Targeting Apurinic/Apyrimidinic Endonuclease 1 and 8-Oxoguanine DNA Glycosylase as Therapeutic Strategy in Acute Myeloid Leukaemia

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Abstract

Acute myeloid leukaemia (AML) is an extremely heterogeneous disease characterised by genomic instability, epigenetic changes and a high oxidative stress burden that drives disease progression and plays an essential role in prognosis and treatment response. AML treatment is challenging and the majority of AML patients suffer relapse, particularly elderly patients. Targeting DNA repair mechanisms in cancer is a proven approach that can potentiate the anti-cancer activity of chemotherapeutic agents to yield better outcomes. The evidence suggests that the APE1 and OGG1 components of the base excision DNA repair pathway may be essential to cancer cell survival, and based on these reports it is hypothesised that targeting APE1 and OGG1 will have therapeutic value in AML.

In order to test this hypothesis, *APE1* and *OGG1* gene expression was silenced in several AML cell lines using small hairpin RNA (shRNA) interference and cells were investigated for effects on proliferation, cloning efficiency, cell cycle, apurinic/apyrimidinic (AP) site accumulation and sensitivity to anti-leukaemic chemotherapy. Moreover, wildtype AML cells were treated with APE1 inhibitors methoxyamine (MX), E3330 and APE1 inhibitor III (APE1-III), and the effect on cell proliferation and cell cycle profile was investigated as single agents and in combination with anti-leukaemic chemotherapy.

APE1 and OGG1 proteins were expressed in all AML cell lines investigated, but protein levels were not correlated with mRNA gene expression, suggesting that post-translational modification may regulate both proteins.

APE1 shRNA knockdown slowed cellular proliferation and reduced cloning efficiency of AML cell lines HL-60, AML3 and U937. APE1 knockdown did not potentiate the sensitivity of AML cell lines to chemotherapeutic agents, including temozolomide, Ara-C, daunorubicin, clofarabine, fludarabine and etoposide. In contrast, chemotherapy-induced cell killing induced was antagonised by APE1 knockdown in AML cells. APE1 inhibition using MX, E3330 and APE1-III showed some single agent activity, with evidence of reduced proliferation and clonogenicity, but potentiation of chemotherapy-induced cytotoxicity was not evident.

APE1 knockdown had no discernible effect on cell cycle kinetics. In contrast, APE1 inhibition using methoxyamine significantly induced cell cycle blockade in S phase, but no alteration in cell cycle profile was evident following APE1 inhibition with E3330 or APE1-III.

RNA sequencing of APE1 knockdown cells identified several genes that were significantly upregulated, including many involved in cell cycle regulation and genes that may contribute to leukaemogenesis, including PAX5, CDKN1A and FOXO1.

Targeting OGG1 using shRNA had no effect on proliferation of HL-60 and U937 AML cell lines, and only a very modest effect on colony formation in semi-solid soft agar. Furthermore, OGG1 silencing had no effect on cellular sensitivity to anti-leukaemic chemotherapy agents, and also did not affect cell cycle kinetics.

In conclusion, the data presented in this thesis indicate that APE1, but not OGG1, may have potential therapeutic value as a single agent, but not in combination with established anti-leukaemic drugs. Additionally, the extensive genetic heterogeneity of AML suggests that targeting APE1 may have a utility in some but not all subtypes of AML. OGG1 may provide prognostic value in AML, but appears not to be a suitable therapeutic target.

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Abbreviations

8-oxoG	8-oxoguanine
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AP site	Apurinic/Apyrimidinic site
APE1	Apurinic/Apyrimidinic endonuclease 1
APE1-III	APE1 inhibitor III
APML	Acute promyelocytic leukaemia
Ara-C	Cytarabine
ARP	Aldehyde Reactive Probe
ATM	Ataxia telangiectasia mutated
ATO	arsenic trioxide
ATRA	all-trans retinoic acid
BER	Base excision repair
BCA	bicinchoninic acid
BRCA 1, 2	Breast cancer, early onset
BSA	bovine serum albumin
CAPS	N-cyclohexyl-3-aminopropanesulphonic acid
Cdk5	Cyclin-dependent kinase 5
cDNA	complementary DNA
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CEBPα	CCAAT/enhancer binding protein
CFO	colony formation assay
CLL	chronic lymphocytic leukaemia
CNS	Central nervous system

CRISPR	Clustered regularly interspaced short palindromic repeats
CUX1	Cut Like Homeobox 1
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsRNA	double strand RNA
E3330	E-3-[2-(5,6-dimethoxy-3-methyl-1,4-benzoquinonyl)]-2-nonylpropenoic acid
EDTA	Ethylenediaminetetraacetic acid
ELN	European Leukaemia Net
FAB	French American british
FAM	Carboxyfluorescein
FBS	Fetal bovine serum
FEN-1	Flap endonuclease 1
FLT3	FMS-like tyrosine kinase-3
FOXO1	Forkhead box protein O1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GIA	growth inhibition assay
gRNA	guide RNA
H_2O_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor 1-alpha
HSC	Haematopoietic stem cells
IDH	Isocitrate dehydrogenase 1

ITD	internal tandem duplications
LIG1	Ligase 1
M	Molar
MAPK 1, 3	Mitogen-Activated Protein Kinase
MGMT	O6-Methylguanine-DNA methyltransferase
MDM2	Mouse double minute 2
MDR1	Multi-drug resistance 1
MDS	Myelodysplastic syndrom
mg	milligram
mM	millimolar
MMR	mismatch repair
MOI	multiplicity of infection
mRNA	messenger RNA
MTT	Thiazolyl Blue Tetrazolium Bromide
MUTYH	MutY DNA Glycosylase
MX	Methoxyamine
NEIL1, 2 and 3	Nei endonuclease VIII like 1, 2, and 3
NF-κB	nuclear factor kappaB
NHEJ	Non-homologous end joining
NICR	Northern institute for cancer research
NPM1	Nucleophosmin 1
OGG1	8-Oxoguanine glycosylase
PBS	Phosphate buffer saline
PARP1	Poly ADP-ribose polymerase 1
PAX5	Paired Box 5

PCNA	Proliferating cell nuclear antigen
PCR	polymerase chain reaction
PI	propidium iodide
PKC	Protein kinase C
PMSF	phenylmethanesulfonyl fluoride
PNKP	polynucleotide kinase/phosphatase
POL-β	Polymerase beta
POL-δ	polymerase delta
POL-ε	Polymerase Epsilon
PUA	phosphor-α,β-unsaturated aldehyde
PVDF	polyvinylidene difluoride
RB	Retinoblastoma
REF-1	redox factor-1
RIN	RNA integrity number
RNA	ribonucleic acid
ROS	reactive oxygen species
RAD 51	Radiation sensitivity abnormal 51
RPMI	Roswell Park Memorial Institute
RT	room temperature
RT-PCR	real time PCR
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
TARDIS	trapped in agarose DNA immunostaining

TCGA	The Cancer Genome Atlas
TDK	tyrosine kinase domain
TET2	Tet Methylcytosine Dioxygenase 2
TF	transcription factors
TMZ	Temozolomide
TOP2A	Topoisomerase II Alpha
TOP2B	Topoisomerase II Beta
Tris	Tris(hydroxymethyl)aminomethane
UBR3	Ubiquitin protein ligase E3 component N-recognin 3
v/v	Volume/volume
w/v	Weight/volume
WBC	white blood cells
WHO	World health organisation
XRCC1	X-ray repair cross-complementing 1
YB-1	Y box binding protein 1

Chapter 1. Introduction

1.1. Acute Myeloid Leukaemia

Multipotent haematopoietic stem cells (HSC) in bone marrow give rise to all of the different type of blood cell. This occurs via a complex molecular cellular process of cell replication and differentiation. There are two types of HSC in bone marrow including lymphoid and myeloid precursors.

Acute myeloid leukaemia (AML) is a group of heterogeneous disorders characterised by uncontrolled proliferation and blocked differentiation of myeloid lineage cells. Subsequently, this leads to accumulation of immature myeloid blast cells in the bone marrow and peripheral blood. It is frequently accompanied by decreased numbers and function of other blood cell components, including erythrocytes and platelet, which consequently lead to anaemia and haemorrhage. Frequent genetic alterations and chromosomal translocations have been implicated in AML pathogenesis. The mechanisms by which some of these alterations cause the disease is now becoming clearer, although for other alterations there is only a limited understanding of causal mechanisms.

1.1.1. AML incidence and epidemiology

AML occurs in all age groups but the incidence increases with age and the median age at diagnosis is 70 years (Figure 1.1). AML is the most frequent leukaemia in neonates but occurs at relatively low frequency in childhood and adolescent. AML accounts for about 33% of all leukaemia cases in the UK, with higher incidence in men compared to women, with a ratio of 12:10 (Cancer research UK website).

1.1.2. AML clinical manifestation and diagnosis

The most common clinical features of AML are anaemia, neutropenia and/or thrombocytopaenia. This is predominantly due to infiltration of bone marrow with malignant blast cells, which result in inadequate production, differentiation and maturation of all blood cells components; white blood cells, red blood cells and thrombocytes. Anaemia is a common clinical presentation of AML patients; which leads to fatigue, general weakness, pallor and other common symptoms and signs of anaemia. Tendency to bleed is also another common clinical feature of AML, caused by the inadequate production and decreased survival of platelets.

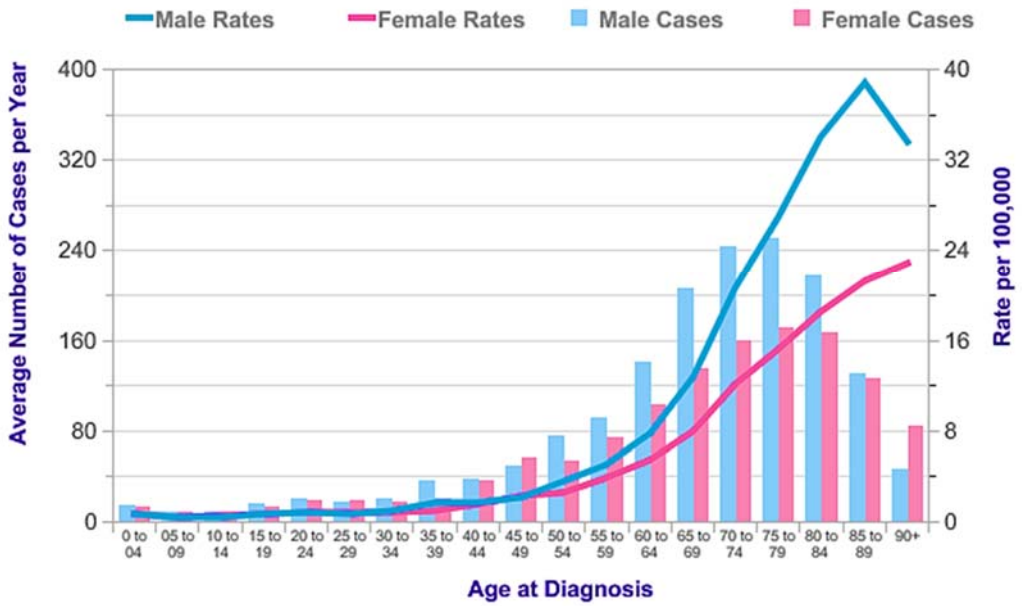


Figure 1.1: Incidence of AML in the UK in different age and sex groups.

This graph shows the average of AML incidence in different age and sex groups in the UK between 2011 and 2013. Over 50% of case diagnosed with AML were over 70 years. The graph also demonstrate higher incidence rate in males compared to females. (Graph adapted from www.cancerresearchuk.org)

Organ dysfunction might occur due to infiltration of myeloblasts from peripheral blood to systems such as lung, brain and central nervous system (CNS). AML patients are also susceptible to infections at the time of presentation, but however, major infections are not likely to happen before diagnosis and this might be reflected by high total leukocyte count.

