with cell cycle arrest in the G0/G1 phase. Both PSM and AXO extract clearly showed a degree of morphological maturation with a reduction of the nucleo/cytoplasmic ratio of the HL-60 cells, and an increase in nitroblue tetrazolium-positive cells. Furthermore, PSM treated cells showed an increase in the expression of the differentiation markers CD11b- and CD14-positive cells, whilst AXO-treated HL-60 cells showed an increase in the expression of CD11b, suggesting that the extracts were able to stimulate myeloid differentiation. Finally, PSM and AXO extracts caused upregulation of transcription factor genes directly implicated in cell lineage commitment and maturation (*CEBPA, CEBPB, CEBPE, SPI1*), in cell cycle regulatory genes *(CDKN1A and CDKN2C*) and downregulation of the oncogene *c-MYC*. The data clearly show the potential anticancer activity of PSM and AXO on HL-60 cells and suggest that it could help develop promising therapeutic agents for the treatment of AML.

The PSM and AXO extract were also tested on the KG-1a cell line (results not shown). The preliminary data showed no significant reduction of metabolic activity after treatment with both the regenerative and non-regenerative extracts. This could be explained by the fact that

KG-1a cells, unlike the HL-60 cell line, exhibit a primitive population of $CD34^+$ $CD38^-$ and the cell line is resistant to differentiation (Greenfield et al., 2012) and thus only the HL-60 cell line was used for further studies.

In this discussion, the effects of both PSM and AXO extracts on HL-60 cells will be outlined. Firstly, the morphological changes seen in HL-60 cells after treatment with both extracts will be discussed, including how the morphological changes after 48- and 72-hour treatment compare with known differentiating agents and other extracts. Also, the way how such changes coincide with the expression of myeloid surface antigens will be showcased. Then, the cell cycle results will be highlighted, and compared with examples from other studies and whether cells can still commit to differentiation even though they are not arrested at the G0/G1 phase of the cycle. Next, the importance of regulating the oncogenic TF *c-MYC* mRNA, which is implicated in neoplastic transformation, will be elaborated on. The following section of the discussion will deal with genes responsible for regulating the cell cycle. In this section, the focus will be on two particular genes, *CDKN1A* and *CDKN2C*, and together with results from other studies using known differentiating agents and natural products, the importance of the results will be emphasised, especially in terms of the anti-proliferative effect the extracts exhibit on HL-60 cells. Confirmation of lineage commitment with up-regulation or reactivation of lineage-specific genes (*CEBPA, CEBPB, CEBPE,* and *SPI1*) following treatment with PSM and AXO will be the subject of the next section. Finally, the probable molecules of interest will be discussed together with the possible pathways implicated in the differentiation of HL-60 cells.

In recent decades, AML treatment remained more or less the same, with chemotherapy as the main therapeutic approach. The constant and urgent need to develop alternative drugs for

cancer therapy is not solely due to resistance of cancer cells to therapeutic drugs, but also due to the undesirable and toxic side effects the patients experience including nephrotoxicity, neuropathy, cardiotoxicity, hepatotoxicity, and ototoxicity. Novel targeted therapies might offer the promise of efficacy with reduced toxicity. Therefore, it is urgent to develop novel therapeutic approaches to overcome these problems.

One of the important hallmarks of cancer cells is poor differentiation, and therapeutic agents that cause cells to differentiate hold great promise for cancer treatment (Yan & Liu, 2016. This approach targets the reactivation of the differentiation programs within cancer cells, and in doing so, cells are able to resume the maturation process and eliminate tumour phenotypes (Yan & Liu, 2016). Generally, differentiation agents are considered less toxic than conventional cancer treatments (de Thé, 2018; Yan & Liu, 2016). Ultimately, any bioactive molecule/s that can offer a combinatorial effect by inducing differentiation and apoptosis with reduced toxicity to the patient's normal cells would be at the forefront in the fight against cancer.

Increasing evidence from cancer studies indicate bioactive compounds derived from natural sources may act as potentially promising therapeutic agents in the treatment of human cancers (Mushtaq *et al*., 2018; Khalifa *et al*., 2019; Newman & Cragg, 2020). A PubMed search of 'natural compounds' reveals that in the last decade alone, more than ninety thousand publications have reported on the therapeutic properties of natural compounds. The effects of natural compounds were studied in both communicable and non-communicable diseases including their cytoprotective effects (Mehta *et al*., 2018), drug development against cancer (Sauter, 2020), diabetes (Patle *et al*., 2020), antimicrobial properties (Franco & Vazquez, 2020), and recently as potential agents against the Covid 19 coronavirus (Khan *et al*., 2020), amongst

many other ailments (Woodbury *et al*., 2018). In the last 50 years, there were over two hundred and forty approved anti-cancer drugs out of which almost 40% are derived from natural sources (Newman & Cragg, 2020).

It is well known that for any anticancer agent to be effective and acceptable, it has to meet various criteria, including its effects on normal cells being relatively harmless (Demir *et al*., 2018). So, the effects of PSM and AXO crude extracts on HL-60 cells and that of PSM on normal human proliferating lymphocyte cells was examined. Initially, both regenerative and non-regenerative extracts were used for treatment of HL-60 cells. Since in the case of both animals the two types of extracts did not show any significant differences in experimental results, subsequent experiments were all carried out using the non-regenerative extracts due to ease of collection and reduction in experimental manipulations. The PSM extract exhibited a selective anti-proliferative effect of 2.7-fold against HL-60 cells compared to activated lymphocytes. This suggests that PSM extract could include promising candidates for cancer therapy. Since the AXO extract was determined to be non-toxic, toxicity studies on normal human proliferating lymphocytes were not carried out.

HL-60 cells have a high N/C ratio, a regular basophilic cytoplasmic rim, and lightly stained nucleus due to lack of chromatin condensation. After treatment with both PSM and AXO, the HL-60 cells showed a decreased N/C ratio with a clear cytoplasm and irregular cytoplasmic membrane when compared to untreated cells. Morphologically, HL-60 cells treated with PSM and AXO resembled the appearance of "paramyeloid' cells with a granulocyte-like nucleus, but monocyte-like cytoplasm similar to those described by Zinzar *et al*., 1989. Cells called "paramyeloid" were also described in patients with chronic myelomoncytic leukaemia (Zinzar *et*

al., 1989). Morphologically, these cells had cytoplasmic and nuclear features that resembled an intermediate between monocytes and granulocytes. The cells lacked nuclear indentations and resembled the 'partially differentiated' HL-60 cells seen following treatment with Arsenic trioxide (Cai *et al*., 2000). The reduction of N/C ratio in PSM and AXO treatment and condensation of chromatin after PSM treatment are similar to the morphological changes described in other studies following treatment with organic plant extracts such as coumarin (Huang *et al*., 2019), arsantin, a sesquiterpene lactone compound present in *Artemisia santolina* (Kweon *et al*., 2015), and clinically used differentiation inducing agents such as ATRA (Tasseff *et al*., 2017). The morphological changes seen after treatment with PSM at 48 h and AXO at 72 h are similar to those reported by another study on HL-60 cell differentiation which described an early, intermediate (24-72 h) and late (96-120 h) temporal morphological changes in HL-60 (Ramirez *et al*., 2017).

Overall, PSM treated cells exhibited a monocyte-like cytoplasm and increased expression of CD11b and CD14, typical of monocytic differentiation, however, the cells did not show an increased adherence to plastic typical of monocytic/macrophage differentiation as seen with PMA treatment. Different compounds can induce morphological and behavioural changes to different extent over time (Zinzar *et al*., 1989) and it is possible that the time point chosen in this study was not sufficiently delayed to detect surface adherence. One study showed that PMA treatment of HL-60 cells caused cell adherence typical of macrophages, while the agent VITD3 resulted in monocytic morphology without adherence (Miyaura *et al*., 1985), similar to PSM treated cells. Interestingly, a myeloid differentiation study using inducible PU.1 in PU.1 null myeloid cell progenitors in mice, report that low level PU.1 expression activates a mixed macrophage/neutrophil gene expression, which could explain the mixed- morphological

appearance already described (Laslo *et al*., 2006). This may be similar to what is happening here. AXO treated cells, although appearing similar in morphology to PSM treated cells, showed the expression of CD11b and lack of CD14 which is a granulocytic signature.

PSM- and AXO-treated cells resembled early committed precursors, and as such, this implies that even though the HL-60 leukaemia cells have committed to differentiation, they may continue to divide before acquiring a fully differentiated state. In AXO treatment, this is reflected by a reduced rate of cell proliferation when compared to the untreated control with an increase in the G0/G1 peak with reduction in S- and G2/M-phases but without complete inhibition of proliferation. The accumulation of AXO treated cells at G0/G1 junction is similar to DMSO treated cells in this study and to ATRA and DMSO treated cells in other studies. (Huang *et al*., 2019; Chen *et al*., 2013). HL-60 cells showed similar cell cycle arrest at the G0/G1 phase following treatment with both animal and plant (Lee et al., 2007) natural compounds.

In PSM treatment, there is no clear cell accumulation at the G0/G1 phase. Brown *et al*., 2002 similarly reported that HL-60 cells treated with ATRA committed to differentiation before cell cycle arrest, with differentiating cells not restricted to any particular stage of the cell cycle. Furthermore, HL-60 cells induced to terminal differentiation in response to ATRA traverse two to three cycles and are CD11b positive before halting division (Tasseff *et al*., 2017; Drayson *et al*., 2001). These cells showed elements of innate immune responses, concomitant with the acquisition of differentiated function despite ongoing expansion of cell numbers (Brown *et al*., 2002). Such gain in immune function can also be beneficial in the management of patients with leukaemia and particularly neutropenic patients with the disease.

Numerous anticancer activity studies investigating novel agents focus not solely on the ability to halt the cell cycle, but also to induce apoptosis (Jain *et al*., 2017; Demir *et al*., 2018). The PSM extract induced accumulation of the cells at the sub-G0/G1 phase, which is indicative of DNA fragmentation and cell death with concomitant loss of cells in the S-phase. The apoptotic effect of PSM was also evident morphologically with condensed nuclei stained with Hoechst 33342 and Annexin V/PI using flow cytometry. Exposure to the AXO extract, on the other hand, showed no sub-G0/G1 population suggesting a non-apoptotic mechanism of reduced cell numbers in HL-60 cells.

Even though PSM-treated cells appear to show a degree of morphological maturation, the lack of the G0/G1 peak commonly associated with cell cycle arrest and differentiation questions the differentiation potential of the extract. Is a successful differentiation induction solely detected by morphological terminal differentiation? Can the acquisition of myeloid surface antigens and molecular characteristics distinguishing treated cells from their untreated early progenitors be sufficient? Terminally differentiated cells are usually linked with an arrest in proliferative capacity and a permanent exit from the division cycle (Ruijtenberg & van den Heuvel, 2016) with drugs targeting cell cycle inhibition driving a rapid termination process and demonstrating that the cell cycle is a principle rate-limiting step of differentiation throughout early and late stages (Li & Kirschner, 2014). Overall, it appears that there is an inverse relationship between proliferation and differentiation. Within this context, cells lose their capacity to divide, with a resultant temporal increase at the G0/G1 phase before or during the acquisition of mature characteristics. However, HL-60 cells can in fact differentiate to neutrophils or monocytes when cell cycle progression is suppressed in early/mid-G1 by

quinidine, at the G1/S boundary by thymidine (Studzinski *et al*., 1985), or in S phase by aphidicolin.

Furthermore, HL-60 Cells exposed to VITD3 underwent monocytic differentiation with a series of rapid maturation divisions and shortening of the G1 phase of the cycle (Brown *et al*., 1999). On the other hand, Vit D3-treated U937 cells (human monoblastic leukaemia) also displayed a brief burst of increased proliferation rate together with functional activation of numerous genes and proteins linked to proliferation prior to cell cycle arrest and differentiation (Rots *et al*., 1999). Another group investigating the signalling and effector mechanisms during differentiation of U937 cells suggested that separate pathways regulate differentiation and p21CIP1 expression/cell cycle arrest in ceramide-stimulated cells (Ragg *et al*., 1998). Also, differentiation of HL-60 using natural grape extracts was one in which the G1 to S transition was unhindered (Della Ragione *et al*., 1998). Overall, it appears neither position in, nor transit through, the cell cycle greatly influences a cell's ability to initiate the gene expression programs that underlie differentiation.

The differentiation at different cell cycle phases is not restricted to leukaemia cell lines. Other differentiation processes, including adipocyte development (Ullah *et al*., 2015) and B-lymphocyte maturation, also involve concurrent proliferation and differentiation. Tang *et al., 1999* inhibited proliferation of oligodendrocyte precursors *in vitro* by overexpression of the cdk-inhibitor p27 protein, resulting in cell cycle arrest without expression of differentiation proteins. This suggests that cell-cycle arrest alone is not sufficient for differentiation, and that other signalling pathways are also needed. Chen *et al*., 1999 demonstrated that differentiated myocytes incorporated bromodeoxyuridine (BrdU) into nuclei, further demonstrating that entry

into the S phase of the cell cycle can still occur in these cells. Also, studies reveal that induction of differentiation can occur when cells are arrested at G2/M phase with cancer cells lines including the human colorectal adenocarcinoma cell line Caco2 maturing following G2/M cell cycle arrest (Zarrilli *et al*., 1999). Taken together, it appears differentiating agents regulating proliferation and maturation, seem at least in part, to be independently controlled until a cell is terminally differentiated after which it exits the cell cycle. Hence, differentiation can be stimulated in HL-60 cells with the cell's ability to execute the gene expression programs that initiate differentiation regardless of whether the cells continue to proliferate normally or are arrested at various points in the cell cycle. So, the path leading to differentiation does not necessarily result in an abrupt halt to the cell cycle. This suggests that active maintenance of cell-cycle arrest is an important feature of the differentiated state.

The reduced proportion of PSM treated cells in the S phase and increased Sub-G0/G1 can be indicative of cells that are no longer traversing the S- to G2/M phase transition and hence have undergone cell cycle arrest as reported in another study (Ragg *et al*., 1998) prior to induction of apoptosis. This could be attributed to a mechanism similar to chemotherapeutic drugs whereby following treatment, DNA breakages accumulate primarily at the S- phase of the cell cycle, unable to proceed to the G2/M phase, with resultant apoptotic cell death (Martino *et al*., 2019). However, this is purely speculative as assessment of double stranded breaks in chromatin with the appearance of DNA breakage markers (ϒH2AX in chromatin) typically seen after treatment of cancer cells with chemotherapeutic drugs was not performed in this work.

The idea that therapeutic regulation of TF activity might be important in overcoming the differentiation block in myeloid leukaemia has already been proven by several experiments

using human leukaemia cell lines and mouse models (Rosenbauer & Tenen, 2007). The overexpression of the TF *c-MYC* mRNA and its protein product has been associated with neoplastic transformation in a variety of cancers (Felsher, 2010). Perturbation of *c-MYC* levels appears necessary for modulating the growth of malignant cells, particularly those of hematopoietic origin (Sirinian *et al*., 2003). In HL-60 cells, *c-MYC* is amplified and numerous studies underlined the importance of *c-MYC* inhibition in suppression of proliferation, which coincides with increased mRNA expression of TFs associated with differentiation (Ren *et al*., 2017). Regulation of *c-MYC* has been reported in leukaemia cells during differentiation by ATRA (Dimberg *et al*., 2002). The effect of different natural compounds including flavonoids on *c-MYC* expression and regulation in HL-60 cells has also been extensively studied (Yu *et al*., 2016; Huang *et al*., 2010).

AXO treatment of HL-60 cells resulted in a significant reduction of *c-MYC* mRNA expression and similar to DMSO treatment, it appears that AXO exhibits potent anti-proliferative characteristics. This *c-MYC* message reduction is also in agreement with other results where HL-60 cells were treated using granulocytic (ATRA) and monocytic (VITD) differentiating agents (Salvatori *et al*., 2011; Xu *et al*., 2009; Jiang *et al*., 2008; Eckhardt *et al*., 1994). Discussing *c-MYC* over-expression Pan *et al*., 2014, reported that inhibition of *c-MYC* rescued the sensitivity to cytotoxic drugs in drug resistant leukaemia cells, and restrained the colony formation ability and promoted differentiation of primary leukaemia patient cells. The transient increase in expression of *SPI1 (PU.1)* and reduction in *c-MYC* expression at 24 hours in PSM treated cells, could indicate that PSM-induced HL-60 differentiation is unstable, with possible retro differentiation and/or the accumulation of PSM resistant HL-60 cells occurring. A loss of labile PSM components may be responsible for this incomplete effect and a constant/repeat PSM

stimulation may therefore be required to maintain growth arrest and differentiation. Similar unstable results were described following treatment of U937 cells with PMA (Otte *et al*., 2011). Recovery of *c-MYC* expression has been reported in other studies whereby *c-MYC* expression was sharply reduced within a few hours during differentiation with nicotinamide and DMSO-treated HL-60 cells but increased again, and was thereafter maintained albeit at low levels (Ida *et al*., 2008; Siebenlist *et al*., 1988). These studies suggested that two distinct mechanisms of transcriptional control operate on *c-MYC* during agent-induced differentiation of HL-60 cells with an early reversible downregulation and a late non-reversible downregulation (Siebenlist *et al*., 1988).

In haematological malignancies, alterations of cyclin dependent kinase inhibitors (CDKIs) gene expression has been extensively investigated (Abou Zahr & Borthakur, 2017; Ghelli Luserna di Rora' *et al*., 2017). G1 phase CKIs might play a significant role not only as tumour suppressor genes but also in differentiation cell fate. To evaluate the biological roles of CDK inhibitors during the differentiation of HL-60 cells, mRNA expression levels of *CDKN1A* and *CDKN2C* following treatment of HL-60 cells with PSM and AXO were evaluated. Both PCM and AXO showed an increase in *CDKN1A* after 24 h and 72 h respectively. In another study, following treatment of HL-60 cells with the differentiating agents PMA and RA, there was an increase in the expression of the *CDKN1A* protein product, $p21^{WAF1}$.

The sustained increase of *CDKN1A* after treatment with AXO implies that this may be a means for relative cell cycle arrest and activation of differentiation in HL-60 cells (which lack functional p53). Interestingly, p53-null cancer cells usually respond less efficiently to direct cytotoxic chemotherapy (Heintz *et al*., 2017). Similarly, upregulation of *CDKN1A* protein product, p21WAF1, was reported to occur during cell cycle arrest and terminal differentiation

of HL-60 cells by ATRA (Horie *et al*., 2004). Treatment of HL-60 cells with phenolic compounds also caused inhibition of cell cycle progression and upregulation of p21WAF1 with induction of differentiation (Fabiani *et al*., 2008). One example is seen following treatment of HL-60 cells with acteoside (plant extract) which resulted in an increase in the protein and mRNA levels of CDKIs including p21 with the binding of p21 to CDK4 and CDK6, resulting in the reduction of CDK2, CDK4 and CDK6 activities which are important for cell cycle progression. A similar mechanism could explain the cell cycle arrest seen following treatment of HL-60 cells with AXO where the increase in *CDKN1A* represses the expression of CDKs implicated in cell cycle progression.

DMSO treatment in this study showed an early upregulation of *CDKN1A* followed by sustained down-regulation over 48- and 72 h treatment. PMA treatment showed an early upregulation followed by a return to baseline levels after 48 h treatment. These results are similar to others reported where in ATRA-resistant HL-60 cells treated with VITD3, the levels of p21*WAF1/CIP1* transcripts increased about twofold after 6 hours, and then decreased toward baseline levels over the remaining 42 hours (Muto *et al*., 1999). In another study transient expression of p21 without the addition of a differentiating agent, also resulted in the appearance of morphologically mature cells that expressed both CD11b and CD14 (Liu *et al*., 1996).

Miranda et al., 2002 demonstrated the involvement of the mitogen activated protein kinase (MAPK) pathway in myeloid differentiation with induction of monocytic differentiation in HL-60 cells by treatment with PMA leading to rapid and sustained activation of MEK (MAP-1/-2), extracellular signal-regulated kinases (ERK-1 and ERK-2), while induction of granulocytic

differentiation by RA caused similar activation of MEK-1/-2 and ERK-2, but not ERK-1. MAPK cascades are key signalling pathways that regulate a wide variety of cellular processes, including proliferation, differentiation, apoptosis and stress responses and the differentiation potential following treatment of HL-60 cells with AXO and PCM could involve the MAP kinase pathway.

AXO treatment also showed a sustained increase in *CDKN2C*. Schwaller *et al*., 1997 demonstrated that granulocytic differentiation following treatment of cells with DMSO was accompanied by increase of *CDKN2C* protein product p18INK4C. However, during PMA-induced monocytic differentiation of leukaemic HL-60 cells, variable expression of particular G1-CKIs was reported whereby p15INK4B was upregulated, and p18INK4C and p19INK4D expression were downregulated. These results are suggestive of a contrasting and complex role of G1-specific cell cycle inhibitors during myeloid differentiation and could partially explain the discordance in PSM treated cells with upregulation of myeloid TFs and the transient up-regulation of *CDKN1A*.

Regulating the activity of TFs can promote both differentiation and cell cycle arrest. This modulation of transcription factors has been used in the experimental treatment of leukaemia (Rosenbauer & Tenen, 2007). Gene expression of myeloid TFs in this study complemented the morphological and surface marker picture, with AXO-treated HL-60 cells exhibiting a granulocytic-like gene signature. Similar to Marchwicka and Marcinkowska, 2018, a strong and sustained expression of *CEBPB*, accompanied by the sustained expression of *CEBPE*, lead to granulocytic differentiation. The increase in C/EBPβ expression was also reported following ATRA- and DMSO- induced differentiation of HL-60 cells (Zhao *et al*., 2018; Gianni *et al*., 2017). AXO-treated HL-60 cells resulted in over expression of *SPI1* (PU.1) and *c-MYC* repression. The

down regulation of *c-MYC* as reported by several studies (Ida *et al*., 2014; Shima *et al*., 1989) causes arrest of leukaemia cell proliferation and ultimately sustains myeloid differentiation of AML cells.

It appears that there is a weak and transient up-regulation of *CEBPA* in both PSM- and AXO-treated cells. This is similar to studies that described transient upregulation of *CEBPA* in the early phase of VITD3 induced differentiation, with a marked and persistent upregulation of C/EBPβ until the late phase of differentiation in HL-60 cells (Marchwicka & Marcinkowska, 2018; Marcinkowska *et al*., 2006). In another study, expression of CD14 and *CEBPE*, as seen following treatment of HL-60 cells with PSM, were described as markers of monocytic differentiation (Ramirez *et al*., 2017). Interestingly, Li *et al*., 2019 demonstrated that *CEBPE* expression is an independent predictor not only for overall survival but also reduced relapse rate of AML patients and a reduction in *CEBPE* expression is associated with high relapse rate. This may be due to its being related to terminal granulocytic differentiation.

These results are consistent with others showing that upregulation or reactivation of TFs can lead to cell cycle arrest and differentiation of leukaemic cells, and could be a novel strategy to treat AML (Takei & Kobayashi,2019). It has also been reported that differentiating agents such as VITD3 activate intracellular signalling pathways including protein kinase C (PKC) pathway (Simpson et al., 1998), calcium-dependent pathways (Sergeev, 2005), the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) (PI3/AKT) kinase pathway (Zhang et al., 2006), and the MAP kinase pathways (Miranda et al., 2002). In myeloid leukaemia cells, activation of one or more of these signal transduction pathways eventually leads to regulation of differentiation of transcription factors, and it is likely that the repression of *c-MYC* and increase in the expression

of several myeloid transcription factors following treatment of HL-60 cells with AXO and PSM is also regulated by at least some of these pathways.

Deregulated apoptosis is another hallmark of cancer. The anti-apoptotic group of *BCL-2* family proteins are frequently overexpressed in many cancers, with cancer cells resisting apoptosis and ultimately treatment failure. *BCL2* expression is significantly up-regulated in newly diagnosed AML and more so in relapsed AML patients (Zhou *et al*., 2019). Targeting apoptotic pathways is another strategy to treat AML (Sillar & Enjeti, 2019). Clinical results over the last few years already offer strong evidence that inhibition of *BCL2* is likely to offer valuable new agents for the treatment of AML (Konopleva & Letai, 2018). Therefore, developing various kinds of effective drugs that promote apoptosis in cancer cells might be a promising approach to aid in the treatment of cancer.

PSM treatment has already shown that the extract causes partial HL-60 cell differentiation and apoptotic effects. The apoptotic effects were further confirmed by a sustained down-regulation of *BCL2* and a sustained over expression of *BAX* expression following PSM treatment of HL-60 cells. A combinatorial chemotherapeutic drug treatment (doxorubicin and amifostine) of HL-60 cells similarly showed a decrease in *BCL2* and an increase in *BAX* expression when compared with the cells treated only with doxorubicin (Rozalski *et al*., 2005).

In summary, based on these results [Figure 5-1 (A and B)], it is likely that PSM and AXO extracts contain different biologically active molecules, with synergistic and/or additive antineoplastic effects. The AXO extract might contain biologically active molecules similar to

those derived from other amphibians including biogenic amines, bufodienolides (bufogenines and bufotoxins), alkaloids, peptides, and proteins that are potentially useful as anticancer agents. Interestingly, similar to AXO extract, the steroid bufalin (isolated from amphibian skin) seems to reduce the expression of c-MYC in HL60 cells. Whereas reduction of c-MYC following AXO treatment results in cell differentiation, bufalin induces apoptosis in HL60 cells. Furthermore, the PSM extract could have molecules that induce apoptosis of leukaemia cells. In fact, mass spectrometry based quantitative methods identified differential enrichment of 35 amines, 7 thiol metabolites and 4 nucleotides from both intact and regenerating planarians (Natarajan et al., 2015). Further characterization of the extracts in this study should help identify the active molecules, which may possibly be suitable drug candidates to develop novel therapeutics.

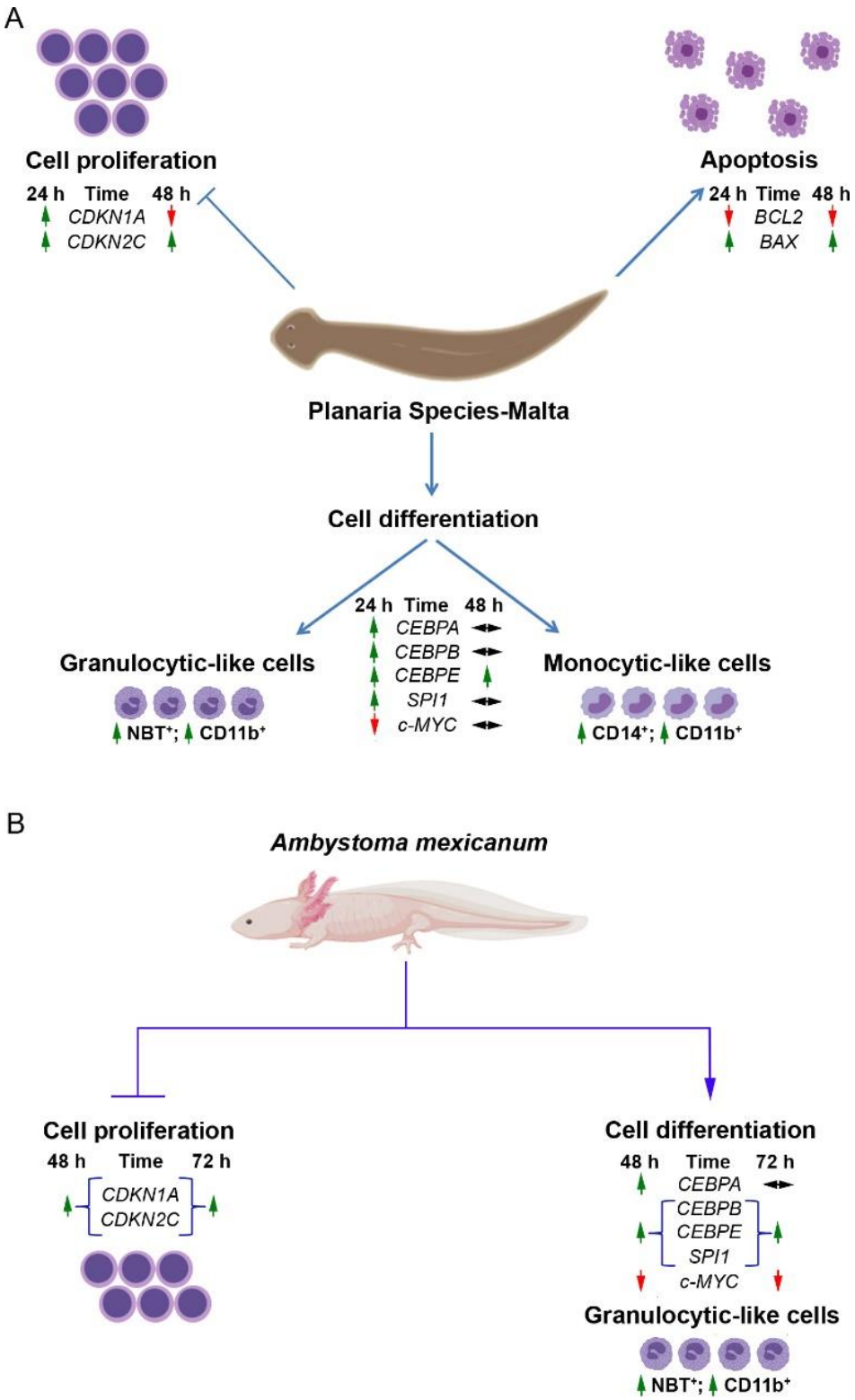

Figure 5-1: Schematic representation of the effects of PSM (A) and AXO (B) extract on HL-60 cells. Possible mechanisms include inducing apoptosis and cell differentiation, and reducing cell proliferation. Green arrows represent increased expression; red arrows indicate decreased expression; black double-headed arrows indicate unchanged expression.

Chapter 6

Limitations

Initially, cancer cell lines were tested with planaria conditioned media (PCM). Planarians were dissociated using a hand-held homogenizer and cells were incubated at 25 °C. The culture media used to grow the planaria cells is an isosmotic medium developed by Schurmann & Peter, 2001. PCM was collected after 7 days and filter sterilised before treating the cell lines. However, the rate of contamination was very high. This is very common when culturing primary cells and could be due to the presence of Bacteroidetes and Proteobacteria in healthy planarians (Arnold et al., 2016). Also, the influence of different culture media effected the behaviour of neoblasts (Schurmann & Peter, 2001), and this could pose a problem in terms of the effect of PCM on cancer cell lines since depending on the media, cell extract may behave differently. Moreover, cell cultures do not reflect the regenerative microenvironment. Thus, further experiments were carried out using crude extracts from both regenerative and non-regenerative planarians. This not only eliminated infections due to symbiotic bacteria, but unlike cultures of planaria cells in media, the protocol of collecting the regenerative tissue ensures the regenerative process is conserved and any active molecules or secondary metabolites secreted during regeneration are present in the crude extract preparation.

6.1 The effect of age and repeated amputations on axolotls

Following limb amputations performed in this study, and due to the fact that blastema size does not exceed 1-2 mm, repeated amputations were required to obtain a sufficient amount of extract to carry out the study. It has been reported that repeat amputations together with the increasing age of the axolotls render the process of regeneration less efficient (Bryant et al., 2017). Repeated amputations of limbs may result in a wound healing response, which interferes with the regenerative program in axolotls. In this study, the time-line chosen for collection of

regenerative extracts coincided with the re-differentiation process sited in literature (Gerber et al., 2018; McGann *et al*., 2001). Overall, even though young axolotls (4-6 months) were used in this study, the collection time point coinciding with re-differentiation of blastema cells depended on literature and could not be verified. Moreover, when further amputations were carried out in this study, full restoration of the limb exceeded the time line reported in literature (Vieira et al., 2020).

Chapter 7

Future Directions

In the future, a systematic approach will be adopted. One of the first steps will be the morphological and molecular characterization of the planaria species in Malta. This will help not only in species identification but also to check whether different species exhibit different effects on cancer cell lines. The second step will involve the identification of the bioactive molecules in both extracts and analyse the behaviour of the molecules and whether they have a synergistic, antagonistic, and or additive effects on human leukaemia cell lines including CSCs derived from the same cell lines *in vitro*. Equally important is to study the effects on normal cells as well.

Following target drug candidate identification, the molecular mechanisms relative to apoptosis, differentiation, and cell cycle regulation will be studied/confirmed. Any epigenetic mechanisms of inducing their effects including DNA methylation and histone modification will be analysed. mRNA, miRNA and proteomic profiles may also be studied if deemed relevant. Eventually, the bioactive molecules will be tested on primary cells from cancer patients. Ultimately, *in vivo* studies will be performed to validate the results obtained *in vitro.*

The above future targets can expand the knowledge on biological and molecular mechanisms by which the extracts regulate the processes of proliferation, apoptosis and differentiation and may lead to the development of new therapeutic strategies that help overcome not only the block in differentiation but also drug resistance often attributed to CSCs.

Chapter 8

References

1. Abou Zahr, A., & Borthakur, G. (2017). Emerging cell cycle inhibitors for acute myeloid leukemia. *Expert opinion on emerging drugs*, *22*(2), 137–148. https://doi.org/10.1080/14728214.2017.1330885
2. Adamidi, C., Wang, Y., Gruen, D., Mastrobuoni, G., You, X., Tolle, D., Dodt, M., Mackowiak, S. D., Gogol-Doering, A., Oenal, P., Rybak, A., Ross, E., Sánchez Alvarado, A., Kempa, S., Dieterich, C., Rajewsky, N., & Chen, W. (2011). De novo assembly and validation of planaria transcriptome by massive parallel sequencing and shotgun proteomics. *Genome research*, *21*(7), 1193–1200. https://doi.org/10.1101/gr.113779.110
3. Agata, K., Saito, Y., & Nakajima, E. (2007). Unifying principles of regeneration I: Epimorphosis versus morphallaxis. *Development, growth & differentiation*, *49*(2), 73–78. https://doi.org/10.1111/j.1440-169X.2007.00919.x
4. Al Bitar, S., & Gali-Muhtasib, H. (2019). The Role of the Cyclin Dependent Kinase Inhibitor $p21^{cip1/waf1}$ in Targeting Cancer: Molecular Mechanisms and Novel Therapeutics. *Cancers*, *11*(10), 1475. https://doi.org/10.3390/cancers11101475
5. Alatrash, G., & Molldrem, J. J. (2011). Vaccines as consolidation therapy for myeloid leukemia. *Expert review of hematology*, *4*(1), 37–50. https://doi.org/10.1586/ehm.10.80
6. Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H., & Boyd, M. R. (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer research*, *48*(3), 589–601.
7. Allis, C. D., & Jenuwein, T. (2016). The molecular hallmarks of epigenetic control. *Nature reviews. Genetics*, *17*(8), 487–500. https://doi.org/10.1038/nrg.2016.59
8. Amin, A., Gali-Muhtasib, H., Ocker, M., & Schneider-Stock, R. (2009). Overview of major classes of plant-derived anticancer drugs. *International journal of biomedical science : IJBS*, *5*(1), 1–11.

9. Anders, B., Veltri, L., Kanate, A. S., Shillingburg, A., Shah, N., Craig, M., & Cumpston, A. (2017). Outcomes of Six-Dose High-Dose Cytarabine as a Salvage Regimen for Patients with Relapsed/Refractory Acute Myeloid Leukemia. *Advances in hematology*, *2017*, 6464972. https://doi.org/10.1155/2017/6464972
10. Anelli, L., Pasciolla, C., Zagaria, A., Specchia, G., & Albano, F. (2017). Monosomal karyotype in myeloid neoplasias: a literature review. *OncoTargets and therapy*, *10*, 2163–2171. https://doi.org/10.2147/OTT.S133937
11. Anjum, K., Abbas, S. Q., Shah, S. A., Akhter, N., Batool, S., & Hassan, S. S. (2016). Marine Sponges as a Drug Treasure. *Biomolecules & therapeutics*, *24*(4), 347–362. https://doi.org/10.4062/biomolther.2016.067
12. Aponte, P. M., & Caicedo, A. (2017). Stemness in Cancer: Stem Cells, Cancer Stem Cells, and Their Microenvironment. *Stem cells international*, *2017*, 5619472. https://doi.org/10.1155/2017/5619472
13. Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., Bloomfield, C. D., Cazzola, M., & Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*, *127*(20), 2391–2405. https://doi.org/10.1182/blood-2016-03-643544
14. Arshad, S., Naveed, M., Ullia, M., Javed, K., Butt, A., Khawar, M., & Amjad, F. (2020). Targeting STAT-3 signaling pathway in cancer for development of novel drugs: Advancements and challenges. *Genetics and molecular biology*, *43*(1), e20180160. https://doi.org/10.1590/1678-4685-GMB-2018-0160
15. Assi, R., Kantarjian, H., Ravandi, F., & Daver, N. (2018). Immune therapies in acute myeloid leukemia: a focus on monoclonal antibodies and immune checkpoint inhibitors. *Current opinion in hematology*, *25*(2), 136–145. https://doi.org/10.1097/MOH.0000000000000401

16. Audia, J. E., & Campbell, R. M. (2016). Histone Modifications and Cancer. *Cold Spring Harbor perspectives in biology*, *8*(4), a019521. https://doi.org/10.1101/cshperspect.a019521

17. Avellino, R., & Delwel, R. (2017). Expression and regulation of C/EBPα in normal myelopoiesis and in malignant transformation. *Blood*, *129*(15), 2083–2091. https://doi.org/10.1182/blood-2016-09-687822

18. Avellino, R., Havermans, M., Erpelinck, C., Sanders, M. A., Hoogenboezem, R., van de Werken, H. J., Rombouts, E., van Lom, K., van Strien, P. M., Gebhard, C., Rehli, M., Pimanda, J., Beck, D., Erkeland, S., Kuiken, T., de Looper, H., Gröschel, S., Touw, I., Bindels, E., & Delwel, R. (2016). An autonomous CEBPA enhancer specific for myeloid-lineage priming and neutrophilic differentiation. *Blood*, *127*(24), 2991–3003. https://doi.org/10.1182/blood-2016-01-695759

19. Bachmann M. (2019). The UniCAR system: A modular CAR T cell approach to improve the safety of CAR T cells. *Immunology letters*, *211*, 13–22. https://doi.org/10.1016/j.imLet.2019.05.003

20. Balderman, S. R., Li, A. J., Hoffman, C. M., Frisch, B. J., Goodman, A. N., LaMere, M. W., Georger, M. A., Evans, A. G., Liesveld, J. L., Becker, M. W., & Calvi, L. M. (2016). Targeting of the bone marrow microenvironment improves outcome in a murine model of myelodysplastic syndrome. *Blood*, *127*(5), 616–625. https://doi.org/10.1182/blood-2015-06-653113

21. Ball, B., & Stein, E. M. (2019). Which are the most promising targets for minimal residual disease-directed therapy in acute myeloid leukemia prior to allogeneic stem cell transplant?. *Haematologica*, *104*(8), 1521–1531. https://doi.org/10.3324/haematol.2018.208587

22. Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell research*, *21*(3), 381–395. https://doi.org/10.1038/cr.2011.22

23. Barbosa, K., Deshpande, A., Chen, B. R., Ghosh, A., Sun, Y., Dutta, S., Weetall, M., Dixon, J., Armstrong, S. A., Bohlander, S. K., & Deshpande, A. J. (2019). Acute myeloid leukemia driven by the CALM-AF10 fusion gene is dependent on BMI1. *Experimental hematology*, *74*, 42–51.e3. https://doi.org/10.1016/j.exphem.2019.04.003