1.1.3. Disease phenotype and classification

AML is an extremely heterogeneous disease and several systems are used to classify AML cases into subgroups. Classification systems predominantly depend on morphological, immunological, cytochemical, cytogenetic and molecular features of AML cells. The first attempt to classify AML was in 1976 by introducing French-American-British (FAB) classification system (Bennett *et al.*, 1976). This was followed by the proposal of a new classification system by the World Health Organisation (WHO) in 2001, which was subsequently revised in 2008 and 2016 (Vardiman *et al.*, 2002; Vardiman *et al.*, 2009; Arber *et al.*, 2016).

1.1.3.1. FAB classification

The FAB classification system introduced 8 subtypes (M0-M7) of AML depending on microscopic morphology and maturation of myeloid cells in peripheral blood and bone marrow after routine staining (Table 1.1). The FAB system also attempted to correlate cytogenetic alterations (such as translocations, inversions and deletions) with specific subtypes, but this was only possible in the M3 subtype (APL: Acute promyelocytic leukaemia) which is characterised by the t(15:17) PLM-RARα translocation.

1.1.3.2. WHO classification

Although the FAB classification is still widely used, it has some limitations which led to introducing WHO classification. The WHO classification divides AML into subtypes based on cellular morphology, cytogenetics, molecular genetics and immunological biomarkers, to generate more informative classification that can also be used for prognostication (Table 1.2) (Vardiman *et al.*, 2009).

Subclass	Description
M0	Acute myeloblastic leukaemia minimally differentiated
M1	Acute myeloblastic leukaemia without maturation
M2	Acute myeloblastic leukaemia with maturation
M3	Hypergranular promyelocytic leukaemia
M4	•Acute myelomonocytic leukaemia •Acute myelomonocytic leukaemia with bone marrow eosinophilia (M4 Eo)
M5	Acute monocytic leukaemia •Undifferentiated monoblastic (M5a) •Well-differentiated promonocytic-monocytic (M5b)
M6	Acute erythroleukaemia
M7	Acute megakaryocytic leukaemia

Table 1.1: Overview of French-American-British (FAB) classification of AML. Adapted from (Bennett *et al.*, 1976)

Type	Description
AML with recurrent genetic abnormalities	AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11 APL with PML-RARα AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A AML with t(6;9)(p23;q34.1);DEK-NUP214 AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1 Provisional entity: AML with BCR-ABL1 AML with mutated NPM1 AML with biallelic mutations of CEBPα Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes	Prior history of myelodysplastic syndrome (MDS) MDS-related cytogenetic abnormality Multilineage dysplasia
Therapy-related myeloid neoplasms	
Acute myeloid leukaemia, not otherwise specified	AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukaemia Acute monoblastic/monocytic leukaemia Pure erythroid leukaemia Acute megakaryoblastic leukaemia Acute basophilic leukaemia Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis (TAM) Myeloid leukaemia associated with Down syndrome

Table 1.2: AML classification based on the World Health Organisation (WHO) system. Adapted from Arber et al., 2016

1.1.4. Genetic mutations and mechanisms of pathogenesis

AML is an aggressive and extremely heterogeneous disease. Recent developments in molecular techniques and whole genome sequencing have improved our understanding of the leukaemogenesis process. Chromosomal translocations and other mutations in AML cause perturbation in genes involved in regulation of proliferation, differentiation and apoptosis of haematopoietic progenitors. Mutations in leukaemia are divided into 3 groups according to their anticipated role in leukaemogenesis (Table 1.3). Specifically, mutations that confer a proliferative advantage on AML cells were classified as class I mutation, and include mutations in FLT3, KIT and RAS (Dombret, 2011). Class II mutations are associated with blocked differentiation of myeloid precursors; and include mutations in CEBPα, RUNX1, NPM1 and CBFβ (Renneville *et al.*, 2008). Mutations that occur in genes related to epigenetic regulation are classified as class III; and are often associated with worse outcome and frequently observed in older patients (Dombret, 2011).

In the next sections (1.1.4.1– 1.1.4.5), common recurrent mutations and molecular alterations in AML will be briefly discussed.

1.1.4.1. Acquired somatic alteration

1.1.4.1.1. Insertion/deletion (Indel) mutations

FLT3 mutations

FLT3 (FMS-like tyrosine kinase like receptor 3) mutation is the most frequent mutation observed in AML and confers a poor prognosis and increased risk of relapse. FLT3 is expressed on immature haematopoietic progenitors (both myeloid and lymphoid) and plays an essential role in normal haematopoiesis, regulating haematopoietic cell proliferation and differentiation (Small, 2006). FLT3 expression is gradually lost upon differentiation, but could be detected in mature dendritic cells (Small, 2006). Two distinct mutations occur in the functional domain of the FLT3 receptor and are reported in AML. The first and most frequent is internal tandem duplication (FLT3-ITD) and the second type of mutation is caused by insertion/deletion or missense mutation in the tyrosine kinase domain (FLT3-TKD).

Mutation class	Class I	Class II	Class II
Genes	FLT3	RUNX1	TET2
	KIT	NPM1	IDH1/2
	JAK2	CEBPα	DNMT
	PTPN11	MLL	ASXL1
	NRAS	RARα	EZH2
	KRAS	CBFβ	

Table 1.3: classification of genetic mutations observed in AML.

Genetic mutations in AML are classified into three distinct categories. Class I include mutations in genes involved in activation of signalling pathways that affect proliferative advantage of haematopoietic cells. Class II mutations affect transcription factors that control haematopoietic cells differentiation. Class III affect genes involved in epigenetic regulation.

1.1.4.2. Acquired point mutations

NPM1

Nucleophosmin 1 (NPM1) is a multifunctional protein that functions in the nucleolus and is also involved in ribosomal protein assembly and transport, control of centrosome duplication, and regulation of the tumour suppressor ARF (Falini and Martelli, 2011). Point mutations in exon 12 in *NPM1* have been reported primarily in haematological malignancies and ascribed as early leukaemogenic event in 35% of AML cases with normal karyotype (Webersinke *et al.*, 2014).

IDH1 and IDH2 mutations

Isocitrate dehydrogenases 1/2 (IDH) are a metabolic enzymes required to catalyse oxidation of isocitrate to yield α-ketoglutarate, which is an essential intermediate in the Krebs cycle and an important co-substrate for cellular metabolic functions (Levis, 2013). Recurrent somatic mutations in cytosolic IDH1 or its mitochondrial homolog IDH2 were primarily identified in colorectal cancer, glioblastoma and other brain tumours (Ward *et al.*, 2012; Walker and Marcucci, 2015). In AML, IDH1 mutation was first identified following DNA sequencing of cytogenetically normal AML and found to be a recurrent event in a small group of patients, subsequently confirmed in larger cohort study along with novel mutation in IDH2 (Mardis *et al.*, 2009; Marcucci *et al.*, 2010). IDH1/2 mutations occur in approximately 17% of newly diagnosed AML cases and they correlate with normal karyotype AML and *NPM1* mutations (Abbas *et al.*, 2010).

CEBPα

The CCAAT/enhancer binding protein α (CEBPα) is a transcription factor essential for myeloid differentiation and is specifically expressed in myelomonocytic cells (Renneville *et al.*, 2008). Mutation in CEBPα usually associated with AML FAB M1, M2 and M4 subtypes and is reported in 11-19% of AML cases (Renneville *et al.*, 2008). Two types of mutations are frequently observed in AML: frameshift mutation in the N-terminal region of the protein results in a truncated form of the CEBPα protein; and insertion/deletion mutations in C-terminal result in deficient DNA binding. Some AML patients present with a single mutation, while others have multiple mutations, including mutation in both the N-terminal and C-terminal. CEBPα mutations are implicated in leukaemogenesis and function by blocking

granulocytic differentiation, activation of proliferation signalling pathways and upregulation of genes involved in erythroid linage differentiation (Castilla, 2008; Marcucci *et al.*, 2008).

RAS

Mutations of RAS oncogene family members frequently occur in variety of cancer types. There are three functional RAS genes, including K-RAS (Kirsten), H-RAS (Harvey) and N-RAS (from neuroblastoma cell line) (Renneville *et al.*, 2008). RAS gene family members encode guanine-nucleotide binding proteins crucial in regulation of signalling transduction pathways required for proliferation, differentiation and apoptosis (Renneville *et al.*, 2008). Mutation in RAS genes activates RAS activity and constitutively activates downstream signalling pathways. Mutation in N-RAS occur in 10%-15% of AML cases, whereas K-RAS mutation occurs in approximately 5% of AML cases (Renneville *et al.*, 2008).

RUNX1

RUNX1 (Runt related transcription factor 1) also named AML1 or CBFA2, is a transcription factor that plays an essential role in regulation of haematopoietic differentiation. RUNX1 is mutated in 12%-16% of AML via either chromosomal translocation, point mutation or gene amplification (Renneville *et al.*, 2008). RUNX1 is highly correlated with AML in males, older age and M0/M1 FAB subtypes (Tang *et al.*, 2009). Patient with *RUNX1* mutations have lower complete remission rates with shorter disease free survival (DFS) and overall survival compared to patients with wildtype *RUNX1* (Walker and Marcucci, 2015).

1.1.4.3. Epigenetic alterations

Epigenetic alterations are defined as inheritable changes occurring in gene expression without an alteration in the DNA coding sequence (Egger *et al.*, 2004). Epigenetic changes occur through two mechanisms, including DNA methylation and alterations in histone modification pattern, where these play a role in silencing of critical genes involved in normal cellular metabolism (Wouters and Delwel, 2016). Epigenetic regulation is a part of physiological development and can become dysregulated in some AML cases.

DNA methylation involves the addition of a methyl group to the carbon at position 5 of cytosine in CpG dinucleotides to yield 5-methylcytosine (Wouters and Delwel, 2016). Cytosine hypermethylation is associated with silencing of tumour suppressor genes and can

contribute to leukaemogenesis via this mechanism (Wouters and Delwel, 2016). Several recurrent mutated genes have been reported in AML and have been associated with epigenetic alterations, including *TET2, IDH1, IDH2 and DNMT3A* (Delhommeau *et al.*, 2009; Schoofs *et al.*, 2014). These mutations have some prognostic value and possibly play key roles in AML pathogenesis. Certain translocation/genetic mutations in AML, such as AML1-ETO, PML-RARα and NPM1, are associated with highly distinctive methylation pattern (Figueroa *et al.*, 2010b; 'Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia,' 2013).

TET2

The ten-eleven translocation 2 (TET2) is one of three members of TET protein family and it plays a critical role in the regulation of demethylation of 5-methylcytosin to 5-hydroxymethylcytosine in DNA. TET2 is mutated recurrently in a variety of myeloid malignancies including myelodysplastic syndrome (MDS), AML and myeloproliferative neoplasm (Nakajima and Kunimoto, 2014). TET2 mutation occurs in 7% to 23% of AML cases and its impact on prognosis is still controversial (Gaidzik *et al.*, 2012). However, a study on 427 patients found that *TET2* mutation confers adverse prognosis in patients with favourable normal cytogenetic karyotype (Metzeler *et al.*, 2011). TET2 mutation dysregulates hydroxylation of 5-methylctosine and leads to hyper-methylation of genes required for normal cellular function (Nakajima and Kunimoto, 2014).

1.1.4.4. Dysregulation of miRNA and gene expression

MicroRNAs (miRNA) are a small noncoding RNAs involved in post-transcriptional regulation of gene expression via degradation of mRNA or inhibition of translation by binding at the 3' UTR region (Chuang *et al.*, 2015). Alterations in miRNA expression have been associated with AML progression and prognosis (Garzon *et al.*, 2008). Various miRNA expression patterns have been reported in different AML cytogenetic groups; hence different cytogenetic groups harbour distinct miRNA expression patterns (Jongen-Lavrencic *et al.*, 2008; Chen *et al.*, 2010). Examples of commonly altered miRNAs in AML include let-7, miR-17-92, miR-155, miR-181, miR-191, miR-9 and miR-196a/miR-196b (Chen *et al.*, 2010; Marcucci *et al.*, 2011a).