24. Bartels, M., Govers, A. M., Fleskens, V., Lourenço, A. R., Pals, C. E., Vervoort, S. J., van Gent, R., Brenkman, A. B., Bierings, M. B., Ackerman, S. J., van Loosdregt, J., & Coffer, P. J. (2015). Acetylation of C/EBPε is a prerequisite for terminal neutrophil differentiation. *Blood*, *125*(11), 1782–1792. https://doi.org/10.1182/blood-2013-12-543850

25. Barzkar, N., Tamadoni Jahromi, S., Poorsaheli, H. B., & Vianello, F. (2019). Metabolites from Marine Microorganisms, Micro, and Macroalgae: Immense Scope for Pharmacology. *Marine drugs*, *17*(8), 464. https://doi.org/10.3390/md17080464

26. Bates, D., & Eastman, A. (2017). Microtubule destabilising agents: far more than just antimitotic anticancer drugs. *British journal of clinical pharmacology*, *83*(2), 255–268. https://doi.org/10.1111/bcp.13126

27. Bely, A. E., & Nyberg, K. G. (2010). Evolution of animal regeneration: re-emergence of a field. *Trends in ecology & evolution*, *25*(3), 161–170. https://doi.org/10.1016/j.tree.2009.08.005

28. Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A., Gralnick, H. R., & Sultan, C. (1976). Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *British journal of haematology*, *33*(4), 451–458. https://doi.org/10.1111/j.1365-2141.1976.tb03563.x

29. Ben-Shmuel, A., Biber, G., & Barda-Saad, M. (2020). Unleashing Nben-atural Killer Cells in the Tumor Microenvironment-The Next Generation of Immunotherapy?. *Frontiers in immunology*, *11*, 275. https://doi.org/10.3389/fimmu.2020.00275

30. Blunt, J. W., Carroll, A. R., Copp, B. R., Davis, R. A., Keyzers, R. A., & Prinsep, M. R. (2018). Marine natural products. *Natural product reports*, *35*(1), 8–53. https://doi.org/10.1039/c7np00052a

31. Boilly, B., Faulkner, S., Jobling, P., & Hondermarck, H. (2017). Nerve Dependence: From Regeneration to Cancer. *Cancer cell*, *31*(3), 342–354. https://doi.org/10.1016/j.ccell.2017.02.005

32. Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine*, *3*(7), 730–737. https://doi.org/10.1038/nm0797-730

33. Bose, P., Dai, Y., & Grant, S. (2014). Histone deacetylase inhibitor (HDACI) mechanisms of action: emerging insights. *Pharmacology & therapeutics*, *143*(3), 323–336. https://doi.org/10.1016/j.pharmthera.2014.04.004

34. Breems, D. A., Van Putten, W. L., De Greef, G. E., Van Zelderen-Bhola, S. L., Gerssen-Schoorl, K. B., Mellink, C. H., Nieuwint, A., Jotterand, M., Hagemeijer, A., Beverloo, H. B., & Löwenberg, B. (2008). Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *26*(29), 4791–4797. https://doi.org/10.1200/JCO.2008.16.0259

35. Brockmann, M., Poon, E., Berry, T., Carstensen, A., Deubzer, H. E., Rycak, L., Jamin, Y., Thway, K., Robinson, S. P., Roels, F., Witt, O., Fischer, M., Chesler, L., & Eilers, M. (2013). Small molecule inhibitors of aurora-a induce proteasomal degradation of N-myc in

childhood neuroblastoma. *Cancer cell*, *24*(1), 75–89. https://doi.org/10.1016/j.ccr.2013.05.005

36. Brown, G., Choudhry, M. A., Durham, J., Drayson, M. T., andMichell, R. H. (1999). Monocytically differentiating HL60 cells proliferate rapidly before they mature.Exp. Cell Res.253,511–518.

37. Brown, G., Drayson, M. T., Durham, J., Toellner, K. M., Hughes, P. J., Choudhry, M. A., Taylor, D. R., Bird, R., & Michell, R. H. (2002). HL-60 cells halted in G1 or S phase differentiate normally. *Experimental cell research*, *281*(1), 28–38. https://doi.org/10.1006/excr.2002.5654

38. Brunet, E., & Jasin, M. (2018). Induction of Chromosomal Translocations with CRISPR-Cas9 and Other Nucleases: Understanding the Repair Mechanisms That Give Rise to Translocations. *Advances in experimental medicine and biology*, *1044*, 15–25. https://doi.org/10.1007/978-981-13-0593-1_2

39. Bryant, D. M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M. B., Payzin-Dogru, D., Lee, T. J., Leigh, N. D., Kuo, T. H., Davis, F. G., Bateman, J., Bryant, S., Guzikowski, A. R., Tsai, S. L., Coyne, S., Ye, W. W., Freeman, R. M., Jr, Peshkin, L., Tabin, C. J., Regev, A., … Whited, J. L. (2017). A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. *Cell reports*, *18*(3), 762–776. https://doi.org/10.1016/j.celrep.2016.12.063

40. Burda, P., Laslo, P., & Stopka, T. (2010). The role of PU.1 and GATA-1 transcription factors during normal and leukemogenic hematopoiesis. *Leukemia*, *24*(7), 1249–1257. https://doi.org/10.1038/leu.2010.104

41. Buscarlet, M., Provost, S., Zada, Y. F., Barhdadi, A., Bourgoin, V., Lépine, G., Mollica, L., Szuber, N., Dubé, M. P., & Busque, L. (2017). *DNMT3A* and *TET2* dominate clonal

hematopoiesis and demonstrate benign phenotypes and different genetic predispositions. *Blood*, *130*(6), 753–762. https://doi.org/10.1182/blood-2017-04-777029

42. Cai, X., Shen, Y. L., Zhu, Q., Jia, P. M., Yu, Y., Zhou, L., Huang, Y., Zhang, J. W., Xiong, S. M., Chen, S. J., Wang, Z. Y., Chen, Z., & Chen, G. Q. (2000). Arsenic trioxide-induced apoptosis and differentiation are associated respectively with mitochondrial transmembrane potential collapse and retinoic acid signaling pathways in acute promyelocytic leukemia. *Leukemia*, *14*(2), 262–270. https://doi.org/10.1038/sj.leu.2401650
43. Calixto J. B. (2019). The role of natural products in modern drug discovery. *Anais da Academia Brasileira de Ciencias*, *91 Suppl 3*, e20190105. https://doi.org/10.1590/0001-3765201920190105
44. Campbell, L. J., Suárez-Castillo, E. C., Ortiz-Zuazaga, H., Knapp, D., Tanaka, E. M., & Crews, C. M. (2011). Gene expression profile of the regeneration epithelium during axolotl limb regeneration. *Developmental dynamics : an official publication of the American Association of Anatomists*, *240*(7), 1826–1840. https://doi.org/10.1002/dvdy.22669
45. *Cancer Facts & Figures 2020*. Published online January 8, 2020. American Cancer Society, Atlanta, Ga.
46. Cao, H., Sethumadhavan, K., & Bland, J. M. (2018). Isolation of Cottonseed Extracts That Affect Human Cancer Cell Growth. *Scientific reports*, *8*(1), 10458. https://doi.org/10.1038/s41598-018-28773-4
47. Capp J. P. (2019). Cancer Stem Cells: From Historical Roots to a New Perspective. *Journal of oncology*, *2019*, 5189232. https://doi.org/10.1155/2019/5189232
48. Carabet, L. A., Rennie, P. S., & Cherkasov, A. (2018). Therapeutic Inhibition of Myc in Cancer. Structural Bases and Computer-Aided Drug Discovery Approaches. *International journal of molecular sciences*, *20*(1), 120. https://doi.org/10.3390/ijms20010120

49. Castelli, G., Pelosi, E., & Testa, U. (2016). Targeted therapies in the treatment of adult acute myeloid leukemias: current status and future perspectives. *International journal of hematologic oncology*, *5*(4), 143–164. https://doi.org/10.2217/ijh-2016-0011

50. Castelli, G., Pelosi, E., & Testa, U. (2019). Emerging Therapies for Acute Myelogenus Leukemia Patients Targeting Apoptosis and Mitochondrial Metabolism. *Cancers*, *11*(2), 260. https://doi.org/10.3390/cancers11020260

51. Castillo-Lara, S., & Abril, J. F. (2018). PlanNET: homology-based predicted interactome for multiple planarian transcriptomes. *Bioinformatics (Oxford, England)*, *34*(6), 1016–1023. https://doi.org/10.1093/bioinformatics/btx738

52. Ceredig, R., Rolink, A. G., & Brown, G. (2009). Models of haematopoiesis: seeing the wood for the trees. *Nature reviews. Immunology*, *9*(4), 293–300. https://doi.org/10.1038/nri2525

53. Chamberlin, S. R., Blucher, A., Wu, G., Shinto, L., Choonoo, G., Kulesz-Martin, M., & McWeeney, S. (2019). Natural Product Target Network Reveals Potential for Cancer Combination Therapies. *Frontiers in pharmacology*, *10*, 557. https://doi.org/10.3389/fphar.2019.00557

54. Charni, M., Aloni-Grinstein, R., Molchadsky, A., & Rotter, V. (2017). p53 on the crossroad between regeneration and cancer. *Cell death and differentiation*, *24*(1), 8–14. https://doi.org/10.1038/cdd.2016.117

55. Chen, G., & Lee, E. Y. (1999). Phenotypic differentiation without permanent cell-cycle arrest by skeletal myocytes with deregulated E2F-1. *DNA and cell biology*, *18*(4), 305–314. https://doi.org/10.1089/104454999315367

56. Chen, H., Zhang, B., Yuan, X., Yao, Y., Zhao, H., Sun, X., & Zheng, Q. (2013). Isoliquiritigenin-induced effects on Nrf2 mediated antioxidant defence in the HL-60 cell monocytic differentiation. *Cell biology international*, *37*(11), 1215–1224. https://doi.org/10.1002/cbin.10156

57. Chen, S. S., Chen, J. F., Johnson, P. F., Muppala, V., & Lee, Y. H. (2000). *CDKN2C*eta, when expressed from the *CDKN2C*lpha gene locus, can functionally replace *CDKN2C*lpha in liver but not in adipose tissue. *Molecular and cellular biology*, *20*(19), 7292–7299. https://doi.org/10.1128/mcb.20.19.7292-7299.2000

58. Cheng, Y., He, C., Wang, M., Ma, X., Mo, F., Yang, S., Han, J., & Wei, X. (2019). Targeting epigenetic regulators for cancer therapy: mechanisms and advances in clinical trials. *Signal transduction and targeted therapy*, *4*, 62. https://doi.org/10.1038/s41392-019-0095-0

59. Choudhari, A. S., Mandave, P. C., Deshpande, M., Ranjekar, P., & Prakash, O. (2020). Phytochemicals in Cancer Treatment: From Preclinical Studies to Clinical Practice. *Frontiers in pharmacology*, *10*, 1614. https://doi.org/10.3389/fphar.2019.01614

60. Collins, S. J., Ruscetti, F. W., Gallagher, R. E., & Gallo, R. C. (1979). Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. *The Journal of experimental medicine*, *149*(4), 969–974. https://doi.org/10.1084/jem.149.4.969

61. Corces, M. R., Buenrostro, J. D., Wu, B., Greenside, P. G., Chan, S. M., Koenig, J. L., Snyder, M. P., Pritchard, J. K., Kundaje, A., Greenleaf, W. J., Majeti, R., & Chang, H. Y. (2016). Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nature genetics*, *48*(10), 1193–1203. https://doi.org/10.1038/ng.3646

62. Corces, M. R., Chang, H. Y., & Majeti, R. (2017). Preleukemic Hematopoietic Stem Cells in Human Acute Myeloid Leukemia. *Frontiers in oncology*, *7*, 263. https://doi.org/10.3389/fonc.2017.00263

63. Coutermarsh-Ott, S. L., Broadway, K. M., Scharf, B. E., & Allen, I. C. (2017). Effect of Salmonella enterica serovar Typhimurium VNP20009 and VNP20009 with restored chemotaxis on 4T1 mouse mammary carcinoma progression. *Oncotarget*, *8*(20), 33601–33613. https://doi.org/10.18632/oncotarget.16830

64. Creutzig, U., Büchner, T., Sauerland, M. C., Zimmermann, M., Reinhardt, D., Döhner, H., & Schlenk, R. F. (2008). Significance of age in acute myeloid leukemia patients younger than 30 years: a common analysis of the pediatric trials AML-BFM 93/98 and the adult trials AMLCG 92/99 and AMLSG HD93/98A. *Cancer*, *112*(3), 562–571. https://doi.org/10.1002/cncr.23220

65. Dahl, R., Walsh, J. C., Lancki, D., Laslo, P., Iyer, S. R., Singh, H., & Simon, M. C. (2003). Regulation of macrophage and neutrophil cell fates by the PU.1:*CDKN2C*lpha ratio and granulocyte colony-stimulating factor. *Nature immunology*, *4*(10), 1029–1036. https://doi.org/10.1038/ni973

66. Dall'Agnese, A., & Puri, P. L. (2016). Could we also be regenerative superheroes, like salamanders?. *BioEssays : news and reviews in molecular, cellular and developmental biology*, *38*(9), 917–926. https://doi.org/10.1002/bies.201600015

67. Damia, G., & Broggini, M. (2004). Cell cycle checkpoint proteins and cellular response to treatment by anticancer agents. *Cell cycle (Georgetown, Tex.)*, *3*(1), 46–50.

68. Damiani, D., & Tiribelli, M. (2019). Molecular landscape in adult acute myeloid leukemia: where we are where we going?. *Journal Of Laboratory And Precision Medicine, 4*. Retrieved from http://jlpm.amegroups.com/article/view/4976

69. Daver, N., Schlenk, R. F., Russell, N. H., & Levis, M. J. (2019). Targeting FLT3 mutations in AML: review of current knowledge and evidence. *Leukemia*, *33*(2), 299–312. https://doi.org/10.1038/s41375-018-0357-9

70. Dingli D., Traulsen, A., & Pacheco, J.M. (2012) Evolutionary Dynamics of Mutations in Hematopoietic Stem Cells and Beyond. M.A. Hayat (ed.), *Stem Cells and Cancer Stem Cells*, 3, DOI 10.1007/978-94-007-2415-0_11

71. De Kouchkovsky, I., & Abdul-Hay, M. (2016). 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood cancer journal*, *6*(7), e441. https://doi.org/10.1038/bcj.2016.50

72. de Stanchina, E., Querido, E., Narita, M., Davuluri, R. V., Pandolfi, P. P., Ferbeyre, G., & Lowe, S. W. (2004). PML is a direct p53 target that modulates p53 effector functions. *Molecular cell*, *13*(4), 523–535. https://doi.org/10.1016/s1097-2765(04)00062-0

73. de Thé H. (2018). Differentiation therapy revisited. *Nature reviews. Cancer*, *18*(2), 117–127. https://doi.org/10.1038/nrc.2017.103

74. Deborde, S., & Wong, R. J. (2017). How Schwann cells facilitate cancer progression in nerves. *Cellular and molecular life sciences: CMLS*, *74*(24), 4405–4420. https://doi.org/10.1007/s00018-017-2578-x

75. Degos L. (2003). The history of acute promyelocytic leukaemia. *British journal of haematology*, *122*(4), 539–553. https://doi.org/10.1046/j.1365-2141.2003.04460.x

76. Delgado, M. D., & León, J. (2010). Myc roles in hematopoiesis and leukemia. *Genes & cancer*, *1*(6), 605–616. https://doi.org/10.1177/1947601910377495

77. Demir, S., Turan, I., Aliyazicioglu, R., Yaman, S. O., & Aliyazicioglu, Y. (2018). *Primula vulgaris* extract induces cell cycle arrest and apoptosis in human cervix cancer cells. *Journal of pharmaceutical analysis*, *8*(5), 307–311. https://doi.org/10.1016/j.jpha.2018.05.003

78. di Masi, A., Cilli, D., Berardinelli, F., Talarico, A., Pallavicini, I., Pennisi, R., Leone, S., Antoccia, A., Noguera, N. I., Lo-Coco, F., Ascenzi, P., Minucci, S., & Nervi, C. (2016). PML nuclear body disruption impairs DNA double-strand break sensing and repair in APL. *Cell death & disease*, *7*(7), e2308. https://doi.org/10.1038/cddis.2016.115

79. Di Rosa, M., Malaguarnera, M., Nicoletti, F., & Malaguarnera, L. (2011). Vitamin D3: a helpful immuno-modulator. *Immunology*, *134*(2), 123–139. https://doi.org/10.1111/j.1365-2567.2011.03482.x

80. Dick J. E. (2005). Acute myeloid leukemia stem cells. *Annals of the New York Academy of Sciences*, *1044*, 1–5. https://doi.org/10.1196/annals.1349.001

81. Dimberg, A., Bahram, F., Karlberg, I., Larsson, L. G., Nilsson, K., & Oberg, F. (2002). Retinoic acid-induced cell cycle arrest of human myeloid cell lines is associated with sequential down-regulation of c-Myc and cyclin E and posttranscriptional up-regulation of p27(Kip1). *Blood*, *99*(6), 2199–2206. https://doi.org/10.1182/blood.v99.6.2199

82. Döhner, H., Estey, E., Grimwade, D., Amadori, S., Appelbaum, F. R., Büchner, T., Dombret, H., Ebert, B. L., Fenaux, P., Larson, R. A., Levine, R. L., Lo-Coco, F., Naoe, T., Niederwieser, D., Ossenkoppele, G. J., Sanz, M., Sierra, J., Tallman, M. S., Tien, H. F., Wei, A. H., … Bloomfield, C. D. (2017). Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*, *129*(4), 424–447. https://doi.org/10.1182/blood-2016-08-733196

83. Döhner, H., Weisdorf, D. J., & Bloomfield, C. D. (2015). Acute Myeloid Leukemia. *The New England journal of medicine*, *373*(12), 1136–1152. https://doi.org/10.1056/NEJMra1406184

84. Doulatov, S., Notta, F., Eppert, K., Nguyen, L. T., Ohashi, P. S., & Dick, J. E. (2010). Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nature immunology*, *11*(7), 585–593. https://doi.org/10.1038/ni.1889

85. Drayson, M. T., Michell, R. H., Durham, J., & Brown, G. (2001). Cell proliferation and CD11b expression are controlled independently during HL-60 cell differentiation initiated by 1,25 alpha-dihydroxyvitamin D(3) or all-trans-retinoic acid. *Experimental cell research*, *266*(1), 126–134. https://doi.org/10.1006/excr.2001.5200

86. Duong, M. T., Qin, Y., You, S. H., & Min, J. J. (2019). Bacteria-cancer interactions: bacteria-based cancer therapy. *Experimental & molecular medicine*, *51*(12), 1–15. https://doi.org/10.1038/s12276-019-0297-0

87. Dvorak H. F. (2015). Tumors: wounds that do not heal-redux. *Cancer immunology research*, *3*(1), 1–11. https://doi.org/10.1158/2326-6066.CIR-14-0209

88. Dybdal-Hargreaves, N. F., Risinger, A. L., & Mooberry, S. L. (2015). Eribulin mesylate: mechanism of action of a unique microtubule-targeting agent. *Clinical cancer research : an official journal of the American Association for Cancer Research*, *21*(11), 2445–2452. https://doi.org/10.1158/1078-0432.CCR-14-3252

89. Eckhardt, S. G., Dai, A., Davidson, K. K., Forseth, B. J., Wahl, G. M., & Von Hoff, D. D. (1994). Induction of differentiation in HL60 cells by the reduction of extrachromosomally amplified c-myc. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(14), 6674–6678. https://doi.org/10.1073/pnas.91.14.6674

90. Eisfeld, A. K., Kohlschmidt, J., Mrózek, K., Blachly, J. S., Walker, C. J., Nicolet, D., Orwick, S., Maharry, S. E., Carroll, A. J., Stone, R. M., de la Chapelle, A., Wang, E. S., Kolitz, J. E., Powell, B. L., Byrd, J. C., & Bloomfield, C. D. (2018). Mutation patterns identify adult patients with de novo acute myeloid leukemia aged 60 years or older who respond favorably to standard chemotherapy: an analysis of Alliance studies. *Leukemia*, *32*(6), 1338–1348. https://doi.org/10.1038/s41375-018-0068-2

91. El-Deiry W. S. (2016). p21(WAF1) Mediates Cell-Cycle Inhibition, Relevant to Cancer Suppression and Therapy. *Cancer research*, *76*(18), 5189–5191. https://doi.org/10.1158/0008-5472.CAN-16-2055

92. Elliott, S. A., & Sánchez Alvarado, A. (2013). The history and enduring contributions of planarians to the study of animal regeneration. *Wiley interdisciplinary reviews. Developmental biology*, *2*(3), 301–326. https://doi.org/10.1002/wdev.82

93. Ely, C. M., Leftwich, J. A., Chenevix-Trench, G., Hall, R. E., & Westin, E. H. (1987). Altered regulation of c-myc in an HL-60 differentiation resistant subclone, HL-60-1E3. *Cancer research*, *47*(17), 4595–4600.

94. Epperly, R., Gottschalk, S., & Velasquez, M. P. (2020). A Bump in the Road: How the Hostile AML Microenvironment Affects CAR T Cell Therapy. *Frontiers in oncology*, *10*, 262. https://doi.org/10.3389/fonc.2020.00262

95. Ercolano, G., De Cicco, P., & Ianaro, A. (2019). New Drugs from the Sea: Pro-Apoptotic Activity of Sponges and Algae Derived Compounds. *Marine drugs*, *17*(1), 31. https://doi.org/10.3390/md17010031

96. Estey, EH. (2018). Acute myeloid leukemia: 2019 update on risk-stratification and management. *Am J Hematology, 93*, 1267267– 1291. https://doi.org/10.1002/ajh.25214

97. Fabiani, R., Rosignoli, P., De Bartolomeo, A., Fuccelli, R., & Morozzi, G. (2008). Inhibition of cell cycle progression by hydroxytyrosol is associated with upregulation of cyclin-dependent protein kinase inhibitors p21(WAF1/Cip1) and p27(Kip1) and with induction of differentiation in HL60 cells. *The Journal of nutrition*, *138*(1), 42–48. https://doi.org/10.1093/jn/138.1.42

98. Falchi, L., Verstovsek, S., Ravandi-Kashani, F., & Kantarjian, H. M. (2016). The evolution of arsenic in the treatment of acute promyelocytic leukemia and other myeloid neoplasms: Moving toward an effective oral, outpatient therapy. *Cancer*, *122*(8), 1160–1168. https://doi.org/10.1002/cncr.29852

99. Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., La Starza, R., Diverio, D., Colombo, E., Santucci, A., Bigerna, B., Pacini, R., Pucciarini, A., Liso, A., Vignetti, M., Fazi, P., Meani, N., Pettirossi, V., Saglio, G., Mandelli, F., … GIMEMA Acute Leukemia Working Party (2005). Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *The New England journal of medicine*, *352*(3), 254–266. https://doi.org/10.1056/NEJMoa041974

100. Farber S, D'Angio G, Evans A, Mitus A. Clinical studies on actinomycin D with special reference to Wilms' tumor in children. *Ann N Y Acad Sci*. 1960;89:421-425. doi:10.1111/j.1749-6632.1960.tb20165.xliu

101. Farkas, J. E., & Monaghan, J. R. (2017). A brief history of the study of nerve dependent regeneration. *Neurogenesis (Austin, Tex.)*, *4*(1), e1302216. https://doi.org/10.1080/23262133.2017.1302216

102. Felsher D. W. (2010). MYC Inactivation Elicits Oncogene Addiction through Both Tumor Cell-Intrinsic and Host-Dependent Mechanisms. *Genes & cancer*, *1*(6), 597–604. https://doi.org/10.1177/1947601910377798

103. Figueroa, M. E., Lugthart, S., Li, Y., Erpelinck-Verschueren, C., Deng, X., Christos, P. J., Schifano, E., Booth, J., van Putten, W., Skrabanek, L., Campagne, F., Mazumdar, M., Greally, J. M., Valk, P. J., Löwenberg, B., Delwel, R., & Melnick, A. (2010). DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer cell*, *17*(1), 13–27. https://doi.org/10.1016/j.ccr.2009.11.020

104. Filmus, J., & Buick, R. N. (1985). Relationship of c-myc expression to differentiation and proliferation of HL-60 cells. *Cancer research*, *45*(2), 822–825.

105. Flores-Romero, H., Landeta, O., Ugarte-Uribe, B., Cosentino, K., García-Porras, M., García-Sáez, A. J., & Basañez, G. (2019). BFL1 modulates apoptosis at the membrane level through a bifunctional and multimodal mechanism showing key differences with BCLXL. *Cell death and differentiation*, *26*(10), 1880–1894. https://doi.org/10.1038/s41418-018-0258-5

106. Foster J. A. (1963). Induction of neoplasms in planarians with carcinogens. *Cancer research*, *23*, 300–303.

107. Fouad, Y. A., & Aanei, C. (2017). Revisiting the hallmarks of cancer. *American journal of cancer research*, *7*(5), 1016–1036.

108. Franco, C., & Hess, S. (2015). Recent proteomic advances in developmental, regeneration, and cancer governing signaling pathways. *Proteomics*, *15*(5-6), 1014–1025. https://doi.org/10.1002/pmic.201400368

109. Franco, C. M., & Vázquez, B. I. (2020). Natural Compounds as Antimicrobial Agents. *Antibiotics (Basel, Switzerland)*, *9*(5), 217. https://doi.org/10.3390/antibiotics9050217

110. Friedman A. D. (2007). Transcriptional control of granulocyte and monocyte development. *Oncogene*, *26*(47), 6816–6828. https://doi.org/10.1038/sj.onc.1210764

111. Friedman A. D. (2015). C/EBPα in normal and malignant myelopoiesis. *International journal of hematology*, *101*(4), 330–341. https://doi.org/10.1007/s12185-015-1764-6

112. Friedmann-Morvinski, D., & Verma, I. M. (2014). Dedifferentiation and reprogramming: origins of cancer stem cells. *EMBO reports*, *15*(3), 244–253. https://doi.org/10.1002/embr.201338254

113. Fu, I., Cai, Y., Geacintov, N. E., Zhang, Y., & Broyde, S. (2017). Nucleosome Histone Tail Conformation and Dynamics: Impacts of Lysine Acetylation and a Nearby Minor Groove Benzo[a]pyrene-Derived Lesion. *Biochemistry*, *56*(14), 1963–1973. https://doi.org/10.1021/acs.biochem.6b01208

114. Fukuoka, T., Kawai, A., Takahara, T., Mori, M., Roeder, R. G., Hasegawa, N., & Ito, M. (2019). PML-RARα induces all-trans retinoic acid-dependent transcriptional activation through interaction with MED1. *Transcription*, *10*(3), 147–156. https://doi.org/10.1080/21541264.2019.1624467

115. Gallagher, R., Collins, S., Trujillo, J., McCredie, K., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F., & Gallo, R. (1979). Characterization of the continuous,

differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood*, *54*(3), 713–733.

116. Gambino, G., Falleni, A., Nigro, M., Salvetti, A., Cecchettini, A., Ippolito, C., Guidi, P., & Rossi, L. (2020). Dynamics of interaction and effects of microplastics on planarian tissue regeneration and cellular homeostasis. *Aquatic toxicology (Amsterdam, Netherlands)*, *218*, 105354. https://doi.org/10.1016/j.aquatox.2019.105354

117. Garcia, P. B., & Attardi, L. D. (2014). Illuminating p53 function in cancer with genetically engineered mouse models. *Seminars in cell & developmental biology*, *27*, 74–85. https://doi.org/10.1016/j.semcdb.2013.12.014

118. Genovese, G., Kähler, A. K., Handsaker, R. E., Lindberg, J., Rose, S. A., Bakhoum, S. F., Chambert, K., Mick, E., Neale, B. M., Fromer, M., Purcell, S. M., Svantesson, O., Landén, M., Höglund, M., Lehmann, S., Gabriel, S. B., Moran, J. L., Lander, E. S., Sullivan, P. F., Sklar, P., … McCarroll, S. A. (2014). Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *The New England journal of medicine*, *371*(26), 2477–2487. https://doi.org/10.1056/NEJMoa1409405

119. Gerber, T., Murawala, P., Knapp, D., Masselink, W., Schuez, M., Hermann, S., Gac-Santel, M., Nowoshilow, S., Kageyama, J., Khattak, S., Currie, J. D., Camp, J. G., Tanaka, E. M., & Treutlein, B. (2018). Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration. *Science (New York, N.Y.)*, *362*(6413), eaaq0681. https://doi.org/10.1126/science.aaq0681

120. Ghelli Luserna di Rora', A., Iacobucci, I., & Martinelli, G. (2017). The cell cycle checkpoint inhibitors in the treatment of leukemias. *Journal of hematology & oncology*, *10*(1), 77. https://doi.org/10.1186/s13045-017-0443-x

121. Ghiaur, G., Wroblewski, M., & Loges, S. (2015). Acute Myelogenous Leukemia and its Microenvironment: A Molecular Conversation. *Seminars in hematology*, *52*(3), 200–206. https://doi.org/10.1053/j.seminhematol.2015.03.003

122. Ghosh, A., Barba, P., & Perales, M. A. (2020). Checkpoint inhibitors in AML: are we there yet?. *British journal of haematology*, *188*(1), 159–167. https://doi.org/10.1111/bjh.16358

123. Gianni, M., Fratelli, M., Bolis, M., Kurosaki, M., Zanetti, A., Paroni, G., Rambaldi, A., Borleri, G., Rochette-Egly, C., Terao, M., & Garattini, E. (2017). RARα2 and PML-RAR similarities in the control of basal and retinoic acid induced myeloid maturation of acute myeloid leukemia cells. *Oncotarget*, *8*(23), 37041–37060. https://doi.org/10.18632/oncotarget.10556

124. Glienke, W., Maute, L., Wicht, J., & Bergmann, L. (2009). Wilms' tumour gene 1 (WT1) as a target in curcumin treatment of pancreatic cancer cells. *European journal of cancer (Oxford, England : 1990)*, *45*(5), 874–880. https://doi.org/10.1016/j.ejca.2008.12.030

125. Godwin, J. W., Debuque, R., Salimova, E., & Rosenthal, N. A. (2017). Heart regeneration in the salamander relies on macrophage-mediated control of fibroblast activation and the extracellular landscape. *NPJ Regenerative medicine*, *2*, 22. https://doi.org/10.1038/s41536-017-0027-y

126. Godwin, J. W., Pinto, A. R., & Rosenthal, N. A. (2013). Macrophages are required for adult salamander limb regeneration. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(23), 9415–9420. https://doi.org/10.1073/pnas.1300290110

127. Goldman, S. L., Hassan, C., Khunte, M., Soldatenko, A., Jong, Y., Afshinnekoo, E., & Mason, C. E. (2019). Epigenetic Modifications in Acute Myeloid Leukemia: Prognosis, Treatment, and Heterogeneity. *Frontiers in genetics*, *10*, 133. https://doi.org/10.3389/fgene.2019.00133

128. Göös, H., Fogarty, C. L., Sahu, B., Plagnol, V., Rajamäki, K., Nurmi, K., Liu, X., Einarsdottir, E., Jouppila, A., Pettersson, T., Vihinen, H., Krjutskov, K., Saavalainen, P., Järvinen, A., Muurinen, M., Greco, D., Scala, G., Curtis, J., Nordström, D., Flaumenhaft, R., … Seppänen, M. (2019). Gain-of-function CEBPE mutation causes noncanonical autoinflammatory inflammasomopathy. *The Journal of allergy and clinical immunology*, *144*(5), 1364–1376. https://doi.org/10.1016/j.jaci.2019.06.003

129. Goyert, S. M., Ferrero, E., Rettig, W. J., Yenamandra, A. K., Obata, F., & Le Beau, M. M. (1988). The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. *Science (New York, N.Y.)*, *239*(4839), 497–500. https://doi.org/10.1126/science.2448876

130. Grafone, T., Palmisano, M., Nicci, C., & Storti, S. (2012). An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment. *Oncology reviews*, *6*(1), e8. https://doi.org/10.4081/oncol.2012.e8

131. Grohme, M. A., Schloissnig, S., Rozanski, A., Pippel, M., Young, G. R., Winkler, S., Brandl, H., Henry, I., Dahl, A., Powell, S., Hiller, M., Myers, E., & Rink, J. C. (2018). The genome of Schmidtea mediterranea and the evolution of core cellular mechanisms. *Nature*, *554*(7690), 56–61. https://doi.org/10.1038/nature25473

132. Grondin, B., Lefrancois, M., Tremblay, M., Saint-Denis, M., Haman, A., Waga, K., Bédard, A., Tenen, D. G., & Hoang, T. (2007). c-Jun homodimers can function as a context-specific coactivator. *Molecular and cellular biology*, *27*(8), 2919–2933. https://doi.org/10.1128/MCB.00936-06

133. Guo H, Ma O, Friedman AD. The Cebpa +37-kb enhancer directs transgene expression to myeloid progenitors and to long-term hematopoietic stem cells. *J Leukoc Biol*. 2014;96(3):419-426. doi:10.1189/jlb.2AB0314-145R

134. Gupta, P., Gurudutta, G. U., Saluja, D., & Tripathi, R. P. (2009). PU.1 and partners: regulation of haematopoietic stem cell fate in normal and malignant haematopoiesis. *Journal of cellular and molecular medicine*, *13*(11-12), 4349–4363. https://doi.org/10.1111/j.1582-4934.2009.00757.x

135. Gupta, S., Kass, G. E., Szegezdi, E., & Joseph, B. (2009). The mitochondrial death pathway: a promising therapeutic target in diseases. *Journal of cellular and molecular medicine*, *13*(6), 1004–1033. https://doi.org/10.1111/j.1582-4934.2009.00697.x

136. Hagstrom, D., Zhang, S., Ho, A., Tsai, E. S., Radić, Z., Jahromi, A., Kaj, K. J., He, Y., Taylor, P., & Collins, E. S. (2018). Planarian cholinesterase: molecular and functional characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity. *Archives of toxicology*, *92*(3), 1161–1176. https://doi.org/10.1007/s00204-017-2130-7

137. Halene, S., Gaines, P., Sun, H., Zibello, T., Lin, S., Khanna-Gupta, A., Williams, S. C., Perkins, A., Krause, D., & Berliner, N. (2010). *CDKN2C*psilon directs granulocytic-vs-monocytic lineage determination and confers chemotactic function via Hlx. *Experimental hematology*, *38*(2), 90–103. https://doi.org/10.1016/j.exphem.2009.11.004

138. Hao, T., Li-Talley, M., Buck, A., & Chen, W. (2019). An emerging trend of rapid increase of leukemia but not all cancers in the aging population in the United States. *Scientific reports*, *9*(1), 12070. https://doi.org/10.1038/s41598-019-48445-1

139. Harada, Y., Nagata, Y., Kihara, R., Ishikawa, Y., Asou, N., Ohtake, S., Miyawaki, S., Sakura, T., Ozawa, Y., Usui, N., Kanamori, H., Ito, Y., Imai, K., Suehiro, Y., Kobayashi, S., Kitamura, K., Sakaida, E., Onizuka, M., Takeshita, A., Ishida, F., … Japan Adult Leukemia Study Group

JALSG (2018). Prognostic analysis according to the 2017 ELN risk stratification by genetics in adult acute myeloid leukemia patients treated in the Japan Adult Leukemia Study Group (JALSG) AML201 study. *Leukemia research*, *66*, 20–27. https://doi.org/10.1016/j.leukres.2018.01.008

140. Harrison, J. S.,gann Wang, X., & Studzinski, G. P. (2016). The role of VDR and BIM in potentiation of cytarabine-induced cell death in human AML blasts. *Oncotarget*, *7*(24), 36447–36460. https://doi.org/10.18632/oncotarget.8998

141. Hasemann, M. S., Lauridsen, F. K., Waage, J., Jakobsen, J. S., Frank, A. K., Schuster, M. B., Rapin, N., Bagger, F. O., Hoppe, P. S., Schroeder, T., & Porse, B. T. (2014). C/EBPα is required for long-term self-renewal and lineage priming of hematopoietic stem cells and for the maintenance of epigenetic configurations in multipotent progenitors. *PLoS genetics*, *10*(1), e1004079. https://doi.org/10.1371/journal.pgen.1004079

142. Hayes M. J. (2017). Sulphated glycosaminoglycans support an assortment of planarian rhabdite structures. *Biology open*, *6*(5), 571–581. https://doi.org/10.1242/bio.024554

143. Hientz, K., Mohr, A., Bhakta-Guha, D., & Efferth, T. (2017). The role of p53 in cancer drug resistance and targeted chemotherapy. *Oncotarget*, *8*(5), 8921–8946. https://doi.org/10.18632/oncotarget.13475

144. Hirai, H., Yokota, A., Tamura, A., Sato, A., & Maekawa, T. (2015). Non-steady-state hematopoiesis regulated by the C/EBPβ transcription factor. *Cancer science*, *106*(7), 797–802. https://doi.org/10.1111/cas.12690

145. Hofmann, S., Schubert, M. L., Wang, L., He, B., Neuber, B., Dreger, P., Müller-Tidow, C., & Schmitt, M. (2019). Chimeric Antigen Receptor (CAR) T Cell Therapy in Acute Myeloid

Leukemia (AML). *Journal of clinical medicine*, *8*(2), 200. https://doi.org/10.3390/jcm8020200

146. Hong, D., Gupta, R., Ancliff, P., Atzberger, A., Brown, J., Soneji, S., Green, J., Colman, S., Piacibello, W., Buckle, V., Tsuzuki, S., Greaves, M., & Enver, T. (2008). Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. *Science (New York, N.Y.)*, *319*(5861), 336–339. https://doi.org/10.1126/science.1150648

147. Horie, N., Mori, T., Asada, H., Ishikawa, A., Johnston, P. G., & Takeishi, K. (2004). Implication of CDK inhibitors p21 and p27 in the differentiation of HL-60 cells. *Biological & pharmaceutical bulletin*, *27*(7), 992–997. https://doi.org/10.1248/bpb.27.992

148. https://clinicaltrials.gov/ct2/results?cond=amL&term=car&cntry=&state=&city=&dist=https://news.harvard.edu/gazette/story/2020/01/solid-vaccine-eliminates-acute-myeloid-leukemia-in-mice/

149. Hu, W., Wang, G., Huang, D., Sui, M., & Xu, Y. (2019). Cancer Immunotherapy Based on Natural Killer Cells: Current Progress and New Opportunities. *Frontiers in immunology*, *10*, 1205. https://doi.org/10.3389/fimmu.2019.01205

150. Huang, S. T., Wang, C. Y., Yang, R. C., Chu, C. J., Wu, H. T., & Pang, J. H. (2010). Wogonin, an active compound in Scutellaria baicalensis, induces apoptosis and reduces telomerase activity in the HL-60 leukemia cells. *Phytomedicine : international journal of phytotherapy and phytopharmacology*, *17*(1), 47–54. https://doi.org/10.1016/j.phymed.2009.06.005

151. Hwang, D., Kim, M., Park, H., Jeong, M. I., Jung, W., & Kim, B. (2019). Natural Products and Acute Myeloid Leukemia: A Review Highlighting Mechanisms of Action. *Nutrients*, *11*(5), 1010. https://doi.org/10.3390/nu11051010

152. IC50 Calculator | AAT Bioquest. Available online: https://www.aatbio.com/tools/ic50calculator (accessed on 5 April 2020).

153. Ida, C., Ogata, S., Okumura, K., & Taguchi, H. (2008). Changes in the gene expression of C-myc and CD38 in HL-60 cells during differentiation induced by nicotinic acid-related compounds. *Bioscience, biotechnology, and biochemistry*, *72*(3), 868–871. https://doi.org/10.1271/bbb.70585

154. Imperato, M. R., Cauchy, P., Obier, N., & Bonifer, C. (2015). The RUNX1-PU.1 axis in the control of hematopoiesis. *International journal of hematology*, *101*(4), 319–329. https://doi.org/10.1007/s12185-015-1762-8

155. Ingram A. J. (1971). The reactions to carcinogens in the axolotl (Ambystoma mexicanum) in relation to the "regeneration field control" hypothesis. *Journal of embryology and experimental morphology*, *26*(3), 425–441.