The microRNA miR-17-92 cluster is one of the most studied miRNA clusters. This family contains seven unique members (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1), which are dysregulated in a number of haematological and solid cancers (Mogilyansky and Rigoutsos, 2013). miR-17-92 is an important regulator of cell cycle and proliferation, and plays a key role in monocytopoiesis and megakaryopoiesis during haematopoiesis (Chen *et al.*, 2010). A number of studies have demonstrated overexpression of miR-17-92 in mixed-lineage leukaemia (MLL) rearranged leukaemia, which is thought to contribute to leukaemogenesis through inhibition of normal haematopoiesis and down-regulation of target genes promoting cell differentiation and apoptosis (Marcucci *et al.*, 2011b).

1.1.4.5. Multistage carcinogenesis (Vogelsteins model)

It is well known that cancer development and progression is driven by mutational events that occur in normal cells. Therefore, it is likely that multistage carcinogenesis evolves in cells with a mutator phenotype. This theory was first established in colorectal cancer by finding an association between hereditary nonpolyposis colorectal cancer and defects in DNA mismatch repair conferring a mutator phenotype (Parsons *et al.*, 1993). This observation highlights the importance of genome integrity and DNA repair; hence dysregulation of genes involved in the DNA damage response or chromosomal stability can lead to increased mutation, genomic instability, and eventually cancer transformation.

In the context of leukaemia, chromosomal translocations play an important role in leukaemogenesis (Dohner and Dohner, 2008; Mrozek and Bloomfield, 2008; Chen *et al.*, 2010). Such chromosomal translocations can lead to the generation of chimeric fusion proteins or insertion of genes close to promoter or enhancer elements, which contribute to genomic instability and disease progression. The multistage leukaemogenesis theory is widely demonstrated in AML, where a series of mutational events lead to proliferation of malignant myeloid progenitors with blockage of myeloid differentiation. As such, the presence of single mutational events such as FLT3, IDH1/2 or CEBPα is not enough to cause leukaemia, but further cooperating mutations are required in order to drive leukemic transition.

The AML1-ETO fusion protein resulting from the t(8;21) chromosomal translocation was shown to block myeloid differentiation and deregulate genes involved in DNA repair and stem cell maintenance (Alcalay *et al.*, 2003). Thus, the AML1-ETO oncoprotein induces a

mutator phenotype in blast cells and increase the rate of secondary mutations (Forster *et al.*, 2016).

1.1.5. Prognostication of AML

Prognostic factors can be divided into two main categories; patient related factors and disease related factors. Patient related factors are crucial to predict treatment related early death and indicate the ability of patients to tolerate the intensive chemotherapy (Erba, 2007) . Such factors include patient age, performance status, comorbidity of other diseases and impaired organ function (Erba, 2007). Disease related factors include white blood cells (WBC) count, molecular genetic alterations, and previous exposure to cytotoxic therapy or prior myelodysplastic syndrome (Döhner *et al.*, 2015). Determination of treatment options and predicting resistance to conventional chemotherapy are dependent on both patient and disease prognostic factors. However, the European Leukemia Net (ELN) introduced an AML prognostication classification system where patients are classified into four categories depending on mutational profile and cytogenetic alterations (Dohner *et al.*, 2010). This classification include favourable, intermediate-I, intermediate-II, and adverse subgroups (Table 1.4).

Prognosis	Cytogenetic abnormality
Favourable	- t(15;17)(q22;q21) - t(8;21)(q22;q22); RUNX1-RUNX1T1 (Regardless of additional cytogenetic abnormalities) - inv(16)(p13q22) or t(16;16)(p13;q22); CBFB-MYH11 - Mutated NPM1 without FLT3-ITD (normal karyotype) - Biallelic mutated CEBPα (normal karyotype)
Intermediate I	- Mutated NPM1 and FLT3-ITD (normal karyotype) - Wild-type NPM1 and FLT3-ITD (normal karyotype) - Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate II	- t(9;11)(p22;q23); MLLT3-KMT2A - Cytogenetic abnormalities not classified as favourable or adverse
Adverse	- abn(3q) excluding t(3;5)(q21~25;q31~35) - inv(3)(q21q26) or t(3;3)(q21;q26) - add(5q), del(5q), −5 - −7, add(7q)/del(7q), [Excluding cases with favourable karyotype] - t(6;11)(q27;q23) - t(10;11)(p11~13;q23) - t(11q23); [excluding t(9;11)(p21~22;q23) and t(11;19)(q23;p13)] - t(9;22)(q34;q11) - −17/abn(17p) - Complex (≥ 4 unrelated abnormalities)

Table 1.4: Prognostic classification of AML based on cytogenetic alterations.

European LeukemiaNet (ELN) Guidelines recommended classification of AML prognostic factors into favourable, intermediate I and II, and adverse subgroups depending on cytogenetic alterations. Adapted from (Döhner *et al.*, 2015).

1.1.6. Treatment of AML

Treatment of AML is challenging and disease relapse is potential common problem, particularly in elderly patients and patients with unfavourable prognosis. Despite the fact that AML treatment is difficult, it is curable in 35 to 40% of people younger than 60 years old, and 5 to 15% of people older than 60 years old (Döhner *et al.*, 2015). Moreover, treatment outcome in old patients, who are unable to tolerate chemotherapy without undesirable side effect, is still unsatisfactory with only 5-10 month median survival rate (Döhner *et al.*, 2015).

1.1.6.1. Remission induction regimens and consolidation therapy

AML treatment has not changed significantly for several decades with the exception of acute promyelocytic leukaemia (APML) which is treated with all-trans-retinoic acid (ATRA) and arsenic trioxide (Coombs *et al.*, 2015). The aim of the treatment is to eliminate blast cells below detectable levels by morphological analysis in peripheral blood and less than 5% in bone marrow. Remission induction therapy is followed by consolidation therapy to maintain complete remission by further cycles of chemotherapy and possibly bone marrow transplantation. Newly diagnosed patients are stratified according to the ELN prognostication system to determine their eligibility for intensive remission induction chemotherapy and to assess treatment options. Remission induction therapy starts with continuous infusion of cytarabine (Ara-C) for 7 days (100-200 mg/m^2) in combination with 3 days of an anthracycline, such as daunorubicin (60 mg/m^2) or idarubicin (10-12 mg/m^2) (Döhner *et al.*, 2015). Complete remission is usually achieved in the first cycle for patients aged less than 60 years (Döhner *et al.*, 2015), however a substantial number of patients will relapse within two years with chemoresistant disease. Patients older than 60 years with favourable or intermediate prognosis, with no coexisting conditions are likely to benefit from the standard induction therapy. Conversely, older patients with adverse cytogenetics with/without coexisting conditions may not benefit the standard induction therapy, but may be treated with low dose Ara-C, hypomethylating agents or hydroxyurea plus supportive care.

However, recent clinical studies have attempted to improve and optimise the outcome of remission induction therapy by stratifying patient into subgroups according to their age and genetic risk, and combining the classical remission induction regimen with targeted therapy. A recent key study performed by the Medical Research Council aimed to improve induction and consolidation outcome in newly diagnosed younger AML patients (aged 0 – 73 years) by comparing conventional treatment course versus new drugs combinations (Burnett *et al.*,

2013). The overall rates of remission between different treatment combinations were similar, but the combination of fludarabine, cytarabine, granulocyte colony-stimulating factor, and idarubicin (FLAG-Ida) significantly reduced relapse rate, and improved disease-free survival rate (Burnett *et al.*, 2013).

With the exception of acute promyelocytic leukaemia, current AML treatment still widely relies on intensive chemotherapy followed by either consolidation or allogeneic stem cell transplantation. However, the field of AML treatment is markedly progressing in terms of finding new therapeutic agents and standard dose optimisation. To this end, it seems that the most effective treatment approach for AML involves conventional treatment in combination with drugs specifically targeting upregulated/activated signal transduction molecules.

1.1.6.2. Nucleoside analogues

Nucleoside analogues have been used clinically for several decades and their ability to target vital cellular mechanisms has made them the cornerstone of AML treatment. Nucleoside analogues are chemically modified molecules developed to resemble normal DNA precursor nucleosides. Consequently, they are integrated into genomic DNA during DNA replication leading to inhibition of DNA synthesis via replication fork collapse. However, the mechanism of action of nucleoside analogues is cell cycle specific, where incorporation of fraudulent nucleosides into DNA during S phase in actively proliferating cells induces cell cycle checkpoint signalling and apoptosis. Nucleoside analogues can inhibit DNA synthesis via disruption of ribonucleotide reductase enzymatic activity, which required to catalyse formation of deoxyribonucleotide triphosphate (dNTP) (Galmarini *et al.*, 2001). Consequently, this result in reduction of dNTP synthesis that is required for DNA synthesis. Accumulating evidences suggest another mechanism by which nucleoside analogues induce cytotoxicity in leukaemia cells; by inhibition of DNA synthesis during DNA repair. Early studies showed that induction of DNA damage by ultraviolet (UV) enhanced incorporation of fludarabine into DNA and inhibited ongoing DNA repair performed by nucleotide excision repair by causing irreversible damage leading to the activation of PARP or P53 mediated apoptosis (Sandoval *et al.*, 1996).

1.1.6.2.1. Cytarabine (Ara-C)

Ara-C is the backbone of AML remission induction treatment. It is a pyrimidine nucleoside analogue which resembles the structure of deoxycytidine in DNA, with a unique hydroxyl group in a β-D-configuration on the 2'-carbon of the deoxyribose ring (Figure 1.2). There are three potential mechanisms by which Ara-C induce cytotoxicity to cancer cells, including incorporation into replicating DNA leading to chain termination, inhibition of DNA repair and inhibition of topoisomerase I enzymes (Gmeiner *et al.*, 2003) . Ara-C is taken up into cells through specific nucleoside transporters and converted through a series of phosphorylation events into Ara-C triphosphate (Matsuda and Sasaki, 2004). This is followed by incorporation into actively replicating DNA instead of deoxycytidine, which induces significant conformational perturbations at the site of incorporation. Consequently, this process prevents and inhibits DNA polymerase binding, and induces stalled replication and chain termination, leading to activation of intra-S phase checkpoints and apoptosis. Recent study have demonstrated that Ara-C is a substrate for polymerase β (an enzyme involved in base excision repair (BER)), suggesting that a component of Ara-C-induced cytotoxicity might be mediated be BER (Prakasha Gowda *et al.*, 2010). During BER, DNA glycosylases recognise and remove damaged base/bases leaving a gap in the DNA backbone; abasic site (AP site). AP sites are cleaved by APE1 and with gap filling mediated by polymerase β. The resulting nick is sealed by the joint actions of XRCC1 and DNA ligase as described in section 1.2.1.1. However, during DNA replication and during BER, the fraudulent pyrimidine nucleoside bases of Ara-C are incorporated into the DNA by polymerase β, which slows ligation by XRCC1 and DNA ligases (Prakasha Gowda *et al.*, 2010), and can eventually lead to stalled replication and fork collapse (Ewald *et al.*, 2008; Prakasha Gowda *et al.*, 2010). This process triggers the S phase DNA damage checkpoint, blocks DNA synthesis and causes cells to accumulate in the S phase of the cell cycle (Ewald *et al.*, 2008; Prakasha Gowda *et al.*, 2010).

1.1.6.2.2. Clofarabine

Clofarabine is a next generation nucleoside analogue which was developed on the basis of previous experience with other nucleoside analogue such as fludarabine and cladribine to achieve higher efficacy and lower toxicity (Figure 1.2) (Zhenchuk *et al.*, 2009). Clofarabine is similar to Ara-C in terms of mechanism of action, by which it incorporate into DNA leading to termination of chain elongation and inhibition of DNA synthesis. It also reported that

clofarabine induces damage to the mitochondrial membrane which triggers apoptosis via release of cytochrome c and other pro-apoptotic factors (Zhenchuk *et al.*, 2009). Preclinical and clinical data demonstrate that clofarabine has a broad anticancer in haematological and solid tumours. Clofarabine is currently used for the treatment of elderly AML patients who are unable to tolerate intensive chemotherapy and patients with refractory AML. Recent clinical trial data demonstrates that clofarabine in combination with low dose Ara-C increases the complete remission rate in elderly patients and those with refractory AML with minimal toxicity, but with no significant improvements in overall survival (Buckley *et al.*, 2015).

1.1.6.2.3. Fludarabine

Fludarabine or 9-β-D-arabinosyl-2-fluoroadenine (Figure 1.2) is an adenosine analogue commonly used for the treatment of chronic lymphocytic leukaemia (CLL). Similar to Ara-C and Clofarabine, fludarabine inhibits DNA synthesis. Currently its use in combination with other drugs such as Ara-C, daunorubicin, clofarabine and/or other drugs is still under investigation in clinical trials. The UK AML15 Medical Research Council clinical trial showed that fludarabine in combination with Ara-C, granulocyte colony-stimulating factor, and idarubicin significantly increase remission rate and reduced the risk of relapse particularly in patients with favourable and intermediate risk karyotype (Burnett *et al.*, 2013).

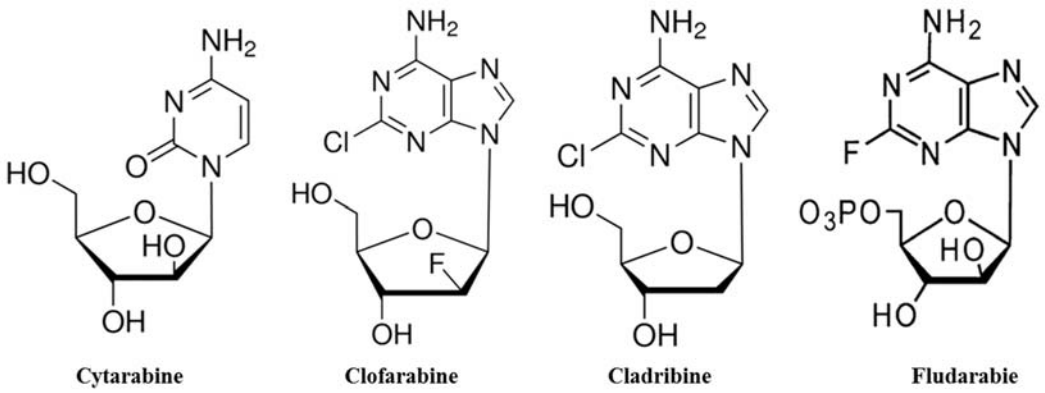


Figure 1.2: Chemical structure of nucleoside analogues commonly used in AML treatment.

1.1.6.3. Anthracyclines

Anthracyclines such as daunorubicin, doxorubicin and idarubicin have been used for over 4 decades as AML remission induction chemotherapy. Anthracycline are administered for 3 days along with continues infusion of Ara-C for 7 days. Despite the extensive clinical utility of anthracyclines in AML treatment, the mechanism of their action is still not fully elusive. However, several mechanisms of action were proposed and all of them act through induction of DNA damage. Mechanisms of anthracyclines action include: DNA intercalation, generation of free radicals to induce DNA damage, DNA alkylation, interfering with DNA unwinding during DNA replication by inhibition of topoisomerase II leading to DNA damage induction and apoptosis (Gewirtz, 1999; Minotti *et al.*, 2004). Anthracyclines also act on cancer cells by lipid peroxidation of cell membrane (Gewirtz, 1999).

More recent studies investigated the benefit of anthracycline dose adjustment/intensification to improve the complete remission rates and overall survival. The UK NCRI AML17 trial was initiated to compare the benefit of high dose daunorubicin (90 mg/m^2) versus 60 mg/m^2 in AML induction therapy in 1206 patients. No evidence were found for an overall benefit in patients treated with high daunorubicin dose. Furthermore, there was high mortality within 60 days in the patient group treated with high dose daunorubicin, which eventually lead to prematurely termination of this study (Burnett *et al.*, 2015).

1.1.6.4.Topoisomerase II poisons

Topoisomerase II (TOP2) enzymes are crucial for normal DNA metabolism during DNA replication, transcription and recombination, and function primarily by removing knots and relaxing supercoiled DNA during replication (Allan and Travis, 2005). Two distinct isoforms of TOP2 enzymes are expressed in human cells including TOP2α (TOP2A) and TOP2β (TOP2B), which share 70% of their amino acid sequence, but are encoded by two different genes on chromosomes 17q21–22 and 3p24, respectively (Pendleton *et al.*, 2014). TOP2 enzymes are tightly regulated by post-translational modification, which can become dysregulated in cancer (Chikamori *et al.*, 2010). However, TOP2 poisons, such as etoposide, doxorubicin and mitoxantrone, are widely used useful anticancer treatments, but are associated with treatment-related secondary AML, and particularly *MLL* gene translocation at 11q23, *PML-RARα* t(15;17) and *AML1-ETO* t(8,21) (Cowell and Austin, 2012). Etoposide is not routinely used in induction therapy because addition to conventional chemotherapy does not significantly improve overall survival (Bishop *et al.*, 1990; Hann *et al.*, 1997; Burnett *et*

al., 2013). However, patients with refractory/relapsed AML can benefit from etoposide treatment with good tolerability (Abbi *et al.*, 2015; Thol *et al.*, 2015). Doxorubicin (the anthracycline and a TOP2 poison as well) is now not used routinely in AML treatment due to its association with cardiotoxicity, and alternative anthracyclines provided better response and outcome in AML patients (Minotti *et al.*, 2004). Mitoxantrone is anthracycline that also has anti-TOP2 activity. It is commonly used in remission induction regimens, and also as post-remission therapy in combination with Ara-C or other chemotherapeutic agent.

1.1.6.5. Differentiation therapy (ATRA and ATO)

ATRA (all-trans-retinoic acid) is the first successful targeted therapy in AML where it is used as a differentiation therapy for M3 FAB acute promyelocytic leukaemia (APL) classification AML patients. This particular subtype is uniquely characterised by the presence of the t(15;17)(q22;q21) translocation that expresses the promyelocytic leukaemia (PML)/retinoic acid receptor α (RARα) fusion protein. ATRA specifically disrupts the PML/RARα fusion protein and abrogates the cell differentiation blockade and induces terminal differentiation of granulocytic cells (Wang and Chen, 2008). Risk of relapse is extremely high when APL is treated with ATRA as a single agent, especially in the subgroup of APL patients with t(11;17)- associated APL which expresses the promyelocytic leukaemia zinc finger (PLZF)/RARα fusion oncoprotein (Petrie *et al.*, 2009). Treatment with a combination of ATRA and arsenic trioxide (ATO) significantly abrogates relapse risk and overcomes resistance issues, yielding high complete remission rates and improved overall survival (Petrie *et al.*, 2009). Furthermore, treatment with a combination of ATRA/ATO with standard induction chemotherapy improves the clinical outcome in APL patient with complete remission rates of 90% to 95% and 5 years overall survival approaching 100% (Wang and Chen, 2008).

1.1.6.6. Hypomethylating agents

Current treatment regimens for AML include intensive chemotherapy in order to eliminate myeloid blasts in the bone marrow and peripheral blood. But there is a large category of patients who either do not respond to available treatment due to genetic/epigenetic heterogeneity of the disease, and/or due to an inability of those patients to tolerate intensive treatment.

Hypomethylating agents benefit MDS patients and elderly AML patients who are not eligible for intensive chemotherapy or who have refractory AML. There are two mechanisms by which hypomethylating agents mediate cytotoxicity. The first mechanism occurs through inhibition of methyltransferase activity and preventing further DNA hypermethylation (Ewald *et al.*, 2008). The second mechanism is via their function as nucleoside analogues and incorporation into DNA, leading to DNA damage which triggers DNA repair pathways, cell cycle checkpointing and apoptosis (Jiemjit *et al.*, 2008; Palii *et al.*, 2008). Azacitidine and decitabine, for example, are hypomethylating agent as well as acting as nucleoside analogues due to their ability to incorporate into DNA and mediate cell death via apoptosis (Ewald *et al.*, 2008). Both agents were assessed in a number of clinical trial which consistently reported improvement in overall survival and tolerability with minimal side effect in elderly AML patients > 60 years with blast counts between 20-30%. A recent clinical trial (the international phase 3 AZA-AML-001 study) reported that azacitidine increased median overall survival by 3.8 months compared to current commonly used AML treatments (Dombret *et al.*, 2015).

1.1.6.7. Consolidation Therapy

Consolidation or post-remission therapy aims to minimise relapse and destroy residual remaining blast cells that were not killed during remission induction. Decision making regarding consolidation therapy is facilitated by genetic profiling; with a decision to either proceed to bone marrow HSC transplantation for high risk AML or conventional consolidation chemotherapy for low risk AML patients.

Consolidation with intensive chemotherapy in patients younger than 60 years constitutes intermediate dose Ara-C for 2 to 4 cycles. The optimal dose and number of cycles is still an open issue, but doses of 1000 to 1500 mg/m^2 are recommended, and could be used in combined with mitoxantrone in patients with adverse karyotype (Döhner *et al.*, 2015). However, high dose Ara-C did not show benefit for favourable or intermediate-risk disease, compared to traditional consolidation (Burnett *et al.*, 2013). Patients with adverse risk who are not able to tolerate chemotherapy or have no response (refractory disease) are eligible for clinical trials with novel agents. A recent clinical trial showed that combination of amsacrine, cytarabine, etoposide, and then mitoxantrone/cytarabine was superior to standard therapy in this patient subgroup (Burnett *et al.*, 2013).

Allogeneic HSC transplantation following complete remission is the best anti-leukaemic treatment choice to minimise disease relapse. The relative benefits and risks of HSC vary in

different cytogenetic groups defined by the European Leukaemia Net (ELN) (section 1.1.5) (Cornelissen *et al.*, 2012). Reports indicate that only intermediate and adverse risk AML patients significantly benefit from allogeneic HSC transplantation, but in patients with favourable karyotype AML the limited benefits of improved survival and outweighed by the increased risk of toxicity and death associated with the transplant conditioning procedure (Koreth *et al.*, 2009; Cornelissen and Blaise, 2016).

1.1.7. Targeted therapy in AML

AML is heterogeneous disease with curable rate of 35 to 40% of people younger than 60 years old, and 5 to 15% of people older than 60 years old. The backbone of AML treatment is combination of Ara-C with anthracycline, has not changed over 40 years. As such, the majority of patients experience relapse, which is a particular problem in elderly groups and those patients with adverse karyotype. Following complete remission, only a minority of AML patients are eligible for allogenic HSC transplantation, which is associated with high morbidity and mortality. Moreover, the lack of selectivity of the conventional AML treatment is a major disadvantage, and results in undesirable adverse side effects. Owing to such poor outcome, it is clear that current conventional AML treatment has significant limitations, highlighting the clinical need for new targeted therapies with reduced toxicity that are better tolerated in patients with poor performance status.

The development of targeted therapies for AML is an area of significant investment for the research community, with a major shift from focusing on conventional treatment strategies to the development of novel therapies that specifically inhibit proteins or pathways essential to maintenance of the leukaemic clone, but which are either not present or not essential in non-leukaemic cells. Theoretically, this targeted approach preserves normal cells and consequently minimise adverse side effect caused by intensive chemotherapy, increasing chemotherapy efficacy and improves quality of life of AML patients. Identification of targeted therapy relies on exploring specific genetic alterations in AML cells that drive disease progression, cell survival and/or treatment resistance. This is followed by discovery and development of molecularly target drugs that exploit these vulnerabilities. As such, recurrently mutated genes in AML, and which are essential for maintenance of the leukaemia, represent potential therapeutic targets.