156. Inukai, S., Kock, K. H., & Bulyk, M. L. (2017). Transcription factor-DNA binding: beyond binding site motifs. *Current opinion in genetics & development*, *43*, 110–119. https://doi.org/10.1016/j.gde.2017.02.007

157. Isbilen, O., Rizaner, N., & Volkan, E. (2018). Anti-proliferative and cytotoxic activities of Allium autumnale P. H. Davis (Amaryllidaceae) on human breast cancer cell lines MCF-7 and MDA-MB-231. *BMC complementary and alternative medicine*, *18*(1), 30. https://doi.org/10.1186/s12906-018-2105-0

158. Ivankovic, M., Haneckova, R., Thommen, A., Grohme, M. A., Vila-Farré, M., Werner, S., & Rink, J. C. (2019). Model systems for regeneration: planarians. *Development (Cambridge, England)*, *146*(17), dev167684. https://doi.org/10.1242/dev.167684

159. Iwasaki, H., Mizuno, S., Arinobu, Y., Ozawa, H., Mori, Y., Shigematsu, H., Takatsu, K., Tenen, D. G., & Akashi, K. (2006). The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes & development*, *20*(21), 3010–3021. https://doi.org/10.1101/gad.1493506

160. Jain, C. K., Majumder, H. K., & Roychoudhury, S. (2017). Natural Compounds as Anticancer Agents Targeting DNA Topoisomerases. *Current genomics*, *18*(1), 75–92. https://doi.org/10.2174/1389202917666160808125213

161. Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., Lindsley, R. C., Mermel, C. H., Burtt, N., Chavez, A., Higgins, J. M., Moltchanov, V., Kuo, F. C., Kluk, M. J., Henderson, B., Kinnunen, L., Koistinen, H. A., Ladenvall, C., Getz, G., Correa, A., … Ebert, B. L. (2014). Age-related clonal hematopoiesis associated with adverse outcomes. *The New England journal of medicine*, *371*(26), 2488–2498. https://doi.org/10.1056/NEJMoa1408617

162. Jiang, G., Albihn, A., Tang, T., Tian, Z., & Henriksson, M. (2008). Role of Myc in differentiation and apoptosis in HL60 cells after exposure to arsenic trioxide or all-trans retinoic acid. *Leukemia research*, *32*(2), 297–307. https://doi.org/10.1016/j.leukres.2007.06.021

163. Johnson P. F. (2005). Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. *Journal of cell science*, *118*(Pt 12), 2545–2555. https://doi.org/10.1242/jcs.02459

164. Jones S. (2004). An overview of the basic helix-loop-helix proteins. *Genome biology*, *5*(6), 226. https://doi.org/10.1186/gb-2004-5-6-226

165. Jones, L. C., Lin, M. L., Chen, S. S., Krug, U., Hofmann, W. K., Lee, S., Lee, Y. H., & Koeffler, H. P. (2002). Expression of C/EBPbeta from the C/EBPalpha gene locus is sufficient for normal hematopoiesis *in vivo*. *Blood*, *99*(6), 2032–2036. https://doi.org/10.1182/blood.v99.6.2032

166. Kamran, S., Awan, S. A., Ahmad, K. N., & Iqbal, Y. (2019). Acute Myeloid Leukemia with t(8;21)(q22;q22) and Trisomy 4: A Rare Occurrence in a Female Child. *Cureus*, *11*(1), e3885. https://doi.org/10.7759/cureus.3885

167. Kanji, S., Pompili, V. J., & Das, H. (2011). Plasticity and maintenance of hematopoietic stem cells during development. *Recent patents on biotechnology*, *5*(1), 40–53. https://doi.org/10.2174/187220811795655896

168. Karami, A., Tebyanian, H., Goodarzi, V., & Shiri, S. (2015). Planarians: an *In vivo* Model for Regenerative Medicine. *International journal of stem cells*, *8*(2), 128–133. https://doi.org/10.15283/ijsc.2015.8.2.128

169. Kaushansky K. (2018). Hunting for hematopoietic transcriptional networks. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(40), 9818–9820. https://doi.org/10.1073/pnas.1813863115

170. Kavanagh, S., Murphy, T., Law, A., Yehudai, D., Ho, J. M., Chan, S., & Schimmer, A. D. (2017). Emerging therapies for acute myeloid leukemia: translating biology into the clinic. *JCI insight*, *2*(18), e95679. https://doi.org/10.1172/jci.insight.95679

171. Khalifa, S., Elias, N., Farag, M. A., Chen, L., Saeed, A., Hegazy, M. F., Moustafa, M. S., Abd El-Wahed, A., Al-Mousawi, S. M., Musharraf, S. G., Chang, F. R., Iwasaki, A., Suenaga, K., Alajlani, M., Göransson, U., & El-Seedi, H. R. (2019). Marine Natural Products: A Source of Novel Anticancer Drugs. *Marine drugs*, *17*(9), 491. https://doi.org/10.3390/md17090491

172. Khan, R. J., Jha, R. K., Amera, G. M., Jain, M., Singh, E., Pathak, A., Singh, R. P., Muthukumaran, J., & Singh, A. K. (2020). Targeting SARS-CoV-2: a systematic drug repurposing approach to identify promising inhibitors against 3C-like proteinase and 2'-O-ribose methyltransferase. *Journal of biomolecular structure & dynamics*, 1–14. Advance online publication. https://doi.org/10.1080/07391102.2020.1753577

173. Khanna-Gupta, A, & Berliner, N. (2018). *Granulocytopoiesis and Monocytopoiesis. Hematology, 321–333.e1.* doi:10.1016/b978-0-323-35762-3.00027-5

174. Konopleva, M., & Letai, A. (2018). *CDKN2C* inhibition in AML: an unexpected bonus?. *Blood*, *132*(10), 1007–1012. https://doi.org/10.1182/blood-2018-03-828269

175. Kowalczyk, T., Sitarek, P., Skała, E., Toma, M., Wielanek, M., Pytel, D., Wieczfińska, J., Szemraj, J., & Śliwiński, T. (2019). Induction of apoptosis by *in vitro* and *in vivo* plant extracts derived from Menyanthes trifoliata L. in human cancer cells. *Cytotechnology*, *71*(1), 165–180. https://doi.org/10.1007/s10616-018-0274-9

176. Kulling, P. M., Olson, K. C., Olson, T. L., Feith, D. J., & Loughran, T. P., Jr (2017). Vitamin D in hematological disorders and malignancies. *European journal of haematology*, *98*(3), 187–197. https://doi.org/10.1111/ejh.12818

177. Kumar C. C. (2011). Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. *Genes & cancer*, *2*(2), 95–107. https://doi.org/10.1177/1947601911408076

178. Kunchala, P., Kuravi, S., Jensen, R., McGuirk, J., & Balusu, R. (2018). When the good go bad: Mutant NPM1 in acute myeloid leukemia. *Blood reviews*, *32*(3), 167–183. https://doi.org/10.1016/j.blre.2017.11.001

179. Kweon, S. H., Song, J. H., Kim, H. J., Kim, T. S., & Choi, B. G. (2015). Induction of human leukemia cell differentiation via PKC/MAPK pathways by arsantin, a sesquiterpene lactone from Artemisia santolina. *Archives of pharmacal research*, *38*(11), 2020–2028. https://doi.org/10.1007/s12272-015-0609-4

180. Lagunas-Rangel, F. A., Chávez-Valencia, V., Gómez-Guijosa, M. Á., & Cortes-Penagos, C. (2017). Acute Myeloid Leukemia-Genetic Alterations and Their Clinical

Prognosis. *International journal of hematology-oncology and stem cell research*, *11*(4), 328–339.

181. Lallemand-Breitenbach, V., Jeanne, M., Benhenda, S., Nasr, R., Lei, M., Peres, L., Zhou, J., Zhu, J., Raught, B., & de Thé, H. (2008). Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nature cell biology*, *10*(5), 547–555. https://doi.org/10.1038/ncb1717

182. Lambert, S. A., Jolma, A., Campitelli, L. F., Das, P. K., Yin, Y., Albu, M., Chen, X., Taipale, J., Hughes, T. R., & Weirauch, M. T. (2018). The Human Transcription Factors. *Cell*, *175*(2), 598–599. https://doi.org/10.1016/j.cell.2018.09.045

183. Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M. A., & Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*, *367*(6464), 645–648. https://doi.org/10.1038/367645a0

184. Larrosa-Garcia, M., & Baer, M. R. (2017). FLT3 Inhibitors in Acute Myeloid Leukemia: Current Status and Future Directions. *Molecular cancer therapeutics*, *16*(6), 991–1001. https://doi.org/10.1158/1535-7163.MCT-16-0876

185. Laslo, P., Spooner, C. J., Warmflash, A., Lancki, D. W., Lee, H. J., Sciammas, R., Gantner, B. N., Dinner, A. R., & Singh, H. (2006). Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell*, *126*(4), 755–766. https://doi.org/10.1016/j.cell.2006.06.052

186. Laurenti, E., & Göttgens, B. (2018). From haematopoietic stem cells to complex differentiation landscapes. *Nature*, *553*(7689), 418–426. https://doi.org/10.1038/nature25022

187. Lawrence, M. S., Stojanov, P., Polak, P., Kryukov, G. V., Cibulskis, K., Sivachenko, A., Carter, S. L., Stewart, C., Mermel, C. H., Roberts, S. A., Kiezun, A., Hammerman, P. S.,

McKenna, A., Drier, Y., Zou, L., Ramos, A. H., Pugh, T. J., Stransky, N., Helman, E., Kim, J., … Getz, G. (2013). Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*, *499*(7457), 214–218. https://doi.org/10.1038/nature12213

188. Lee, C. T., Huang, Y. W., Yang, C. H., & Huang, K. S. (2015). Drug delivery systems and combination therapy by using vinca alkaloids. *Current topics in medicinal chemistry*, *15*(15), 1491–1500. https://doi.org/10.2174/1568026615666150414120547

189. Lee, J. H., Sung, J. Y., Choi, E. K., Yoon, H. K., Kang, B. R., Hong, E. K., Park, B. K., Kim, Y. N., Rho, S. B., & Yoon, K. (2019). C/EBPβ Is a Transcriptional Regulator of Wee1 at the G_2/M Phase of the Cell Cycle. *Cells*, *8*(2), 145. https://doi.org/10.3390/cells8020145

190. Lei, K., Thi-Kim Vu, H., Mohan, R. D., McKinney, S. A., Seidel, C. W., Alexander, R., Gotting, K., Workman, J. L., & Sánchez Alvarado, A. (2016). Egf Signaling Directs Neoblast Repopulation by Regulating Asymmetric Cell Division in Planarians. *Developmental cell*, *38*(4), 413–429. https://doi.org/10.1016/j.devcel.2016.07.012

191. Ley, T. J., Ding, L., Walter, M. J., McLellan, M. D., Lamprecht, T., Larson, D. E., Kandoth, C., Payton, J. E., Baty, J., Welch, J., Harris, C. C., Lichti, C. F., Townsend, R. R., Fulton, R. S., Dooling, D. J., Koboldt, D. C., Schmidt, H., Zhang, Q., Osborne, J. R., Lin, L., … Wilson, R. K. (2010). DNMT3A mutations in acute myeloid leukemia. *The New England journal of medicine*, *363*(25), 2424–2433. https://doi.org/10.1056/NEJMoa1005143

192. Leyssens, C., Verlinden, L., & Verstuyf, A. (2013). Antineoplastic effects of 1,25(OH)2D3 and its analogs in breast, prostate and colorectal cancer. *Endocrine-related cancer*, *20*(2), R31–R47. https://doi.org/10.1530/ERC-12-0381

193. Li, K., Du, Y., Wei, D. Q., & Zhang, F. (2019). CEBPE expression is an independent prognostic factor for acute myeloid leukemia. *Journal of translational medicine*, *17*(1), 188. https://doi.org/10.1186/s12967-019-1944-x

194. Li, S., Garrett-Bakelman, F. E., Chung, S. S., Sanders, M. A., Hricik, T., Rapaport, F., Patel, J., Dillon, R., Vijay, P., Brown, A. L., Perl, A. E., Cannon, J., Bullinger, L., Luger, S., Becker, M., Lewis, I. D., To, L. B., Delwel, R., Löwenberg, B., Döhner, H., … Mason, C. E. (2016). Distinct evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia. *Nature medicine*, *22*(7), 792–799. https://doi.org/10.1038/nm.4125

195. Li, V. C., & Kirschner, M. W. (2014). Molecular ties between the cell cycle and differentiation in embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(26), 9503–9508. https://doi.org/10.1073/pnas.1408638111

196. Lin, C., & Li, Y. (2013). The role of peptide and DNA vaccines in myeloid leukemia immunotherapy. *Cancer cell international*, *13*(1), 13. https://doi.org/10.1186/1475-2867-13-13

197. Livak, K.J., Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25, 402-408.

198. Liu, M., Lee, M. H., Cohen, M., Bommakanti, M., & Freedman, L. P. (1996). Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes & development*, *10*(2), 142–153. https://doi.org/10.1101/gad.10.2.142

199. Liu, X. F., Xiang, L., Zhou, Q., Carralot, J. P., Prunotto, M., Niederfellner, G., & Pastan, I. (2016). Actinomycin D enhances killing of cancer cells by immunotoxin RG7787 through activation of the extrinsic pathway of apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(38), 10666–10671. https://doi.org/10.1073/pnas.1611481113

200. Liu, Y., Nonnemacher, M. R., & Wigdahl, B. (2009). CCAAT/enhancer-binding proteins and the pathogenesis of retrovirus infection. *Future microbiology*, *4*(3), 299–321. https://doi.org/10.2217/fmb.09.4

201. Londono, R., Sun, A. X., Tuan, R. S., & Lozito, T. P. (2018). TISSUE REPAIR AND EPIMORPHIC REGENERATION: AN OVERVIEW. *Current pathobiology reports*, *6*(1), 61–69. https://doi.org/10.1007/s40139-018-0161-2

202. Iolascon, A., Faienza, M. F., Coppola, B., della Ragione, F., Schettini, F., & Biondi, A. (1996). Homozygous deletions of cyclin-dependent kinase inhibitor genes, p16(INK4A) and p18, in childhood T cell lineage acute lymphoblastic leukemias. *Leukemia*, *10*(2), 255–260.

203. Lourenço, A. R., & Coffer, P. J. (2017). A tumor suppressor role for C/EBPα in solid tumors: more than fat and blood. *Oncogene*, *36*(37), 5221–5230. https://doi.org/10.1038/onc.2017.151

204. Lv, L., Yu, J., & Qi, Z. (2020). Acute myeloid leukemia with inv(16)(p13.1q22) and deletion of the 5'MYH11/3'CBFB gene fusion: a report of two cases and literature review. *Molecular cytogenetics*, *13*, 4. https://doi.org/10.1186/s13039-020-0474-9

205. Mak, K. S., Funnell, A. P., Pearson, R. C., & Crossley, M. (2011). PU.1 and Haematopoietic Cell Fate: Dosage Matters. *International journal of cell biology*, *2011*, 808524. https://doi.org/10.1155/2011/808524

206. Makanae, A., Mitogawa, K., & Satoh, A. (2014). Implication of two different regeneration systems in limb regeneration. *Regeneration (Oxford, England)*, *1*(3), 1–9. https://doi.org/10.1002/reg2.16

207. Mannelli, F., Ponziani, V., Bencini, S., Bonetti, M. I., Benelli, M., Cutini, I., Gianfaldoni, G., Scappini, B., Pancani, F., Piccini, M., Rondelli, T., Caporale, R., Gelli, A. M., Peruzzi, B., Chiarini, M., Borlenghi, E., Spinelli, O., Giupponi, D., Zanghì, P., Bassan, R., … Bosi, A. (2017). *CEBPA*-double-mutated acute myeloid leukemia displays a unique phenotypic

profile: a reliable screening method and insight into biological features. *Haematologica*, *102*(3), 529–540. https://doi.org/10.3324/haematol.2016.151910

208. Marchwicka, A., & Marcinkowska, E. (2018). Regulation of Expression of CEBP Genes by Variably Expressed Vitamin D Receptor and Retinoic Acid Receptor α in Human Acute Myeloid Leukemia Cell Lines. *International journal of molecular sciences*, *19*(7), 1918. https://doi.org/10.3390/ijms19071918

209. Marcinkowska, E., Garay, E., Gocek, E., Chrobak, A., Wang, X., & Studzinski, G. P. (2006). Regulation of *CDKN2C*eta isoforms by MAPK pathways in HL60 cells induced to differentiate by 1,25-dihydroxyvitamin D3. *Experimental cell research*, *312*(11), 2054–2065. https://doi.org/10.1016/j.yexcr.2006.03.003

210. Marinello, J., Delcuratolo, M., & Capranico, G. (2018). Anthracyclines as Topoisomerase II Poisons: From Early Studies to New Perspectives. *International journal of molecular sciences*, *19*(11), 3480. https://doi.org/10.3390/ijms19113480

211. Martino, E., Casamassima, G., Castiglione, S., Cellupica, E., Pantalone, S., Papagni, F., Rui, M., Siciliano, A. M., & Collina, S. (2018). Vinca alkaloids and analogues as anti-cancer agents: Looking back, peering ahead. *Bioorganic & medicinal chemistry letters*, *28*(17), 2816–2826. https://doi.org/10.1016/j.bmcl.2018.06.044

212. Massaro, F., Molica, M., & Breccia, M. (2016). Current first- and second-line treatment options in acute promyelocytic leukemia. *International journal of hematologic oncology*, *5*(3), 105–118. https://doi.org/10.2217/ijh-2016-0010

213. Mathieu, J., Yant, L. J., Mürdter, F., Küttner, F., & Schmid, M. (2009). Repression of flowering by the miR172 target SMZ. *PLoS biology*, *7*(7), e1000148. https://doi.org/10.1371/journal.pbio.1000148

214. Matulja, D., Kolympadi Markovic, M., Ambrožić, G., Laclef, S., Pavelić, S. K., & Marković, D. (2019). Secondary Metabolites from Gorgonian Corals of the Genus *Eunicella*: Structural Characterizations, Biological Activities, and Synthetic Approaches. *Molecules (Basel, Switzerland)*, *25*(1), 129. https://doi.org/10.3390/molecules25010129

215. McCusker, C., & Gardiner, D. M. (2011). The axolotl model for regeneration and aging research: a mini-review. *Gerontology*, *57*(6), 565–571. https://doi.org/10.1159/000323761

216. McGann, C. J., Odelberg, S. J., & Keating, M. T. (2001). Mammalian myotube dedifferentiation induced by newt regeneration extract. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(24), 13699–13704. https://doi.org/10.1073/pnas.221297398

217. McGowan, J. V., Chung, R., Maulik, A., Piotrowska, I., Walker, J. M., & Yellon, D. M. (2017). Anthracycline Chemotherapy and Cardiotoxicity. *Cardiovascular drugs and therapy*, *31*(1), 63–75. https://doi.org/10.1007/s10557-016-6711-0

218. Megías-Vericat, J. E., Ballesta-López, O., Barragán, E., & Montesinos, P. (2019). *IDH1*-mutated relapsed or refractory AML: current challenges and future prospects. *Blood and lymphatic cancer : targets and therapy*, *9*, 19–32. https://doi.org/10.2147/BLCTT.S177913

219. Mehta, J., Rayalam, S., & Wang, X. (2018). Cytoprotective Effects of Natural Compounds against Oxidative Stress. *Antioxidants (Basel, Switzerland)*, *7*(10), 147. https://doi.org/10.3390/antiox7100147

220. Melnick, A., & Licht, J. D. (1999). Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood*, *93*(10), 3167–3215.

221. Meyer, C., Burmeister, T., Gröger, D., Tsaur, G., Fechina, L., Renneville, A., Sutton, R., Venn, N. C., Emerenciano, M., Pombo-de-Oliveira, M. S., Barbieri Blunck, C., Almeida Lopes, B., Zuna, J., Trka, J., Ballerini, P., Lapillonne, H., De Braekeleer, M., Cazzaniga, G., Corral Abascal, L., van der Velden, V., … Marschalek, R. (2018). The MLL recombinome of acute leukemias in 2017. *Leukemia*, *32*(2), 273–284. https://doi.org/10.1038/leu.2017.213

222. Miller, D. M., Thomas, S. D., Islam, A., Muench, D., & Sedoris, K. (2012). c-Myc and cancer metabolism. *Clinical cancer research: an official journal of the American Association for Cancer Research*, *18*(20), 5546–5553. https://doi.org/10.1158/1078-0432.CCR-12-0977

223. Mitsis, T., Efthimiadou, A., Bacopoulou, F., Vlachakis, D., Chrousos, G.P., Eliopoulos, E. Transcription factors and evolution: An integral part of gene expression (Review). (2020). *World Academy of Sciences Journal*, *2*:3-8. doi.org/10.3892/wasj.2020.32

224. Mitton, B., Chae, H. D., Hsu, K., Dutta, R., Aldana-Masangkay, G., Ferrari, R., Davis, K., Tiu, B. C., Kaul, A., Lacayo, N., Dahl, G., Xie, F., Li, B. X., Breese, M. R., Landaw, E. M., Nolan, G., Pellegrini, M., Romanov, S., Xiao, X., & Sakamoto, K. M. (2016). Small molecule inhibition of cAMP response element binding protein in human acute myeloid leukemia cells. *Leukemia*, *30*(12), 2302–2311. https://doi.org/10.1038/leu.2016.139

225. Miyamoto, T., Weissman, I. L., & Akashi, K. (2000). AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(13), 7521–7526. https://doi.org/10.1073/pnas.97.13.7521

226. Miyaura, C., Abe, E., Suda, T., & Kuroki, T. (1985). Alternative differentiation of human promyelocytic leukemia cells (HL-60) induced selectively by retinoic acid and 1 alpha,25-dihydroxyvitamin D3. *Cancer research*, *45*(9), 4244–4248.