1.1.7.1. FLT3 inhibitors

FLT3 mutation is one of the most prevalent mutation in AML, occurring in approximately 30% of AML cases, and is associated with poor prognosis and a high risk of relapse (Pemmaraju *et al.*, 2014). There are two different groups of FLT3 mutations including FLT3 with internal tandem duplications (FLT3-ITD) and point mutations in the tyrosine kinase domain (FLT3-TKD). Due to the fact that FLT3 protein is frequently mutated in AML and confers a poor prognosis, it became a legitimate target for therapy in AML. Several FLT3 inhibitors have been developed and tested in clinical trials, including sorafenib, lestaurtinib, midostaurin, tandutinib and sunitinib. However, the results from clinical trial for the first generation of FLT3 inhibitors were unsatisfactory, primarily due to low specificity for FLT3 and concerns about development of resistance mediated by acquired mutations in FLT3 (Wander *et al.*, 2014). In addition, single agent treatment was only effective at inducing a transient reduction in blast count. Currently, phase III trials are ongoing to test the second generation of FLT3 inhibitors which thought to be highly potent and more specific for the FLT3 kinase (Döhner *et al.*, 2015).

1.1.7.2.Gemtuzumab ozogamicin (Mylotarg®)

Gemtuzumab ozogamicin (Mylotarg®) is a treatment for AML patients with CD33 positive AML and is used at first relapse in those aged over 60 years and not eligible for further intensive chemotherapy. Gemtuzumab is the first approved antibody targeted chemotherapy and is composed of anti-CD33 antibody linked to a calicheamicin derivative, which is a potent antitumor antibiotic. Data from several randomised clinical trials demonstrated that gemtuzumab did not increase the proportion of patients achieving complete remission, but it significantly reduced the risk of relapse and increase the overall survival in patient with favourable and intermediate prognosis (Burnett *et al.*, 2011b; Hills *et al.*, 2014). Despite promising results from initial clinical trials, the drug license was suspended in the United States of America due to high treatment-related mortality, including cardiac failure (Petersdorf *et al.*, 2013).

1.1.7.3. IDH1 and IDH2 inhibitor

IDH1 and IDH2 are crucial metabolic enzymes in the Krebs cycle and both are frequently mutated in AML and contribute to leukaemogenesis (Abbas *et al.*, 2010; Chaturvedi *et al.*,

2013). IDH mutations lead to DNA hypermethylation through disruption of the epigenetic regulator TET2 (Figueroa *et al.*, 2010a). Although IDH mutations are considered legitimate therapeutic targets in AML, very few IDH inhibitors are available for preclinical evaluation. Nevertheless, studies with IDH2 inhibitor AGI-6780 have demonstrated induction of differentiation and suppression of leukaemia cell growth (Wang *et al.*, 2013a). Furthermore, some IDH inhibitors have undergone clinical evaluation in clinical trials, and preliminary results are encouraging (Stein *et al.*, 2014). In early phase I clinical trial, the AG-221 IDH2 inhibitor induced differentiation of leukemic blasts, had a favourable pharmacokinetic profile and led to durable responses with complete remissions of up to 4.5 months when administered as a single agent (Stein *et al.*, 2014).

1.1.7.4. Targeting DNA repair/DNA damage response in AML

It is becoming clearer that chromosomal translocations, genetic aberrations and epigenetic events can disrupt genome stability and activate the DNA damage response in AML, and yet these same alterations also affect treatment efficacy, confer treatment resistance, and promote disease progression. Mutations in genes involved in DNA repair in AML are relatively uncommon with the exception of *TP53*. However, targeting DNA repair in AML is not fully established and still under investigation in preclinical studies and some clinical trials, such as PARP-1 inhibitors for example.

1.1.7.4.1. PARP-1

Poly (ADP-ribose) polymerase 1 (PARP-1) inhibition has demonstrated therapeutic efficacy in cancers harbouring BRCA1 or BRCA2 mutations, including some breast and ovarian cancers. PARP-1 play a crucial role in base excision repair (BER) (see section 1.2.1.1). Specifically, PARP-1 inhibition potentiates the anticancer activity of chemotherapeutic agents that generate DNA damage repaired by PARP-1 and BER, such as temozolomide, topoisomerase I poisons and ionising radiation (Mitchell *et al.*, 2009; Curtin and Szabo, 2013). Several preclinical studies have demonstrated the potential utility of PARP-1 inhibition in AML. One key study demonstrated anti-leukaemic activity of PARP-1 inhibitors, as a single agent, on a number of AML and MDS cell lines in addition to primary AML cells (Gaymes *et al.*, 2009). The addition of decitabine in combination with PARP-1 inhibitors further potentiated cell killing. PARP-1 inhibition led to the accumulation of DNA double

strand breaks which subsequently led to apoptosis (Gaymes *et al.*, 2009). Consistent with this observation, a recent preclinical study confirmed the potential utility of PARP-1 inhibition in AML, and particularly in cells harbouring the AML1-ETO and PML-RARα fusion oncoproteins (Esposito *et al.*, 2015). It appears that sensitivity to PARP-1 inhibition correlated with changes in the expression of genes involved in DNA damage repair; particularly components of homologous recombination, including RAD51, ATM and BRCA1/2 (Aly and Ganesan, 2011; Weil and Chen, 2011). PARP-1 inhibition triggers differentiation and senescence in both human and mouse model with AML1-ETO and PML-RARα; consistent with previous reports that DNA damage induces differentiation in haematopoietic cells (Santos *et al.*, 2014). Therefore, PARP-1 inhibition is a potential target in AML and its utility is still under exploration in phase I clinical trials both with and without temozolomide or carboplatin for the treatment of refractory AML, high-risk myelodysplasia, or aggressive myeloproliferative disorders (clinicaltrials.gov website).

1.2. Targeting base excision repair as a therapeutic strategy in AML

DNA repair systems are important to maintain genomic integrity and prevent ongoing DNA mutation caused by endogenous and exogenous DNA damaging agents such as chemotherapeutic agents and radiation. Several DNA repair pathways are required to maintain genomic integrity, including base excision repair (BER), nucleotide excision repair, mismatch repair, homologous recombination repair, non-homologous end joining (NHEJ) and direct DNA repair pathway.

Base excision repair (BER) is an important DNA repair system required to repair DNA lesions induced by alkylation, oxidation, ionizing radiation as well as deamination. Unrepaired damaged bases can mispair during DNA replication and could become fixed as mutation (Kelley *et al.*, 2014). BER operate in all cell cycle phases through two sub-pathways; short-patch BER and long-patch BER (Branzei and Foiani, 2008). It is not fully understood how short and long patch repair are invoked, but the type of damage and the cell cycle phase may play a crucial role in this process (Fortini and Dogliotti, 2007; Kim and Wilson, 2012). In particular, short-patch pathway is active during the G1 phase to repair single base damage, and long-patch BER is utilised in the repair of DNA damage by replacing a strand of 2-8 bases, primarily during S and G2 phases of the cell cycle (Fortini and Dogliotti, 2007; Branzei and Foiani, 2008).

The potential utility of targeting DNA repair (specifically BER) components has already been demonstrated in preclinical and clinical studies. For example, inhibition of PARP1, a key modulator enzyme in BER, has shown promise in preclinical and clinical studies of germline BRCA1/2 deficient breast and ovarian cancer with dysregulated homologous recombination repair. Although PARP inhibition is promising, recent reports have raised some issues, including the development of PARP inhibition resistance and enhanced myelosuppression (Plummer *et al.*, 2008; Fojo and Bates, 2013). Moreover, the PARP family has 17 members raising issues related to drug selectivity which may limit the clinical utility of PARP inhibitors (Rouleau *et al.*, 2010). However, targeting other components of BER such as APE1, XRCC1 or POLβ also show promise in preclinical studies (Barakat *et al.*, 2012; Li and Wilson, 2014). In particular, targeting APE1 has been extensively investigated leading to the development of several inhibitors (discussed in section 1.2.1.4).

The specific importance of BER for AML cell survival is not fully understood. However, previous studies have highlighted the importance of BER and other DNA repair systems to maintenance of the tumour environment, leading to the hypothesis that BER components (particularly APE1 and OGG1) may play an essential role in AML that could be targeted through inhibition of function. In the following sections (1.2.1 and 1.2.2), the biological roles of APE1 and OGG1 in normal and cancer cells will be discussed, in addition to the potential for targeting these components in a therapeutic setting.

1.2.1. Targeting Apurinic/Apyrimidinic endonuclease 1 (APE1) as a therapeutic strategy in AML

APE1 is an abundant protein in eukaryotic cells with approximately $10^4 – 10^5$ molecules per cell and an approximate half-life of 8 hours (Tell *et al.*, 2009). It is a relatively small protein (36.5 kDa) and consists of 318 amino acids encoded by ~3 kb gene localised on chromosome 14 q11.2-12 (Fritz, 2000). The redox function of APE1 resides in the N-terminal region while the C-terminal portion is responsible for the DNA repair function (Tell *et al.*, 2009). The first 33-35 amino acids of the N-terminal region comprises the nuclear localization sequence (NLS) which is also essential for protein-protein interaction and RNA binding activity of APE1 (Tell *et al.*, 2010a).

APE1 is a multifunctional protein, crucial for cell survival, proliferation and maintenance of genomic stability. It plays a key role in repairing DNA damage induced by oxidative stress and alkylating agents through its function as part of BER (Tell and Wilson, 2010). APE1 also

has a redox regulatory function, which is mediated through interaction and activation of a variety of transcription factors involved in regulation of cell survival and proliferation, including NF-κB, AP-1, Egr-1, HIF-1α and TP53 (Tell *et al.*, 2010a). In addition, recent studies have revealed more functions of APE1, including RNA quality control, regulation of parathyroid hormone through interaction with negative calcium repressing element, angiogenesis and other functions (Bhakat *et al.*, 2009). Furthermore, APE1 is essential for embryonic development, cell survival and viability; homozygous mutation in *APE1* in mice induced embryogenic lethality at day 5.5 (Xanthoudakis *et al.*, 1996).

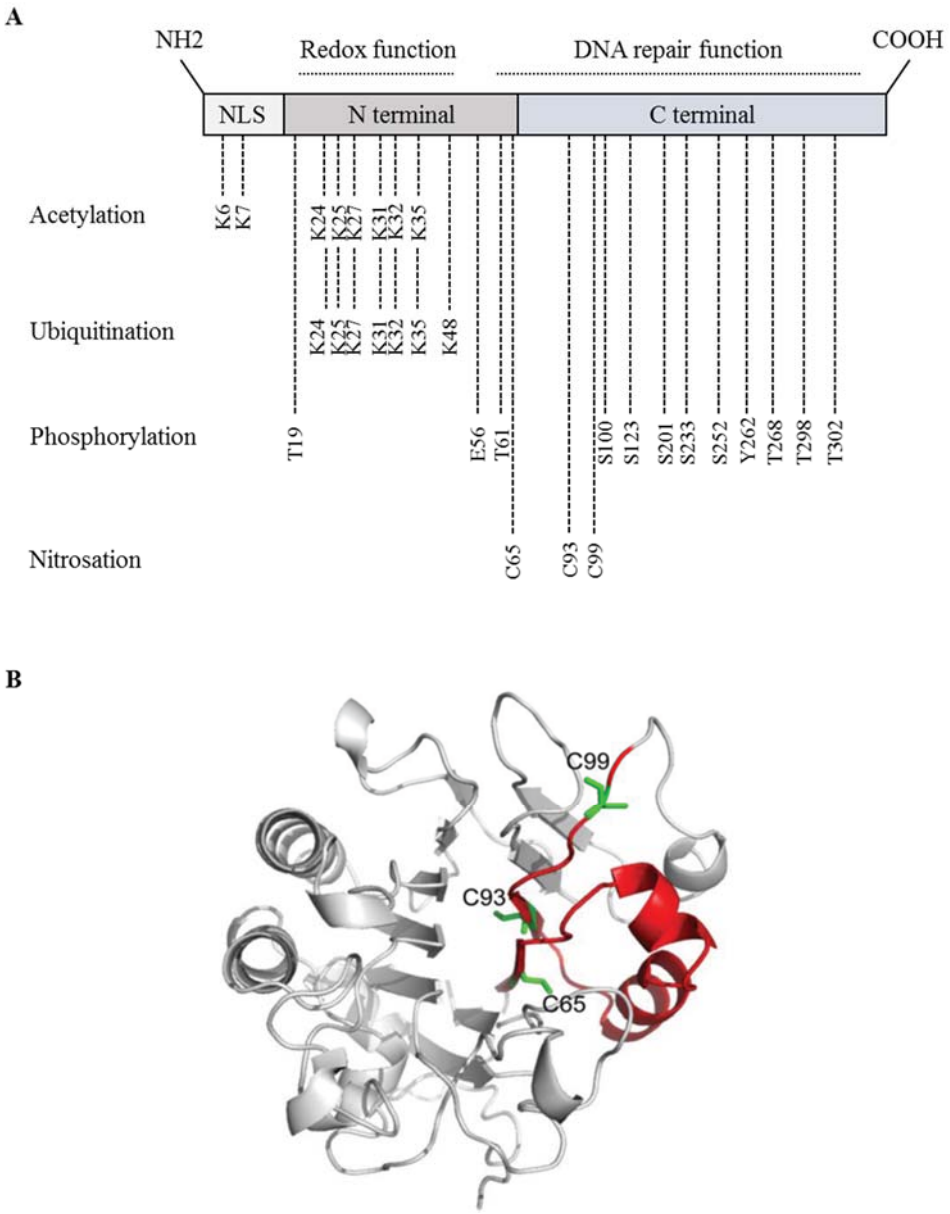


Figure 1.3: The structure of human APE1 protein.

(A) APE1 protein (36.5 kDa) consists of 318 amino acids. The redox function of APE1 resides in the N-terminal region while the C-terminal portion is responsible for the DNA repair function. The first 33-35 amino acids of the N-terminal region comprises the nuclear localization sequence (NLS) which is also essential for protein-protein interaction and RNA binding activity of APE1. Figure adapted from (Dyrkheeva *et al.*, 2016). **(B)** APE1 tertiary structure with illustration of the position of critical redox active cysteines residues C65, C93 and C99. Figure adapted from (Luo *et al.*, 2012).

1.2.1.1.APE1 functions

DNA repair function

Several DNA base lesions caused by alkylating agents and majority of lesions induced by oxidative stress are repaired by BER. The main components of BER include DNA glycosylases, APE1, DNA polymerases and DNA ligases. Other factors are important for recruiting and coordination of BER. However, APE1 is unique in its role in BER and no other enzyme has endonuclease function. Additionally, APE1 also possesses very weak 3' exonuclease activity and 3' phosphodiesterase activity (Li and Wilson, 2014).

BER is initiated by recognition and excision of the damaged base/bases by the action of a DNA glycosylase to create an abasic site (Apurinic/Apyrimidinic site or AP site). Different DNA glycosylases recognise their specific damaged base/bases. There are two types of DNA glycosylases including monofunctional and bifunctional glycosylases (Table 1.5) (Kim and Wilson, 2012). Monofunctional glycosylases only perform base excision of the damaged base and mainly repair alkylated and deaminated bases (Krokan and Bjoras, 2013). Bifunctional glycosylases mainly repair oxidized DNA lesions and exhibit glycosylase activity in addition to AP lyase activity, which create 3' incision to AP site in the phosphodiester backbone of the DNA by either by β-elimination or by β,δ-elimination (Kim and Wilson, 2012; Krokan and Bjoras, 2013).

However, following removal of the damaged base, processing the DNA damage proceed via either short patch or long patch BER depending on the type of damage and glycosylase involved (Figure 1.4) (Kim and Wilson, 2012).

- **Short patch BER**

Short patch BER is engaged in response to single nucleotide damage and is the predominant pathway in proliferating and non-proliferating cells (Akbari et al., 2004; Fortini and Dogliotti, 2007). If the damaged base removed by monofunctionl glycosylases (Table 1.5), processing the DNA damage is processed via short patch BER. Following removal of the damaged base/bases, the AP-endonuclease APE1 processes the resulting AP site by creating a 5' incision in the phosphodiester backbone of the DNA (Kim and Wilson, 2012). This incision generates hydroxyl group (OH) at 3' end and deoxy ribose phosphate at the 5' termini (5'dRP). This followed by recruiting β polymerase to removes 5'dRP by its phosphodiesterase activity and filling the gap with the correct bases (Kim and Wilson, 2012).

Subsequent to this, DNA ligase III and XRCC1 (X-ray cross-species complementing 1) are activated and function to repair the incision and complete the repair process (Figure 1.4) (Kim and Wilson, 2012). Although the role of PARP1 (and PARP2) is BER is not clear evidence suggests that PARP is essential in activation and recruitment of polymerase β, XRCC1 and DNA ligase III (Kelley and Fishel, 2008). In contrast, recent studies suggest that PARP is not essential to BER, but complements BER (Strom et al., 2011).

Incision of the damaged base by the AP lyase activity (β-elimination) of a bifunctional glycosylase, including OGG1, MYH and NTH1, creates a blocking DNA nick phosphor-α,β-unsaturated aldehyde (PUA) at the 3' end. This lesion is refractory to polymerase activity and must be removed by phosphodiesterase activity of APE1 in order to allow polymerase to gap fill. Following processing of the PUA, repair can proceed via gap filling mediated by polymerase β with nick ligation performed by DNA ligase III and XRCC1 via short patch BER.

- **Long patch BER**

Long patch BER specifically repair oxidised and reduced damaged bases (Kim and Wilson, 2012). Removal of damaged bases by bifunctional glycosylases NEIL1/2 or 3 via β,δ-elimination, creates a blocking 3' phosphate (PO4), which resists DNA polymerase mediated repair and requires further processing by polynucleotide kinase/phosphatase (PNKP). PNKP has 3' phosphatase and 5' DNA kinase activities that remove 3'PO4 blocking group (Krokan and Bjoras, 2013). Proliferating cell nuclear antigen (PCNA) and/or polymerases ε and δ subsequently perform strand removal by displacing 3-8 bases (Kim and Wilson, 2012). The displaced strand is subsequently resynthesised by FEN1 (flap endonuclease 1). Ultimately, DNA ligase I completes the repair process by sealing the DNA nick (Figure 1.4) (Kim and Wilson, 2012).

There are several factors that could influence selection of the short or long patch BER pathways. The nature of DNA damage determines which glycosylase initiates the repair process and therefore determines which sub-pathways could be executed (Fortini et al., 1999). For example, the oxidised DNA lesion 2-deoxyribonolactone is refractory to polymerase β lyase activity and therefore requires long patch BER (Sung and Demple, 2006). The differentiation state and the cell cycle stage of the cell may also contribute to sub-pathway

selection. The short patch pathway is executed equally in both dividing and non-dividing cells (Akbari et al., 2004). On the other hand, long patch repair pathway is thought to be primarily active only in dividing cells (Fortini and Dogliotti, 2007; Narciso et al., 2007). Protein-protein interaction that occur during/after excising damaged base may also contribute to pathway selection. XRCC1 is a crucial BER component that plays a key role in coordinating the early steps of BER as well as its role in ligation of the DNA backbone (Vidal et al., 2001; Moor et al., 2015). Cells with mutated XRCC1 exhibit impaired ligation and defective polymerase β-dependent single nucleotide insertion via the short patch pathway (Cappelli et al., 1997).

- **Coordination of BER by APE1**

BER is a tightly regulated process and several factors are involved in the coordination of repair. APE1 is involved in coordination of BER by interaction and recruitment of other BER components. Several glycosylases and factors are reported to directly interact with APE1 to regulate BER, including OGG1, XRCC1, polymerase β, PARP-1 and P53 (Vidal *et al.*, 2001; Sidorenko *et al.*, 2007; Parsons and Dianov, 2013; Moor *et al.*, 2015; Poletto *et al.*, 2016). APE1 interaction with BER enzymes enhances the efficiency of DNA damage repair as well as minimising accumulation of potential cytotoxic DNA repair intermediates.

After excision of a damaged base, DNA glycosylases, such as OGG1, TDG, UDG and NTH1, bind tightly to the created AP site to protect it and the strand break until the recruitment of APE1 to the damage site (Donley *et al.*, 2015). Consequently, APE1 displaces the DNA glycosylase and activates BER downstream effectors such as polymerases, ligases and XRCC1. APE1 also interacts with XRCC1 during processing of AP sites in order to accelerate the repair process and prevent formation of 5’ blocking lesions (dirty ends), which could be cytotoxic (Vidal *et al.*, 2001). Furthermore, APE1 recruitments polymerase β to the AP site in order to accelerate excision of 5′-dRP residues (Bennett *et al.*, 1997).

Although BER is a conserved pathway that maintains genome stability through elimination of a number of alkylated, deaminated and oxidised bases, there is evidence that suggest redundancy in BER to safeguard DNA against cytotoxic and mutagenic lesions. For example, removal of a damaged base by the AP lyase function of bifunctional glycosylases such as OGG1 and NEIL-1, 2 creates a 3’PUA that can be processed via PNKP and repair proceeds via long-patch BER, therefore bypassing APE1 inhibition (Mokkapati *et al.*, 2004;

Wiederhold *et al.*, 2004). Loss of APE1 function could also be compensated by APE2, which exhibits strong 3'-5' exonuclease and 3'phosphodiesterase activities and weak AP endonuclease activity (Tsuchimoto *et al.*, 2001; Burkovics *et al.*, 2006; Burkovics *et al.*, 2009). The status of mismatch DNA repair (MMR) and O^6-methylguanine-DNA methyltransferase (MGMT) could also backup BER and limit DNA damage if BER is disabled. For example, temozolomide (TMZ), a methylating agent, induce O^6-methylguanine, N3-methyladenine and N7-methylguanine DNA adducts that are collectively repaired by MGMT and BER. Specifically, N3-methyladenine and N7-methylguanine DNA adducts are removed and processed by BER and considered relatively inert or weakly pro-cytotoxic (Fronza and Gold, 2004; Shrivastav *et al.*, 2010; Wirtz *et al.*, 2010). O^6-methylguanine lesions are rapidly removed by MGMT, but if unrepaired, these can become mismatched with thymine during DNA replication. Inefficient removal of mismatched bases leads to the accumulation of DNA stand breaks and induction of apoptosis (Zhang *et al.*, 2012). Therefore, TMZ-induced DNA adducts are largely removed and excised by MGMT and their cytotoxicity is primarily dependant on the MMR status of the cell. MMR can also backup BER in removal of mismatch bases such as 5-fluorouracil:G and U:G, which are predominantly removed by BER (Fischer *et al.*, 2007; Schanz *et al.*, 2009)."

Redox/transcriptional regulation function of APE1

In addition to the DNA repair function of APE1, it also functions as a reduction/oxidation signalling protein and is therefore referred to as redox effector factor 1 (REF-1). APE1 reduces functional cysteine (Cys) domains situated in the DNA binding sites of a number of transcription factors such as NF-κB, AP-1, Egr-1, HIF-1α, P53 and other transcription factors (Bhakat *et al.*, 2009). The exact mechanism by which APE1 reduces and interacts with transcription factors has not been fully elucidated. However, there are two functional Cys residues (Cys65 and Cys93) implicated in APE1 redox function (Luo *et al.*, 2012). Cys65 is a buried residue located in the N-terminus on the first beta strand in the fold of a beta sheet in the protein core (Figure 1.3B) (Kelly *et al.,* 2012). Cys93 is also buried inside the protein core, but in the opposite beta sheet to that where Cys65 lies (Figure 1.3B) (Luo *et al.*, 2012). It is thought that the redox function is mediated through a thiol-mediated redox reaction (Luo *et al.*, 2012). This occurs through interaction of Cys65 on APE1 with the functional Cys residues on the target transcription factor to form mixed disulphide bonds which are then attacked and resolved by Cys93 (Luo *et al.*, 2012). The formation of a disulphide bond in

APE1 means that it becomes oxidized and the target transcription factor becomes reduced (Luo *et al.*, 2012).