227. Moarii, M., & Papaemmanuil, E. (2017). Classification and risk assessment in AML: integrating cytogenetics and molecular profiling. *Hematology. American Society of*

Hematology. Education Program, *2017*(1), 37–44. https://doi.org/10.1182/asheducation-2017.1.37

228. Moudi, M., Go, R., Yien, C. Y., & Nazre, M. (2013). Vinca alkaloids. *International journal of preventive medicine*, *4*(11), 1231–1235.

229. Mrózek K. (2008). Cytogenetic, molecular genetic, and clinical characteristics of acute myeloid leukemia with a complex karyotype. *Seminars in oncology*, *35*(4), 365–377. https://doi.org/10.1053/j.seminoncol.2008.04.007

230. Mrózek, K., Harper, D. P., & Aplan, P. D. (2009). Cytogenetics and molecular genetics of acute lymphoblastic leukemia. *Hematology/oncology clinics of North America*, *23*(5), 991–v. https://doi.org/10.1016/j.hoc.2009.07.001

231. Mrózek, K., Heerema, N. A., & Bloomfield, C. D. (2004). Cytogenetics in acute leukemia. *Blood reviews*, *18*(2), 115–136. https://doi.org/10.1016/S0268-960X(03)00040-7

232. Mueller, B. U., Pabst, T., Fos, J., Petkovic, V., Fey, M. F., Asou, N., Buergi, U., & Tenen, D. G. (2006). ATRA resolves the differentiation block in t(15;17) acute myeloid leukemia by restoring PU.1 expression. *Blood*, *107*(8), 3330–3338. https://doi.org/10.1182/blood-2005-07-3068

233. Mushtaq, S., Abbasi, B. H., Uzair, B., & Abbasi, R. (2018). Natural products as reservoirs of novel therapeutic agents. *EXCLI journal*, *17*, 420–451. https://doi.org/10.17179/excli2018-1174

234. Muto, A., Kizaki, M., Yamato, K., Kawai, Y., Kamata-Matsushita, M., Ueno, H., Ohguchi, M., Nishihara, T., Koeffler, H. P., & Ikeda, Y. (1999). 1,25-Dihydroxyvitamin D3 induces differentiation of a retinoic acid-resistant acute promyelocytic leukemia cell line (UF-1) associated with expression of p21(WAF1/CIP1) and p27(KIP1). *Blood*, *93*(7), 2225–2233.

235. Namasu, C. Y., Katzerke, C., Bräuer-Hartmann, D., Wurm, A. A., Gerloff, D., Hartmann, J. U., Schwind, S., Müller-Tidow, C., Hilger, N., Fricke, S., Christopeit, M., Niederwieser, D., & Behre, G. (2017). ABR, a novel inducer of transcription factor C/EBPα, contributes to myeloid differentiation and is a favorable prognostic factor in acute myeloid leukemia. *Oncotarget*, *8*(61), 103626–103639. https://doi.org/10.18632/oncotarget.22093

236. Natarajan, N., Ramakrishnan, P., Lakshmanan, V., Palakodeti, D., & Rangiah, K. (2015). A quantitative metabolomics peek into planarian regeneration. *The Analyst*, *140*(10), 3445–3464. https://doi.org/10.1039/c4an02037e

237. Nerlov C. (2007). The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. *Trends in cell biology*, *17*(7), 318–324. https://doi.org/10.1016/j.tcb.2007.07.004

238. Nerlov, C., & Graf, T. (1998). PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes & development*, *12*(15), 2403–2412. https://doi.org/10.1101/gad.12.15.2403

239. Neuendorff, N. R., Loh, K. P., Mims, A. S., Christofyllakis, K., Soo, W. K., Bölükbasi, B., Oñoro-Algar, C., Hundley, W. G., & Klepin, H. D. (2020). Anthracycline-related cardiotoxicity in older patients with acute myeloid leukemia: a Young SIOG review paper. *Blood advances*, *4*(4), 762–775. https://doi.org/10.1182/bloodadvances.2019000955

240. Newman, D. J., & Cragg, G. M. (2020). Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *Journal of natural products*, *83*(3), 770–803. https://doi.org/10.1021/acs.jnatprod.9b01285

241. Niwa H. (2018). The principles that govern transcription factor network functions in stem cells. *Development (Cambridge, England)*, *145*(6), dev157420. https://doi.org/10.1242/dev.157420

242. Niwa-Kawakita, M., Ferhi, O., Soilihi, H., Le Bras, M., Lallemand-Breitenbach, V., & de Thé, H. (2017). PML is a ROS sensor activating p53 upon oxidative stress. *The Journal of experimental medicine*, *214*(11), 3197–3206. https://doi.org/10.1084/jem.20160301

243. Noguera, N. I., Catalano, G., Banella, C., Divona, M., Faraoni, I., Ottone, T., Arcese, W., & Voso, M. T. (2019). Acute Promyelocytic Leukemia: Update on the Mechanisms of Leukemogenesis, Resistance and on Innovative Treatment Strategies. *Cancers*, *11*(10), 1591. https://doi.org/10.3390/cancers11101591

244. Norsworthy, K. J., DeZern, A. E., Tsai, H. L., Hand, W. A., Varadhan, R., Gore, S. D., Gojo, I., Pratz, K., Carraway, H. E., Showel, M., McDevitt, M. A., Gladstone, D., Ghiaur, G., Prince, G., Seung, A. H., Benani, D., Levis, M. J., Karp, J. E., & Smith, B. D. (2017). Timed sequential therapy for acute myelogenous leukemia: Results of a retrospective study of 301 patients and review of the literature. *Leukemia research*, *61*, 25–32. https://doi.org/10.1016/j.leukres.2017.08.009

245. Nowak, D., Stewart, D., & Koeffler, H. P. (2009). Differentiation therapy of leukemia: 3 decades of development. *Blood*, *113*(16), 3655–3665. https://doi.org/10.1182/blood-2009-01-198911

246. Nowoshilow, S., Schloissnig, S., Fei, J. F., Dahl, A., Pang, A., Pippel, M., Winkler, S., Hastie, A. R., Young, G., Roscito, J. G., Falcon, F., Knapp, D., Powell, S., Cruz, A., Cao, H., Habermann, B., Hiller, M., Tanaka, E. M., & Myers, E. W. (2018). The axolotl genome and the evolution of key tissue formation regulators. *Nature*, *554*(7690), 50–55. https://doi.org/10.1038/nature25458

247. Nurminen, V., Seuter, S., & Carlberg, C. (2019). Primary Vitamin D Target Genes of Human Monocytes. *Frontiers in physiology*, *10*, 194. https://doi.org/10.3389/fphys.2019.00194

248. Nutt, S. L., Metcalf, D., D'Amico, A., Polli, M., & Wu, L. (2005). Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. *The Journal of experimental medicine*, *201*(2), 221–231. https://doi.org/10.1084/jem.20041535

249. Onal, P., Grün, D., Adamidi, C., Rybak, A., Solana, J., Mastrobuoni, G., Wang, Y., Rahn, H. P., Chen, W., Kempa, S., Ziebold, U., & Rajewsky, N. (2012). Gene expression of pluripotency determinants is conserved between mammalian and planarian stem cells. *The EMBO journal*, *31*(12), 2755–2769. https://doi.org/10.1038/emboj.2012.110

250. Ong, T. H., Romanova, E. V., Roberts-Galbraith, R. H., Yang, N., Zimmerman, T. A., Collins, J. J., 3rd, Lee, J. E., Kelleher, N. L., Newmark, P. A., & Sweedler, J. V. (2016). Mass Spectrometry Imaging and Identification of Peptides Associated with Cephalic Ganglia Regeneration in Schmidtea mediterranea. *The Journal of biological chemistry*, *291*(15), 8109–8120. https://doi.org/10.1074/jbc.M115.709196

251. Ossenkoppele, G., & Schuurhuis, G. J. (2016). MRD in AML: does it already guide therapy decision-making?. *Hematology. American Society of Hematology. Education Program*, *2016*(1), 356–365. https://doi.org/10.1182/asheducation-2016.1.356

252. Ostrander, E. L., Kramer, A. C., Mallaney, C., Celik, H., Koh, W. K., Fairchild, J., Haussler, E., Zhang, C., & Challen, G. A. (2020). Divergent Effects of Dnmt3a and Tet2 Mutations on Hematopoietic Progenitor Cell Fitness. *Stem cell reports*, *14*(4), 551–560. https://doi.org/10.1016/j.stemcr.2020.02.011

253. Otte, A., Mandel, K., Reinstrom, G., & Hass, R. (2011). Abolished adherence alters signaling pathways in phorbol ester-induced human U937 cells. *Cell communication and signaling : CCS*, *9*, 20. https://doi.org/10.1186/1478-811X-9-20

254. Oviedo, N. J., & Beane, W. S. (2009). Regeneration: The origin of cancer or a possible cure?. *Seminars in cell & developmental biology*, *20*(5), 557–564. https://doi.org/10.1016/j.semcdb.2009.04.005

255. Pan, X. N., Chen, J. J., Wang, L. X., Xiao, R. Z., Liu, L. L., Fang, Z. G., Liu, Q., Long, Z. J., & Lin, D. J. (2014). Inhibition of c-Myc overcomes cytotoxic drug resistance in acute myeloid leukemia cells by promoting differentiation. *PloS one*, *9*(8), e105381. https://doi.org/10.1371/journal.pone.0105381

256. Papaemmanuil, E., Gerstung, M., Bullinger, L., Gaidzik, V. I., Paschka, P., Roberts, N. D., Potter, N. E., Heuser, M., Thol, F., Bolli, N., Gundem, G., Van Loo, P., Martincorena, I., Ganly, P., Mudie, L., McLaren, S., O'Meara, S., Raine, K., Jones, D. R., Teague, J. W., … Campbell, P. J. (2016). Genomic Classification and Prognosis in Acute Myeloid Leukemia. *The New England journal of medicine*, *374*(23), 2209–2221. https://doi.org/10.1056/NEJMoa1516192

257. Pardoll D. M. (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nature reviews. Cancer*, *12*(4), 252–264. https://doi.org/10.1038/nrc3239

258. Patatanian, E., & Thompson, D. F. (2008). Retinoic acid syndrome: a review. *Journal of clinical pharmacy and therapeutics*, *33*(4), 331–338. https://doi.org/10.1111/j.1365-2710.2008.00935.x

259. Patle, D., Vyas, M., & Khatik, G. L. (2020). A Review on natural products and herbs used in the management of diabetes. *Current diabetes reviews*, 10.2174/1573399816666200408090058. Advance online publication. https://doi.org/10.2174/1573399816666200408090058

260. Paulsson, K., Lassen, C., Kuric, N., Billström, R., Fioretos, T., Tanke, H. J., & Johansson, B. (2003). MYC is not overexpressed in a case of chronic myelomonocytic leukemia with MYC-containing double minutes. *Leukemia*, *17*(4), 813–815. https://doi.org/10.1038/sj.leu.2402841

261. Pearson, B. J., & Sánchez Alvarado, A. (2010). A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. *Development (Cambridge, England)*, *137*(2), 213–221. https://doi.org/10.1242/dev.044297

262. Petruczynik, A., Tuzimski, T., Plech, T., Misiurek, J., Szalast, K., & Szymczak, G. (2019). Comparison of Anticancer Activity and HPLC-DAD Determination of Selected Isoquinoline Alkaloids from *Thalictrum foetidum*, *Berberis* sp. and *Chelidonium majus* Extracts. *Molecules (Basel, Switzerland)*, *24*(19), 3417. https://doi.org/10.3390/molecules24193417

263. Pinsino, A., Thorndyke, M. C., & Matranga, V. (2007). Coelomocytes and post-traumatic response in the common sea star Asterias rubens. *Cell stress & chaperones*, *12*(4), 331–341. https://doi.org/10.1379/csc-288.1

264. Plass, M., Solana, J., Wolf, F. A., Ayoub, S., Misios, A., Glažar, P., Obermayer, B., Theis, F. J., Kocks, C., & Rajewsky, N. (2018). Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science (New York, N.Y.)*, *360*(6391), eaaq1723. https://doi.org/10.1126/science.aaq1723

265. Powell, R. V., Willett, C. R., Goertzen, L. R., & Rashotte, A. M. (2019). Lineage specific conservation of cis-regulatory elements in Cytokinin Response Factors. *Scientific reports*, *9*(1), 13387. https://doi.org/10.1038/s41598-019-49741-6

266. Przybilla, J., Hopp, L., Lübbert, M., Loeffler, M., & Galle, J. (2017). Targeting DNA hypermethylation: Computational modeling of DNA demethylation treatment of acute

myeloid leukemia. *Epigenetics*, *12*(10), 886–896. https://doi.org/10.1080/15592294.2017.1361090

267. Purushothaman, S., Elewa, A., & Seifert, A. W. (2019). Fgf-signaling is compartmentalized within the mesenchyme and controls proliferation during salamander limb development. *eLife*, *8*, e48507. https://doi.org/10.7554/eLife.48507

268. Quail, D. F., & Joyce, J. A. (2013). Microenvironmental regulation of tumor progression and metastasis. *Nature medicine*, *19*(11), 1423–1437. https://doi.org/10.1038/nm.3394

269. Radomska, H. S., Huettner, C. S., Zhang, P., Cheng, T., Scadden, D. T., & Tenen, D. G. (1998). CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Molecular and cellular biology*, *18*(7), 4301–4314. https://doi.org/10.1128/mcb.18.7.4301

270. Ragg, S. J., Kaga, S., Berg, K. A., & Ochi, A. (1998). The mitogen-activated protein kinase pathway inhibits ceramide-induced terminal differentiation of a human monoblastic leukemia cell line, U937. *Journal of immunology (Baltimore, Md. : 1950)*, *161*(3), 1390–1398.

271. Ragione, F. D., Cucciolla, V., Borriello, A., Pietra, V. D., Racioppi, L., Soldati, G., Manna, C., Galletti, P., & Zappia, V. (1998). Resveratrol arrests the cell division cycle at S/G2 phase transition. *Biochemical and biophysical research communications*, *250*(1), 53–58. https://doi.org/10.1006/bbrc.1998.9263

272. Raimundo, I., Silva, S. G., Costa, R., & Keller-Costa, T. (2018). Bioactive Secondary Metabolites from Octocoral-Associated Microbes-New Chances for Blue Growth. *Marine drugs*, *16*(12), 485. https://doi.org/10.3390/md16120485

273. Ramirez, R. N., El-Ali, N. C., Mager, M. A., Wyman, D., Conesa, A., & Mortazavi, A. (2017). Dynamic Gene Regulatory Networks of Human Myeloid Differentiation. *Cell systems*, *4*(4), 416–429.e3. https://doi.org/10.1016/j.cels.2017.03.005

274. Rashidi, A., & Cashen, A. F. (2015). A cytogenetic model predicts relapse risk and survival in patients with acute myeloid leukemia undergoing hematopoietic stem cell transplantation in morphologic complete remission. *Leukemia research*, *39*(1), 77–81. https://doi.org/10.1016/j.leukres.2014.11.007

275. Ravandi, F., Walter, R. B., & Freeman, S. D. (2018). Evaluating measurable residual disease in acute myeloid leukemia. *Blood advances*, *2*(11), 1356–1366. https://doi.org/10.1182/bloodadvances.2018016378

276. Reddien, P. W., & Sánchez Alvarado, A. (2004). Fundamentals of planarian regeneration. *Annual review of cell and developmental biology*, *20*, 725–757. https://doi.org/10.1146/annurev.cellbio.20.010403.095114

277. Reiß, C., Olsson, L., & Hoßfeld, U. (2015). The history of the oldest self-sustaining laboratory animal: 150 years of axolotl research. *Journal of experimental zoology. Part B, Molecular and developmental evolution*, *324*(5), 393–404. https://doi.org/10.1002/jez.b.22617

278. Ren, W., Guo, J., Jiang, F., Lu, J., Ding, Y., Li, A., Liang, X., & Jia, W. (2014). CCAAT/enhancer-binding protein α is a crucial regulator of human fat mass and obesity associated gene transcription and expression. *BioMed research international*, *2014*, 406909. https://doi.org/10.1155/2014/406909

279. Ren, X., Zhang, Z., Tian, J., Wang, H., Song, G., Guo, Q., Tian, J., Han, Y., Liao, Q., Liu, G., Ding, H., & Jiang, G. (2017). The downregulation of c-Myc and its target gene hTERT is associated with the antiproliferative effects of baicalin on HL-60 cells. *Oncology letters*, *14*(6), 6833–6840. https://doi.org/10.3892/ol.2017.7039

280. Ridinger-Saison, M., Boeva, V., Rimmelé, P., Kulakovskiy, I., Gallais, I., Levavasseur, B., Paccard, C., Legoix-Né, P., Morlé, F., Nicolas, A., Hupé, P., Barillot, E., Moreau-Gachelin, F., & Guillouf, C. (2012). Spi-1/PU.1 activates transcription through clustered DNA occupancy in

erythroleukemia. *Nucleic acids research*, *40*(18), 8927–8941. https://doi.org/10.1093/nar/gks659

281. Riley, R. S., June, C. H., Langer, R., & Mitchell, M. J. (2019). Delivery technologies for cancer immunotherapy. *Nature reviews. Drug discovery*, *18*(3), 175–196. https://doi.org/10.1038/s41573-018-0006-z

282. Rink J. C. (2013). Stem cell systems and regeneration in planaria. *Development genes and evolution*, *223*(1-2), 67–84. https://doi.org/10.1007/s00427-012-0426-4

283. Roberts-Galbraith, R. H., Brubacher, J. L., & Newmark, P. A. (2016). A functional genomics screen in planarians reveals regulators of whole-brain regeneration. *eLife*, *5*, e17002. https://doi.org/10.7554/eLife.17002

284. Roe, J. S., & Vakoc, C. R. (2014). C/EBPα: critical at the origin of leukemic transformation. *The Journal of experimental medicine*, *211*(1), 1–4. https://doi.org/10.1084/jem.20132530

285. Rosenbauer, F., & Tenen, D. G. (2007). Transcription factors in myeloid development: balancing differentiation with transformation. *Nature reviews. Immunology*, *7*(2), 105–117. https://doi.org/10.1038/nri2024

286. Rosmarin, A. G., Weil, S. C., Rosner, G. L., Griffin, J. D., Arnaout, M. A., & Tenen, D. G. (1989). Differential expression of CD11b/CD18 (Mo1) and myeloperoxidase genes during myeloid differentiation. *Blood*, *73*(1), 131–136.