Abbreviation	Full name	Damaged base type	Description
AAG/MPG	Alkyl adenine DNA glycosylase/ Methyl purine DNA glycosylase	Alkylated base	Mono-functional glycosylases, i.e. they excise the damaged base only.
UNG	Uracil DNA glycosylase	Deaminated base	
TDG	Thymine DNA glycosylase	Deaminated base	
MBD4	Methyl-CpG-binding domain 4	Deaminated base	
OGG1	8-oxo-guanine glycosylase 1	Oxidized base	Bi-functional glycosylases, i.e. they excise the damaged base/s and have AP lyase activity.
MYH	MutY homolog	Oxidized base	
NTH1	Endonuclease three homolog 1	Oxidized base	
NEIL1	Nei endonuclease VIII-like 1	Oxidized base	
NEIL2	Nei endonuclease VIII-like 2	Oxidized base	
NEIL3	Nei endonuclease VIII-like 3	Oxidized base	

Table 1.5: List of DNA glycosylases involved in base excision repair pathway.

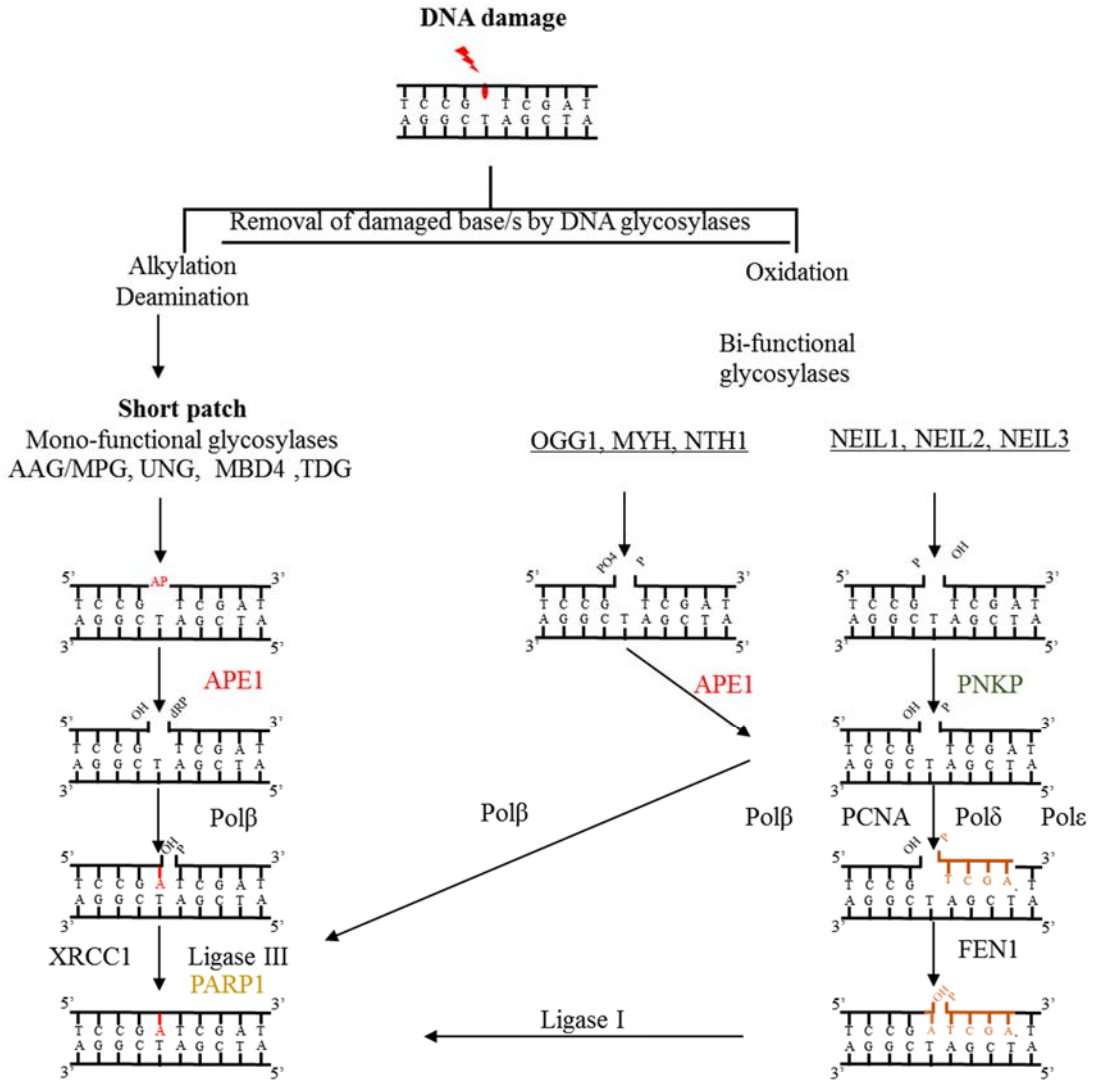


Figure 1.4: Overview of base excision repair (BER) pathway.

First step in BER begins with recognition of DNA damage and excising damaged base by DNA glycosylases. BER process the DNA damage via short or long patch sub-pathways depending on the type and cause of damage as well as the glycosylases involved. Removal of damaged bases result in creating AP site in the DNA backbone. In short patch BER, APE1 is recruited to processes the resulting AP site by creating a 5' incision in the phosphodiester backbone of the DNA. This incision generates hydroxyl group (OH) at 3' end and deoxy ribose phosphate (dRP) or phosphate group at the 5'. This followed by recruiting polymerase β to the site of damage to fill the correct base followed by sealing the gap by XRCC1 and ligase III. If the damaged base is removed by AP lyase activity of DNA glycosylase, this will create phosphor-α,β-unsaturated aldehyde (PUA) at the 3' end which processed by phosphodiesterase activity of A|PE1 followed by filling the gap by polymerase β and sealing the DNA backbone by XRCC1 and ligase III via short patch BER. In long patch pathway, bifunctional glycosylases creates a blocking 3' phosphate (PO4), which resists DNA polymerase mediated repair and requires further processing by polynucleotide kinase/phosphatase (PNKP). The process of DNA repair continue through polymerase β, ε and δ, PCNA and FEN1 which displace strand of 3-8 bases and synthesise a new strand. DNA ligase I completes the repair process by sealing the DNA nick.

DNA repair and redox functions are completely independent from each other and inhibiting or interrupting one function does not affect the other (Bapat *et al.*, 2009). This is because each function is encoded by different structural domains of APE1. The repair function is encoded by C-terminal while the redox function is performed by the N-terminal region of the protein (Bapat *et al.*, 2009).

Recent studies have revealed that targeting the redox activity of APE1 (but not repair activity) using small molecule inhibitors or by introducing an inactivating mutation, hyper-sensitises tumour cells to alkylating agents and radiotherapy (Kelley *et al.*, 2012). Targeting this function is thought to inhibit the interaction between APE1 and transcription factors involved in driving tumour growth, angiogenesis and proliferation of malignant cells (Kelley *et al.*, 2012). As such, inhibiting the redox function of APE1 has become a potentially attractive target for the development anticancer drugs.

Other APE1 functions

The DNA repair and redox functions are not the only important functions of APE1. Recent studies have revealed unexpected novel function of APE1. For example, APE1 has an RNA quality control function mediated through interaction with nucleophosmin 1 (NPM1) (Vascotto *et al.*, 2009b) and exerts endoribonuclease activity on c-MYC mRNA (Barnes *et al.*, 2009; Kim *et al.*, 2010; Kim *et al.*, 2011).

NPM1 is one of the hub proteins located in the nucleolus and is a multifunctional protein involved in ribosomal protein assembly and transport, control of centrosome duplication, and regulation of the tumour suppressor ARF (Falini and Martelli, 2011). The mechanism by which NPM1 interacts with APE1 is unclear, but it is thought that NPM1 directly binds to the N-terminus of APE1 and inhibits its binding to RNA (Tell *et al.*, 2010b). In addition, NPM1 binds rRNA and prevents it from binding to APE1. Oxidative stress is thought to decrease the binding affinity of NPM1 for both APE1 and rRNA, which releases APE1 to exert its RNA function (Tell *et al.*, 2010b). Consequently, after AP site cleavage, the resulting RNA fragments are degraded via the activity of an exosome complex and exoribonuclease 1 (XRN1) (Tell *et al.*, 2010b). Although NPM1 mutation in AML confers relatively favourable prognosis, this mutation might not affect APE1 functions as a recent study demonstrated that the endonuclease activity of APE1 is consistent regardless of the presence or absence NPM1 mutation (Vascotto *et al.*, 2013).

APE1 also possesses a second RNA function, and displays endoribonuclease activity on c-MYC proto-oncogene mRNA (Barnes *et al.*, 2009; Kim *et al.*, 2010; Kim *et al.*, 2011).

Investigations have demonstrated that APE1 has endoribonuclease activity by cleaving specific regions of c-MYC mRNA (Kim *et al.*, 2011). The endoribonuclease activity of APE1 occurs in the absence of Mg^{+2} ions, which are crucial in the DNA endonuclease function of APE1 (Kim *et al.*, 2011). *In vitro* studies clearly demonstrate a role for APE1 in the regulation and stability of c-MYC mRNA; possibly through its endoribonuclease function (Barnes *et al.*, 2009; Kim *et al.*, 2010). Specifically, transient knockdown of APE1 in HeLa cells using siRNA leads to increased c-MYC mRNA level (2 to 5 fold) and an increase in transcript half-life (Barnes *et al.*, 2009). However, the mechanism by which APE1 interacts and regulates mRNA expression is not yet understood.

Dissecting the endoribonuclease function of APE1 has raised many questions surrounding its involvement in RNA metabolism. For example, if APE1 is able to regulate c-MYC gene expression, is it also able to regulate the expression of other genes, including those proteins involved in cancer promotion and progression. Although beyond the scope of this project, it might be useful to identify other targets for the endoribonuclease function of APE1, and whether targeting this activity of APE1 could be exploited for cancer therapy.

As APE1 is developed as a target in cancer treatment, its functions have been investigated extensively in the literature. APE1 was identified as a transcriptional repressor via its ability to bind to negative calcium repressing element and regulate parathyroid hormone gene expression and the human renin gene (Bhakat *et al.*, 2003; Bhakat *et al.*, 2009). APE1 also has been shown to interact with Y-box binding protein 1 (YB-1), leading to transcriptional activation of the multi-drug resistance gene 1 (MDR1) (Sengupta *et al.*, 2011). APE1 also inhibits activation of PARP during repair of single strand breaks induced by oxidative stress (Peddi *et al.*, 2006). Moreover, APE1 is involved in natural killer cell mediated cell cytotoxicity by interaction with granzymes A and K (Guo *et al.*, 2008).

1.2.1.2. Role of APE1 in cancer

Since APE1 is essential for normal cellular functions, its role has been explored in cancer and other human diseases, such as neurodegenerative diseases and cardiovascular diseases (Jeon *et al.*, 2004; Gencer *et al.*, 2012; Maynard *et al.*, 2015). The importance of APE1 is highlighted by its ability to modulate DNA repair via BER and its redox transcriptional regulation of several pathways that are involved in homeostasis of normal cells and survival of cancer cells. Consistent with this, several biological and aetiological studies provide

evidence that APE1 is dysregulated during cancer progression, and that chemotherapy resistance is associated with elevated expression of APE1 in cancer cells.

Although most studies have ascribed APE1 localisation predominantly to the nucleus, where it functions as an endonuclease in BER and as a redox regulator, some tissues shows cytoplasmic localisation or both nuclear and cytoplasmic localisation (Tell *et al.*, 2005; Sheng *et al.*, 2012; Vascotto *et al.*, 2013). Particularly, the localisation of APE1 to the cytoplasm is reported in cell types displaying high metabolic activity such as hepatocytes, spermatocytes and lymphocytes (Tell *et al.*, 2005). Furthermore, APE1 localisation to the cytoplasm has been well documented in many cancers and correlates with poor prognosis (Di Maso *et al.*, 2007; Sheng *et al.*, 2012; Sudhakar *et al.*, 2014), highlighting the importance of APE1 extra-nuclear functions.