287. Rosmarin, A. G., Yang, Z., & Resendes, K. K. (2005). Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis. *Experimental hematology*, *33*(2), 131–143. https://doi.org/10.1016/j.exphem.2004.08.015

288. Ross, K. G., Currie, K. W., Pearson, B. J., & Zayas, R. M. (2017). Nervous system development and regeneration in freshwater planarians. *Wiley interdisciplinary reviews. Developmental biology*, *6*(3), 10.1002/wdev.266. https://doi.org/10.1002/wdev.266

289. Rothenberg, E. V., Hosokawa, H., & Ungerbäck, J. (2019). Mechanisms of Action of Hematopoietic Transcription Factor PU.1 in Initiation of T-Cell Development. *Frontiers in immunology*, *10*, 228. https://doi.org/10.3389/fimmu.2019.00228

290. Rothenberg, E. V., Kueh, H. Y., Yui, M. A., & Zhang, J. A. (2016). Hematopoiesis and T-cell specification as a model developmental system. *Immunological reviews*, *271*(1), 72–97. https://doi.org/10.1111/imr.12417

291. Rothlin, C. V., & Ghosh, S. (2020). Lifting the innate immune barriers to antitumor immunity. *Journal for immunotherapy of cancer*, *8*(1), e000695. https://doi.org/10.1136/jitc-2020-000695

292. Rots, N. Y., Iavarone, A., BromLeigh, V., & Freedman, L. P. (1999). Induced differentiation of U937 cells by 1,25-dihydroxyvitamin D3 involves cell cycle arrest in G1 that is preceded by a transient proliferative burst and an increase in cyclin expression. *Blood*, *93*(8), 2721–2729.

293. Rózalski, M., Mirowski, M., Balcerczak, E., Krajewska, U., Młynarski, W., & Wierzbicki, R. (2005). Induction of caspase 3 activity, BCL2, BAX, and p65 gene expression modulation in human acute promyelocytic leukemia HL-60 cells by doxorubicin with amifostine. *Pharmacological reports : PR*, *57*(3), 360–366.

294. Ruijtenberg, S., & van den Heuvel, S. (2016). Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression. *Cell cycle (Georgetown, Tex.)*, *15*(2), 196–212. https://doi.org/10.1080/15384101.2015.1120925

295. Rush, E. A., Pollock, S. L., Abecassis, I., & Redner, R. L. (2013). Interaction with RXR is necessary for NPM-RAR-induced myeloid differentiation blockade. *Leukemia research*, *37*(12), 1704–1710. https://doi.org/10.1016/j.leukres.2013.09.024

296. Saldívar-González, F. I., Gómez-García, A., Chávez-Ponce de León, D. E., Sánchez-Cruz, N., Ruiz-Rios, J., Pilón-Jiménez, B. A., & Medina-Franco, J. L. (2018). Inhibitors of DNA Methyltransferases From Natural Sources: A Computational Perspective. *Frontiers in pharmacology*, *9*, 1144. https://doi.org/10.3389/fphar.2018.01144

297. Saloman, J. L., Albers, K. M., Rhim, A. D., & Davis, B. M. (2016). Can Stopping Nerves, Stop Cancer?. *Trends in neurosciences*, *39*(12), 880–889. https://doi.org/10.1016/j.tins.2016.10.002

298. Salvatori, B., Iosue, I., Djodji Damas, N., Mangiavacchi, A., Chiaretti, S., Messina, M., Padula, F., Guarini, A., Bozzoni, I., Fazi, F., & Fatica, A. (2011). Critical Role of c-Myc in Acute Myeloid Leukemia Involving Direct Regulation of miR-26a and Histone Methyltransferase EZH2. *Genes & cancer*, *2*(5), 585–592. https://doi.org/10.1177/1947601911416357

299. Sanz, M. A., Fenaux, P., Tallman, M. S., Estey, E. H., Löwenberg, B., Naoe, T., Lengfelder, E., Döhner, H., Burnett, A. K., Chen, S. J., Mathews, V., Iland, H., Rego, E., Kantarjian, H., Adès, L., Avvisati, G., Montesinos, P., Platzbecker, U., Ravandi, F., Russell, N. H., … Lo-Coco, F. (2019). Management of acute promyelocytic leukemia: updated recommendations from an expert panel of the European LeukemiaNet. *Blood*, *133*(15), 1630–1643. https://doi.org/10.1182/blood-2019-01-894980

300. Saunders, A., Faiola, F., & Wang, J. (2013). Concise review: pursuing self-renewal and pluripotency with the stem cell factor Nanog. *Stem cells (Dayton, Ohio)*, *31*(7), 1227–1236. https://doi.org/10.1002/stem.1384

301. Sauter E. R. (2020). Cancer prevention and treatment using combination therapy with natural compounds. *Expert review of clinical pharmacology*, *13*(3), 265–285. https://doi.org/10.1080/17512433.2020.1738218

302. Schwaller, J., Pabst, T., Koeffler, H. P., Niklaus, G., Loetscher, P., Fey, M. F., & Tobler, A. (1997). Expression and regulation of G1 cell-cycle inhibitors (p16INK4A, p15INK4B, p18INK4C, p19INK4D) in human acute myeloid leukemia and normal myeloid cells. *Leukemia*, *11*(1), 54–63. https://doi.org/10.1038/sj.leu.2400522

303. Scimone, M. L., Kravarik, K. M., Lapan, S. W., & Reddien, P. W. (2014). Neoblast specialization in regeneration of the planarian Schmidtea mediterranea. *Stem cell reports*, *3*(2), 339–352. https://doi.org/10.1016/j.stemcr.2014.06.001

304. Scott, L. M., Civin, C. I., Rorth, P., & Friedman, A. D. (1992). A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. *Blood*, *80*(7), 1725–1735.

305. Sergeev I. N. (2005). Calcium signaling in cancer and vitamin D. *The Journal of steroid biochemistry and molecular biology*, *97*(1-2), 145–151. https://doi.org/10.1016/j.jsbmb.2005.06.007

306. Seita, J., & Weissman, I. L. (2010). Hematopoietic stem cell: self-renewal versus differentiation. *Wiley interdisciplinary reviews. Systems biology and medicine*, *2*(6), 640–653. https://doi.org/10.1002/wsbm.86

307. Shafat, M. S., Oellerich, T., Mohr, S., Robinson, S. D., Edwards, D. R., Marlein, C. R., Piddock, R. E., Fenech, M., Zaitseva, L., Abdul-Aziz, A., Turner, J., Watkins, J. A., Lawes, M., Bowles, K. M., & Rushworth, S. A. (2017). Leukemic blasts program bone marrow adipocytes to generate a protumoral microenvironment. *Blood*, *129*(10), 1320–1332. https://doi.org/10.1182/blood-2016-08-734798

308. Shallis, R. M., Wang, R., Davidoff, A., Ma, X., & Zeidan, A. M. (2019). Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges. *Blood reviews*, *36*, 70–87. https://doi.org/10.1016/j.blre.2019.04.005

309. Sharma, S., & Gurudutta, G. (2016). Epigenetic Regulation of Hematopoietic Stem Cells. *International journal of stem cells*, *9*(1), 36–43. https://doi.org/10.15283/ijsc.2016.9.1.36

310. Shieh, S. J., & Cheng, T. C. (2015). Regeneration and repair of human digits and limbs: fact and fiction. *Regeneration (Oxford, England)*, *2*(4), 149–168. https://doi.org/10.1002/reg2.41

311. Shima, H., Nakayasu, M., Aonuma, S., Sugimura, T., & Nagao, M. (1989). Loss of the MYC gene amplified in human HL-60 cells after treatment with inhibitors of poly(ADP-ribose) polymerase or with dimethyl sulfoxide. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(19), 7442–7445. https://doi.org/10.1073/pnas.86.19.7442

312. Shlush, L. I., Zandi, S., Mitchell, A., Chen, W. C., Brandwein, J. M., Gupta, V., Kennedy, J. A., Schimmer, A. D., Schuh, A. C., Yee, K. W., McLeod, J. L., Doedens, M., Medeiros, J. J., Marke, R., Kim, H. J., Lee, K., McPherson, J. D., Hudson, T. J., HALT Pan-Leukemia Gene Panel Consortium, Brown, A. M., … Dick, J. E. (2014). Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*, *506*(7488), 328–333. https://doi.org/10.1038/nature13038

313. Siebenlist, U., Bressler, P., & Kelly, K. (1988). Two distinct mechanisms of transcriptional control operate on c-myc during differentiation of HL60 cells. *Molecular and cellular biology*, *8*(2), 867–874. https://doi.org/10.1128/mcb.8.2.867

314. Sillar, J. R., & Enjeti, A. K. (2019). Targeting Apoptotic Pathways in Acute Myeloid Leukaemia. *Cancers*, *11*(11), 1660. https://doi.org/10.3390/cancers11111660

315. Simboli-Campbell, M., Narvaez, C. J., Tenniswood, M., & Welsh, J. (1996). 1,25-Dihydroxyvitamin D3 induces morphological and biochemical markers of apoptosis in MCF-7 breast cancer cells. *The Journal of steroid biochemistry and molecular biology*, *58*(4), 367–376. https://doi.org/10.1016/0960-0760(96)00055-6

316. Simpson, R. U., O'Connell, T. D., Pan, Q., Newhouse, J., & Somerman, M. J. (1998). Antisense oligonucleotides targeted against protein kinase Cbeta and CbetaII block 1,25-(OH)2D3-induced differentiation. *The Journal of biological chemistry*, *273*(31), 19587–19591. https://doi.org/10.1074/jbc.273.31.19587

317. Sirinian, M. I., Pisegna, S., Paroli, M., Militi, S., Testa, U., & Peschle, C. (2003). Zinc modulates c-Myc/Mad1 balance in human leukemia cells. *Leukemia*, *17*(1), 272–274. https://doi.org/10.1038/sj.leu.2402746

318. Slomiany, B. A., D'Arigo, K. L., Kelly, M. M., & Kurtz, D. T. (2000). *CDKN2C*lpha inhibits cell growth via direct repression of E2F-DP-mediated transcription. *Molecular and cellular biology*, *20*(16), 5986–5997. https://doi.org/10.1128/mcb.20.16.5986-5997.2000

319. Sluys, R., & Riutort, M. (2018). Planarian Diversity and Phylogeny. *Methods in molecular biology (Clifton, N.J.)*, *1774*, 1–56. https://doi.org/10.1007/978-1-4939-7802-1_1

320. Smith, H. S., & Deer, T. R. (2009). Safety and efficacy of intrathecal ziconotide in the management of severe chronic pain. *Therapeutics and clinical risk management*, *5*(3), 521–534. https://doi.org/10.2147/tcrm.s4438

321. Solomon, D. L., Amati, B., & Land, H. (1993). Distinct DNA binding preferences for the c-Myc/Max and Max/Max dimers. *Nucleic acids research*, *21*(23), 5372–5376. https://doi.org/10.1093/nar/21.23.5372

322. Stanton, R. A., Gernert, K. M., Nettles, J. H., & Aneja, R. (2011). Drugs that target dynamic microtubules: a new molecular perspective. *Medicinal research reviews*, *31*(3), 443–481. https://doi.org/10.1002/med.20242

323. Steensma, D. P., Bejar, R., Jaiswal, S., Lindsley, R. C., Sekeres, M. A., Hasserjian, R. P., & Ebert, B. L. (2015). Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood*, *126*(1), 9–16. https://doi.org/10.1182/blood-2015-03-631747

324. Stocum D. L. (2017). Mechanisms of urodele limb regeneration. *Regeneration (Oxford, England)*, *4*(4), 159–200. https://doi.org/10.1002/reg2.92

325. Stoiber, S., Cadilha, B. L., Benmebarek, M. R., Lesch, S., Endres, S., & Kobold, S. (2019). Limitations in the Design of Chimeric Antigen Receptors for Cancer Therapy. *Cells*, *8*(5), 472. https://doi.org/10.3390/cells8050472

326. Strober W. (2015). Trypan Blue Exclusion Test of Cell Viability. *Current protocols in immunology*, *111*, A3.B.1–A3.B.3. https://doi.org/10.1002/0471142735.ima03bs111

327. Studzinski, G. P., Bhandal, A. K., & Brelvi, Z. S. (1985). Cell cycle sensitivity of HL-60 cells to the differentiation-inducing effects of 1-alpha,25-dihydroxyvitamin D3. *Cancer research*, *45*(8), 3898–3905.

328. Šudomová, M., Hassan, S., Khan, H., Rasekhian, M., & Nabavi, S. M. (2019). A Multi-Biochemical and In Silico Study on Anti-Enzymatic Actions of Pyroglutamic Acid against PDE-5, ACE, and Urease Using Various Analytical Techniques: Unexplored Pharmacological Properties and Cytotoxicity Evaluation. *Biomolecules*, *9*(9), 392. https://doi.org/10.3390/biom9090392

329. Suleiman, S., Suleiman, S., Schembri Wismayer, P., & Calleja Agius, J. (2019). The axolotl model for cancer research: a mini-review. *Journal of B.U.ON. : official journal of the Balkan Union of Oncology*, *24*(6), 2227–2231.

330. Suraweera, A., O'Byrne, K. J., & Richard, D. J. (2018). Combination Therapy With Histone Deacetylase Inhibitors (HDACi) for the Treatment of Cancer: Achieving the Full Therapeutic Potential of HDACi. *Frontiers in oncology*, *8*, 92. https://doi.org/10.3389/fonc.2018.00092

331. Suzuki, M., Satoh, A., Ide, H., & Tamura, K. (2005). Nerve-dependent and -independent events in blastema formation during Xenopus froglet limb regeneration. *Developmental biology*, *286*(1), 361–375. https://doi.org/10.1016/j.ydbio.2005.08.021

332. Swapna, L. S., Molinaro, A. M., Lindsay-Mosher, N., Pearson, B. J., & Parkinson, J. (2018). Comparative transcriptomic analyses and single-cell RNA sequencing of the freshwater planarian Schmidtea mediterranea identify major cell types and pathway conservation. *Genome biology*, *19*(1), 124. https://doi.org/10.1186/s13059-018-1498-x

333. Sweeney, C., & Vyas, P. (2019). The Graft-Versus-Leukemia Effect in AML. *Frontiers in oncology*, *9*, 1217. https://doi.org/10.3389/fonc.2019.01217

334. Takei, H., & Kobayashi, S. S. (2019). Targeting transcription factors in acute myeloid leukemia. *International journal of hematology*, *109*(1), 28–34. https://doi.org/10.1007/s12185-018-2488-1

335. Tanaka, E. M., & Reddien, P. W. (2011). The cellular basis for animal regeneration. *Developmental cell*, *21*(1), 172–185. https://doi.org/10.1016/j.devcel.2011.06.016

336. Tang, X. M., Beesley, J. S., Grinspan, J. B., Seth, P., Kamholz, J., & Cambi, F. (1999). Cell cycle arrest induced by ectopic expression of p27 is not sufficient to promote oligodendrocyte differentiation. *Journal of cellular biochemistry*, *76*(2), 270–279. https://doi.org/10.1002/(sici)1097-4644(20000201)76:2<270::aid-jcb10>3.0.co;2-6

337. Tasseff, R., Jensen, H. A., Congleton, J., Dai, D., Rogers, K. V., Sagar, A., Bunaciu, R. P., Yen, A., & Varner, J. D. (2017). An Effective Model of the Retinoic Acid Induced HL-60 Differentiation Program. *Scientific reports*, *7*(1), 14327. https://doi.org/10.1038/s41598-017-14523-5

338. Thao, N. P., Luyen, B. T., Kim, E. J., Kang, J. I., Kang, H. K., Cuong, N. X., Nam, N. H., Kiem, P. V., Minh, C. V., & Kim, Y. H. (2015). Steroidal constituents from the edible sea urchin

Diadema savignyi Michelin induce apoptosis in human cancer cells. *Journal of medicinal food*, *18*(1), 45–53. https://doi.org/10.1089/jmf.2013.3105

339. Thomas, D., & Majeti, R. (2017). Biology and relevance of human acute myeloid leukemia stem cells. *Blood*, *129*(12), 1577–1585. https://doi.org/10.1182/blood-2016-10-696054

340. Toso, J. F., Gill, V. J., Hwu, P., Marincola, F. M., Restifo, N. P., Schwartzentruber, D. J., Sherry, R. M., Topalian, S. L., Yang, J. C., Stock, F., Freezer, L. J., Morton, K. E., Seipp, C., Haworth, L., Mavroukakis, S., White, D., MacDonald, S., Mao, J., Sznol, M., & Rosenberg, S. A. (2002). Phase I study of the intravenous administration of attenuated Salmonella typhimurium to patients with metastatic melanoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *20*(1), 142–152. https://doi.org/10.1200/JCO.2002.20.1.142

341. Truong, B. T., Lee, Y. J., Lodie, T. A., Park, D. J., Perrotti, D., Watanabe, N., Koeffler, H. P., Nakajima, H., Tenen, D. G., & Kogan, S. C. (2003). CCAAT/Enhancer binding proteins repress the leukemic phenotype of acute myeloid leukemia. *Blood*, *101*(3), 1141–1148. https://doi.org/10.1182/blood-2002-05-1374

342. Ullah, I., Subbarao, R. B., & Rho, G. J. (2015). Human mesenchymal stem cells - current trends and future prospective. *Bioscience reports*, *35*(2), e00191. https://doi.org/10.1042/BSR20150025

343. Umesono, Y., & Agata, K. (2009). Evolution and regeneration of the planarian central nervous system. *Development, growth & differentiation*, *51*(3), 185–195. https://doi.org/10.1111/j.1440-169X.2009.01099.x

344. Van Roten, A., Barakat, A., Wouters, A., Tran, T. A., Mouton, S., Noben, J. P., Gentile, L., & Smeets, K. (2018). A carcinogenic trigger to study the function of tumor suppressor genes

in *Schmidtea mediterranea*. *Disease models & mechanisms*, *11*(9), dmm032573. https://doi.org/10.1242/dmm.032573

345. van Tonder, A., Joubert, A. M., & Cromarty, A. D. (2015). Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC research notes*, *8*, 47. https://doi.org/10.1186/s13104-015-1000-8

346. van Vuuren, R. J., Visagie, M. H., Theron, A. E., & Joubert, A. M. (2015). Antimitotic drugs in the treatment of cancer. *Cancer chemotherapy and pharmacology*, *76*(6), 1101–1112. https://doi.org/10.1007/s00280-015-2903-8

347. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology*, *3*(7), RESEARCH0034. https://doi.org/10.1186/gb-2002-3-7-research0034

348. Vangala, R. K., Heiss-Neumann, M. S., Rangatia, J. S., Singh, S. M., Schoch, C., Tenen, D. G., Hiddemann, W., & Behre, G. (2003). The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. *Blood*, *101*(1), 270–277. https://doi.org/10.1182/blood-2002-04-1288

349. Verbiest, T., Bouffler, S., Nutt, S. L., & Badie, C. (2015). PU.1 downregulation in murine radiation-induced acute myeloid leukaemia (AML): from molecular mechanism to human AML. *Carcinogenesis*, *36*(4), 413–419. https://doi.org/10.1093/carcin/bgv016

350. Vetrie, D., Helgason, G. V., & Copland, M. (2020). The leukaemia stem cell: similarities, differences and clinical prospects in CML and AML. *Nature reviews. Cancer*, *20*(3), 158–173. https://doi.org/10.1038/s41568-019-0230-9

351. Vieira, W. A., Wells, K. M., & McCusker, C. D. (2020). Advancements to the Axolotl Model for Regeneration and Aging. *Gerontology*, *66*(3), 212–222. https://doi.org/10.1159/000504294

352. Vila-Farré, M., & C Rink, J. (2018). The Ecology of Freshwater Planarians. *Methods in molecular biology (Clifton, N.J.)*, *1774*, 173–205. https://doi.org/10.1007/978-1-4939-7802-1_3

353. Visani, G., Olivieri, A., Malagola, M., Brunori, M., Piccaluga, P. P., Capelli, D., Pomponio, G., Martinelli, G., Isidori, A., Sparaventi, G., & Leoni, P. (2006). Consolidation therapy for adult acute myeloid leukemia: a systematic analysis according to evidence based medicine. *Leukemia & lymphoma*, *47*(6), 1091–1102. https://doi.org/10.1080/10428190500513595

354. Vitkevičienė, A., Skiauterytė, G., Žučenka, A., Stoškus, M., Gineikienė, E., Borutinskaitė, V., Griškevičius, L., & Navakauskienė, R. (2019). HDAC and HMT Inhibitors in Combination with Conventional Therapy: A Novel Treatment Option for Acute Promyelocytic Leukemia. *Journal of oncology*, *2019*, 6179573. https://doi.org/10.1155/2019/6179573

355. Voss, S. R., Kump, D. K., Putta, S., Pauly, N., Reynolds, A., Henry, R. J., Basa, S., Walker, J. A., & Smith, J. J. (2011). Origin of amphibian and avian chromosomes by fission, fusion, and retention of ancestral chromosomes. *Genome research*, *21*(8), 1306–1312. https://doi.org/10.1101/gr.116491.110

356. Waddington, C. (1935). Cancer and the Theory of Organisers. *Nature,* 135, 606–608. https://doi.org/10.1038/135606a0

357. Wafa, A., Moassass, F., Liehr, T., Al-Ablog, A., & Al-Achkar, W. (2016). Acute promyelocytic leukemia with the translocation t(15;17)(q22;q21) associated with t(1;2)(q42~43;q11.2~12): a case report. *Journal of medical case reports*, *10*, 203. https://doi.org/10.1186/s13256-016-0982-8

358. Walker, A., & Marcucci, G. (2012). Molecular prognostic factors in cytogenetically normal acute myeloid leukemia. *Expert review of hematology*, *5*(5), 547–558. https://doi.org/10.1586/ehm.12.45

359. Walter, M. J., Shen, D., Ding, L., Shao, J., Koboldt, D. C., Chen, K., Larson, D. E., McLellan, M. D., Dooling, D., Abbott, R., Fulton, R., Magrini, V., Schmidt, H., Kalicki-Veizer, J., O'Laughlin, M., Fan, X., Grillot, M., Witowski, S., Heath, S., Frater, J. L., … Graubert, T. A. (2012). Clonal architecture of secondary acute myeloid leukemia. *The New England journal of medicine*, *366*(12), 1090–1098. https://doi.org/10.1056/NEJMoa1106968

360. Walters, J. A., Tang, J. N., Poole, P., & Wood-Baker, R. (2017). Pneumococcal vaccines for preventing pneumonia in chronic obstructive pulmonary disease. *The Cochrane database of systematic reviews*, *1*(1), CD001390. https://doi.org/10.1002/14651858.CD001390.pub4

361. Wang, B., Waters, A. L., Valeriote, F. A., & Hamann, M. T. (2015). An efficient and cost-effective approach to kahalalide F N-terminal modifications using a nuisance algal bloom of Bryopsis pennata. *Biochimica et biophysica acta*, *1850*(9), 1849–1854. https://doi.org/10.1016/j.bbagen.2015.05.004

362. Wang, C. Z., Kazmierczak, R. A., & Eisenstark, A. (2016). Strains, Mechanism, and Perspective: Salmonella-Based Cancer Therapy. *International journal of microbiology*, *2016*, 5678702. https://doi.org/10.1155/2016/5678702

363. Wei, Q., & Frenette, P. S. (2018). Niches for Hematopoietic Stem Cells and Their Progeny. *Immunity*, *48*(4), 632–648. https://doi.org/10.1016/j.immuni.2018.03.024

364. Weiner, D. B., & Nabel, G. J. (2018). Development of Gene-Based Vectors for Immunization. *Plotkin's Vaccines*, 1305–1319.e8. https://doi.org/10.1016/B978-0-323-35761-6.00067-5

365. Whitfield, J. R., Beaulieu, M. E., & Soucek, L. (2017). Strategies to Inhibit Myc and Their Clinical Applicability. *Frontiers in cell and developmental biology*, *5*, 10. https://doi.org/10.3389/fcell.2017.00010

366. Wilkinson, A. C., Nakauchi, H., & Göttgens, B. (2017). Mammalian Transcription Factor Networks: Recent Advances in Interrogating Biological Complexity. *Cell systems*, *5*(4), 319–331. https://doi.org/10.1016/j.cels.2017.07.004

367. Williams, B. A., Law, A., Hunyadkurti, J., Desilets, S., Leyton, J. V., & Keating, A. (2019). Antibody Therapies for Acute Myeloid Leukemia: Unconjugated, Toxin-Conjugated, Radio-Conjugated and Multivalent Formats. *Journal of clinical medicine*, *8*(8), 1261. https://doi.org/10.3390/jcm8081261

368. Williams, P., Basu, S., Garcia-Manero, G., Hourigan, C. S., Oetjen, K. A., Cortes, J. E., Ravandi, F., Jabbour, E. J., Al-Hamal, Z., Konopleva, M., Ning, J., Xiao, L., Hidalgo Lopez, J., Kornblau, S. M., Andreeff, M., Flores, W., Bueso-Ramos, C., Blando, J., Galera, P., Calvo, K. R., … Daver, N. G. (2019). The distribution of T-cell subsets and the expression of immune checkpoint receptors and ligands in patients with newly diagnosed and relapsed acute myeloid leukemia. *Cancer*, *125*(9), 1470–1481. https://doi.org/10.1002/cncr.31896

369. Winters, A. C., & Bernt, K. M. (2017). MLL-Rearranged Leukemias-An Update on Science and Clinical Approaches. *Frontiers in pediatrics*, *5*, 4. https://doi.org/10.3389/fped.2017.00004

370. Wontakal, S. N., Guo, X., Smith, C., MacCarthy, T., Bresnick, E. H., Bergman, A., Snyder, M. P., Weissman, S. M., Zheng, D., & Skoultchi, A. I. (2012). A core erythroid transcriptional network is repressed by a master regulator of myelo-lymphoid differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(10), 3832–3837. https://doi.org/10.1073/pnas.1121019109

371. Wood, K., Tellier, M., & Murphy, S. (2018). DOT1L and H3K79 Methylation in Transcription and Genomic Stability. *Biomolecules*, *8*(1), 11. https://doi.org/10.3390/biom8010011

372. Woodbury, A., McCrary, M. R., & Yu, S. P. (2018). Molecular Targets and Natural Compounds in Drug Development for the Treatment of Inflammatory Pain. *Current drug targets*, *19*(16), 1905–1915. https://doi.org/10.2174/1389450119666180514120438

373. Woolthuis, C. M., & Park, C. Y. (2016). Hematopoietic stem/progenitor cell commitment to the megakaryocyte lineage. *Blood*, *127*(10), 1242–1248. https://doi.org/10.1182/blood-2015-07-607945

374. Wurm, A. A., Zjablovskaja, P., Kardosova, M., Gerloff, D., Bräuer-Hartmann, D., Katzerke, C., Hartmann, J. U., Benoukraf, T., Fricke, S., Hilger, N., Müller, A. M., Bill, M., Schwind, S., Tenen, D. G., Niederwieser, D., Alberich-Jorda, M., & Behre, G. (2017). Disruption of the C/EBPα-miR-182 balance impairs granulocytic differentiation. *Nature communications*, *8*(1), 46. https://doi.org/10.1038/s41467-017-00032-6

375. Xu, B., Liu, P., Li, J., & Lu, H. (2009). All-trans retinoic acid induces Thrombospondin-1 expression in acute promyelocytic leukemia cells though down-regulation of its transcription repressor, c-MYC oncoprotein. *Biochemical and biophysical research communications*, *382*(4), 790–794. https://doi.org/10.1016/j.bbrc.2009.03.105

376. Yamanaka, R., Barlow, C., Lekstrom-Himes, J., Castilla, L. H., Liu, P. P., Eckhaus, M., Decker, T., Wynshaw-Boris, A., & Xanthopoulos, K. G. (1997). Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(24), 13187–13192. https://doi.org/10.1073/pnas.94.24.13187

377. Yan, M., & Liu, Q. (2016). Differentiation therapy: a promising strategy for cancer treatment. *Chinese journal of cancer*, *35*, 3. https://doi.org/10.1186/s40880-015-0059-x

378. Yang, X., Lay, F., Han, H., & Jones, P. A. (2010). Targeting DNA methylation for epigenetic therapy. *Trends in pharmacological sciences*, *31*(11), 536–546. https://doi.org/10.1016/j.tips.2010.08.001

379. Yilmaz, M., Wang, F., Loghavi, S., Bueso-Ramos, C., Gumbs, C., Little, L., Song, X., Zhang, J., Kadia, T., Borthakur, G., Jabbour, E., Pemmaraju, N., Short, N., Garcia-Manero, G., Estrov, Z., Kantarjian, H., Futreal, A., Takahashi, K., & Ravandi, F. (2019). Late relapse in acute myeloid leukemia (AML): clonal evolution or therapy-related leukemia?. *Blood cancer journal*, *9*(2), 7. https://doi.org/10.1038/s41408-019-0170-3

380. Yu, J., Li, Y.,salavt Zhang, D., Wan, D., & Jiang, Z. (2020). Clinical implications of recurrent gene mutations in acute myeloid leukemia. *Experimental hematology & oncology*, *9*, 4. https://doi.org/10.1186/s40164-020-00161-7

381. Yu, Z. Y., Xiao, H., Wang, L. M., Shen, X., Jing, Y., Wang, L., Sun, W. F., Zhang, Y. F., Cui, Y., Shan, Y. J., Zhou, W. B., Xing, S., Xiong, G. L., Liu, X. L., Dong, B., Feng, J. N., Wang, L. S., Luo, Q. L., Zhao, Q. S., & Cong, Y. W. (2016). Natural Product Vibsanin A Induces Differentiation of Myeloid Leukemia Cells through PKC Activation. *Cancer research*, *76*(9), 2698–2709. https://doi.org/10.1158/0008-5472.CAN-15-1616

382. Yuan, R., Hou, Y., Sun, W., Yu, J., Liu, X., Niu, Y., Lu, J. J., & Chen, X. (2017). Natural products to prevent drug resistance in cancer chemotherapy: a review. *Annals of the New York Academy of Sciences*, *1401*(1), 19–27. https://doi.org/10.1111/nyas.13387

383. Yun, C. W., Kim, H. J., & Lee, S. H. (2019). Therapeutic Application of Diverse Marine-derived Natural Products in Cancer Therapy. *Anticancer research*, *39*(10), 5261–5284. https://doi.org/10.21873/anticanres.13721

384. Zarrilli, R., Pignata, S., Apicella, A., Di Popolo, A., Memoli, A., Ricchi, P., Salzano, S., & Acquaviva, A. M. (1999). Cell cycle block at G1-S or G2-M phase correlates with differentiation of Caco-2 cells: effect of constitutive insulin-like growth factor II expression. *Gastroenterology*, *116*(6), 1358–1366. https://doi.org/10.1016/s0016-5085(99)70500-7

385. Zeichner, S. B., Alghamdi, S., Elhammady, G., & Poppiti, R. J. (2014). Prognostic significance of TP53 mutations and single nucleotide polymorphisms in acute myeloid leukemia: a case series and literature review. *Asian Pacific journal of cancer prevention : APJCP*, *15*(4), 1603–1609. https://doi.org/10.7314/apjcp.2014.15.4.1603

386. Zhang, Y., Zhang, J., & Studzinski, G. P. (2006). AKT pathway is activated by 1, 25-dihydroxyvitamin D3 and participates in its anti-apoptotic effect and cell cycle control in differentiating HL60 cells. *Cell cycle (Georgetown, Tex.)*, *5*(4), 447–451. https://doi.org/10.4161/cc.5.4.2467

387. Zhang, C., Zhong, J. F., Stucky, A., Chen, X. L., Press, M. F., & Zhang, X. (2015). Histone acetylation: novel target for the treatment of acute lymphoblastic leukemia. *Clinical epigenetics*, *7*, 117. https://doi.org/10.1186/s13148-015-0151-8

388. Zhao, Y., Zhong, L., Liu, L., Yao, S. F., Chen, M., Li, L. W., Shan, Z. L., Xiao, C. L., Gan, L. G., Xu, T., & Liu, B. Z. (2018). Salinomycin induces apoptosis and differentiation in human acute promyelocytic leukemia cells. *Oncology reports*, *40*(2), 877–886. https://doi.org/10.3892/or.2018.6513

389. Zheng J. Oncogenic chromosomal translocations and human cancer (review). *Oncol Rep*. 2013;30(5):2011-2019. doi:10.3892/or.2013.2677

390. Zhou, G. B., Zhang, J., Wang, Z. Y., Chen, S. J., & Chen, Z. (2007). Treatment of acute promyelocytic leukaemia with all-trans retinoic acid and arsenic trioxide: a paradigm of synergistic molecular targeting therapy. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *362*(1482), 959–971. https://doi.org/10.1098/rstb.2007.2026

391. Zhou, J. D., Zhang, T. J., Xu, Z. J., Gu, Y., Ma, J. C., Li, X. X., Guo, H., Wen, X. M., Zhang, W., Yang, L., Liu, X. H., Lin, J., & Qian, J. (2019). BCL2 overexpression: clinical implication and biological insights in acute myeloid leukemia. *Diagnostic pathology*, *14*(1), 68. https://doi.org/10.1186/s13000-019-0841-1

392. Zhou, J., Zhang, X., Wang, Y., & Guan, Y. (2015). PU.1 affects proliferation of the human acute myeloid leukemia U937 cell line by directly regulating MEIS1. *Oncology letters*, *10*(3), 1912–1918. https://doi.org/10.3892/ol.2015.3404

393. Zielins, E. R., Ransom, R. C., Leavitt, T. E., Longaker, M. T., & Wan, D. C. (2016). The role of stem cells in limb regeneration. *Organogenesis*, *12*(1), 16–27. https://doi.org/10.1080/15476278.2016.1163463

394. Zinzar, S., Ohnuma, T., & Holland, J. F. (1989). Effects of simultaneous and sequential exposure to granulocytic and monocytic inducers on the choice of differentiation pathway in HL-60 promyelocytic leukemia cells. *Leukemia research*, *13*(1), 23–30. https://doi.org/10.1016/0145-2126(89)90027-1

395. Zuna, J., Burjanivova, T., Mejstrikova, E., Zemanova, Z., Muzikova, K., Meyer, C., Horsley, S. W., Kearney, L., Colman, S., Ptoszkova, H., Marschalek, R., Hrusak, O., Stary, J., Greaves, M., & Trka, J. (2009). Covert preleukemia driven by MLL gene fusion. *Genes, chromosomes & cancer*, *48*(1), 98–107. https://doi.org/10.1002/gcc.20622

Chapter 9

Appendix

Ethical Consent

Ethical consent was granted by the Faculty of Medicine and Surgery Research Ethics Committee

Ref No: FRECMDS_1819_002

Scientific contributions related to this thesis

During the course of the PhD, parts of my thesis have been presented in several conferences including oral, abstract, and poster presentations. Also, a review article has been published and another two articles are pending peer review prior to publication. I have also co-authored two publications as part of our research team investigates the effect of different compounds on differentiation of leukaemia cell lines.

Publications

Corresponding author

- **Suleiman, S.,** Suleiman, S., Schembri Wismayer, P., & Calleja Agius, J. (2019). The axolotl model for cancer research: a mini-review. *Journal of B.U.ON.: official journal of the Balkan Union of Oncology*, *24*(6), 2227–2231.
- **Suleiman, S.,** Di Fiore, R., Cassar, A., Formosa, M. M., Calleja-Agius, J., & Schembri-Wismayer, P. (2020). Anticancer effects of an extract from a local planarian species on human acute myeloid leukemia HL-60 cells in vitro. *Biomedicine & pharmacotherapy =*

Biomedecine & pharmacotherapie, *130*, 110549. Advance online publication. https://doi.org/10.1016/j.biopha.2020.110549

- **Suleiman, S.**, Di Fiore, R., Cassar, A., Formosa, M. M., Schembri-Wismayer, P., & Calleja-Agius, J. (2020). Axolotl *Ambystoma mexicanum* extract induces cell cycle arrest and differentiation in human acute myeloid leukemia HL-60 cells. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, *42*(9), 1010428320954735. https://doi.org/10.1177/1010428320954735

Co-Author

- Stoica, S., Magoulas, G. E., Antoniou, A. I., **Suleiman, S.,** Cassar, A., Gatt, L., Papaioannou, D., Athanassopoulos, C. M., & Schembri-Wismayer, P. (2016). Synthesis of minoxidil conjugates and their evaluation as HL-60 differentiation agents. *Bioorganic & medicinal chemistry letters*, *26*(4), 1145–1150. https://doi.org/10.1016/j.bmcl.2016.01.048
- Todorović, T. R., Vukašinović, J., Portalone, G., **Suleiman, S.,** Gligorijević, N., Bjelogrlić, S., Jovanović, K., Radulović, S., Anđelković, K., Cassar, A., Filipović, N. R., & Schembri-Wismayer, P. (2016). (Chalcogen)semicarbazones and their cobalt complexes differentiate HL-60 myeloid leukaemia cells and are cytotoxic towards tumor cell lines. *MedChemComm*, *8*(1), 103–111. https://doi.org/10.1039/c6md00501b
- Ballarin, L.; Rinkevich, B.; Bartscherer, K.; Burzynski, A.; Cambier, S.; Cammarata, M.; Domart-Coulon, I.; Drobne, D.; Encinas, J.; Frank, U.; Geneviere, A.-M.; Hobmayer, B.; Löhelaid, H.; Lyons, D.; Martinez, P.; Oliveri, P.; Peric, L.; Piraino, S.; Ramšak, A.; Rakers, S.; Rentzsch, F.; Rosner, A.; Da Silva, T.H.; Somorjai, I.; **Suleiman, S**.; Coelho, A.V. Maristem–Stem Cells of Marine/Aquatic Invertebrates: From Basic Research to Innovative Applications. *Sustainability* 2018, *10*, 526.

- Schembri-Wismayer, P., Cassar, A., Theuma, K. B., Stipourou. I., Passarella, D., **Suleiman, S**., and Micallef, N. (2016). Xjenza Online, 4:58-63. Xjenza Online – Journal of The Malta Chamber of Scientists www.xjenza.org DOI: 10.7423/XJENZA.2016/1.08

Poster presentations

2014 - Effects of biological extracts on terminal differentiation of solid tumours and leukaemias. CIBICAN conference on molecular pharmacology and mechanisms of new anti-cancer drugs. Cost action CM1106

2018 – Effects of a biological extract from a regenerative organism on leukaemia cell lines. 10th Malta Medical School Conference

Oral presentation

2018 - COST Action 16203 "MARISTEM". Effects of biological extracts on terminal differentiation of HL60 & NB4 leukaemia cell lines.

2018 - Effects of a biological extract from a regenerative organism on leukaemia cell lines. 10th Malta Medical School Conference

Abstracts

2019 - Special Issue: Proceedings of the 19th International Federation of Associations of Anatomists Congress, 9th - 11th August 2019, London, UK https://onlinelibrary.wiley.com/doi/epdf/10.1111/joa.13163. The effect of a regeneration extract from a Mexican Axolotl on the HL-60 Leukaemia cell line.

2020 - EuroLeuk - An Extract from a Local Planarian Species Shows Antitumor Effect in Human Acute Myeloid Leukemia HL-60 Cells