Anti-cancer agents predominantly function by damaging the DNA and targeting signalling pathways that otherwise promote cancer cell survival. As such, the efficacy of such agents could be reduced in cells with functional APE1-mediated DNA repair and redox regulation. Therefore, APE1 could be a potential predictive marker for response to therapy and disease progression. Consistent with this hypothesis, APE1 overexpression was significantly associated with poor prognosis in breast cancer (Woo *et al.*, 2014). Furthermore, APE1 expression was also correlated with poor overall survival of patients with ovarian cancer, and may predict platinum resistance (Al-Attar *et al.*, 2010). Conversely, low APE1 expression can be a potential prognostic marker and sign of aggressive disease as demonstrated in oestrogen receptor positive breast cancer (Abdel-Fatah *et al.*, 2014).

1.2.1.3. APE1 genetic polymorphism and susceptibility to cancer

APE1 is a multifunctional protein and responsible for maintaining genomic stability. As such, any DNA sequence polymorphism could impact on protein function, may increase cancer risk and also be prognostic. The most frequently reported APE1 polymorphism is an amino acid substitution from aspartic acid to glutamic acid in the position 148 (Asp148Glu), encoded by a polymorphism in exon 5 (Karahalil *et al.*, 2012). It is carried by approximately 46% of western Europeans (Wallace *et al.*, 2012). Although this polymorphism does not appear to affect the DNA endonuclease function of APE1, it is thought to confer hypersensitivity to radiation and an increased susceptibility to cancer (Wallace *et al.*, 2012). However, reports about the Asp148Glu variant are inconsistent; Wang and colleagues report an association between Asp148Glu and risk of bladder cancer, while another study reported no significant

association with susceptibility to bladder cancer (Liu *et al.*, 2013). Other polymorphisms include Gln51His, Ile64Val, Leu104Arg, Glu126Asp, Arg237Ala and Asp283Gly (Fishel and Kelley, 2007; Wallace *et al.*, 2012). The latter four polymorphisms are rare and are associated with reduced DNA endonuclease activity (Hadi *et al.*, 2000), although further work is required to determine whether they affect risk of cancer in humans.

Genetic polymorphisms in DNA genes may influence leukaemia susceptibility and/or treatment outcome. Genetic polymorphisms in DNA repair gene such as *XRCC1* and *XPD* has been linked to leukaemia risk and treatment outcome (Allan *et al.*, 2004; Bănescu *et al.*, 2014). Relatively few studies have investigated associations between genetic variations in *APE1* and the risk of developing leukaemia or prognosis. Kuptsova and colleagues investigated *APE1* variants, including Asp148Glu, in primary samples from AML patients, and found no effect on DNA repair capacity. In addition, another study found no significant associations between the Asp148Glu *APE1* variant and event-free survival 320 paediatric patients with acute lymphoblastic leukaemia (Krajinovic *et al.*, 2002). However, a study investigated 105 Chinese children with AML found that the Asp148Glu *APE1* polymorphism was associated with increased risk of leukaemia in children exposed to X-ray radiation (Zhu *et al.*, 2008).

Additional studies are essential to clarify the relationship between DNA repair variants and leukaemia. Specifically, larger studies are needed in order to clarify the role of these polymorphisms as determinants of susceptibility to cancer and prognosis.

1.2.1.4. APE1 inhibitors

APE1 is essential for maintenance of genomic stability and cell survival, and plays a key role in responding to DNA damage under oxidative stress. Even in conditions when exogenous DNA damage is very low, silencing APE1 using RNA interference was enough to decrease cell proliferation, and led to an accumulation of unrepaired cytotoxic AP sites and increased apoptosis (Fung and Demple, 2005). Additionally, increased APE1 expression has been linked with cancer chemotherapy resistance (Bapat *et al.*, 2009). Taken together, these data strongly suggest that targeting APE1 is an attractive strategy for the development of novel cancer treatments. This strategy has been intensively investigated in the last few years. For example, reducing APE1 protein levels using RNA interfering techniques or inhibition of its functions using specific inhibitors, sensitised cancer cells to radiotherapy and

chemotherapeutic agents such as temozolomide (TMZ), methylmethane sulfonate (MMS) and bleomycin (Bapat *et al.*, 2009).

Given the importance of APE1 in both normal and malignant cell function, there is a clear need to identify APE1 inhibitors specific to just one of its many functions. In addition, there is also a need to develop specific APE1 inhibitors with no off-target effects and which are effective in the low micromolar (μM) or nanomolar (nM) concentration range. Recent studies have identified a number of promising compounds thought to specifically inhibit APE1 in the low μM concentration range, but these need further validation, characterisation and optimization before moving forward to clinical use (Al-Safi *et al.*, 2012; Rai *et al.*, 2012; Srinivasan *et al.*, 2012; Raia *et al.*, 2013). Furthermore, inhibitors against specific functions of APE1 would help to identify which are critical for cancer promotion or chemotherapy resistance, as well as identifying which functions might be suitable targets for cancer therapy, i.e. targeting DNA repair or redox function.

Currently, there are two categories of APE1 inhibitors, including DNA repair inhibitors and redox functions inhibitors. Of these, the best studied include DNA repair inhibitors methoxyamine, lucanthone and CRT0044876, and redox function inhibitors E3330, gossypol, soy isoflavones and resveratrol.

1.2.1.4.1. *APE1 DNA repair inhibitors*

Methoxyamine

Methoxyamine (MX) (see Figure 1.5 for chemical structure) is an indirect inhibitor of the DNA repair function of APE1 (Wilson and Simeonov, 2010), which irreversibly binds to AP sites in damaged DNA and blocks APE1 from binding, and thus interrupts BER (Liu and Gerson, 2004; Wilson and Simeonov, 2010). MX reacts with the aldehyde group within the AP site that results in the formation of an intermediate adduct refractory to APE1 lyase activity (Figure 1.6) (Rosa *et al.*, 1991; Liu and Gerson, 2004). AP site binding by methoxyamine leads to an accumulation of AP sites, increasing DNA damage as well as generation of cytotoxic single DNA strand breaks. Preclinical studies revealed that methoxyamine potentiates fludarabine cytotoxicity in HL60 AML cells, primary CLL leukaemia cells and HL-60 xenograft mouse model (Bulgar *et al.*, 2010). Currently, MX in combination with fludarabine is in phase I trials to treat patient with relapsed or refractory haematological malignancies such as chronic myeloid/lymphoid leukaemia and Hodgkin/non-Hodgkin lymphoma (clinicaltrials.gov). Methoxyamine is also in phase 1 trials in

combination with TMZ to treated patient with advanced solid tumours (clinicaltrials.gov). However, MX may have limited clinical utility because it is required in high concentrations, depending on cell type, in order to potentiate cytotoxicity when combined with alkylating agents.

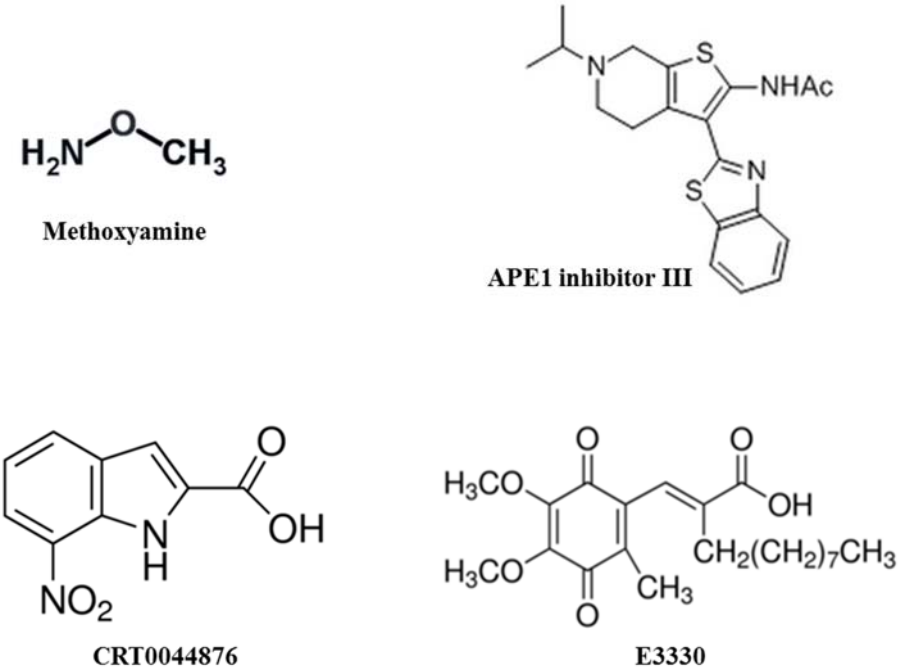


Figure 1.5: Chemical structure of common APE1 inhibitors

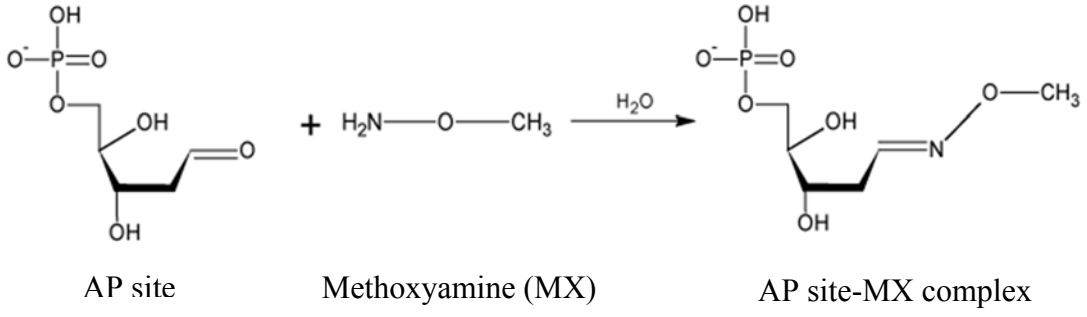


Figure 1.6: MX interaction with AP site (adapted from (Zhu *et al.*, 2012))

APE1 inhibitor III (APE1- III)

The development of APE1 inhibitors for use in the clinic is reliant on understanding the mechanism of action by which they operate (Luo *et al.*, 2008; Tell *et al.*, 2009). Initially, however, the identification of compounds with APE1 inhibitory activity requires the use of high-throughput screening approaches. First attempt to identify APE1 inhibitors was developed and adapted by Madhusudan and colleagues, 2005. This technique is a fluorescence-based assay using purified APE1 and an artificial abasic DNA substrate; tetrahydrofuran (Madhusudan *et al.*, 2005). However, this approach was utilised to identify specific APE1 inhibitor in about 352,498 compounds, which resulted in the identification of a potential APE1 inhibitor III molecule (Rai *et al.*, 2012). APE1 inhibitor III (*N-(3-(benzo[d]thiazol-2-yl)-6-isopropyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridin-2-yl)acetamide*) (APE1-III) (Figure 1.5) and its analogue displayed low μM APE1 inhibition, which resulted in significant accumulation of AP sites in treated cells, and potentiation of a number of chemotherapeutic agents. This compound demonstrated a low inhibitory concentration 50% (IC_{50}) of 2 μM for the endonuclease function of APE1 in whole cell extract and recombinant protein (Rai et al, 2012), which was confirmed in an independent study (Poletto *et al.*, 2015). The pharmacokinetic features of the APE1-III and its analogue (compound 52) was promising with a good cytotoxicity profile. APE1-III crossed the blood-brain barrier more efficiently compared to compound 52, but achieved lower maximum plasma levels, and a shorter half-life time in plasma in a mouse model. However, there has been no further development of these compounds and they have not yet entered clinical trial.

MEF fibroblast cells either wild-type ($NPM1^{+/+}$) or null ($NPM1^{-/-}$) for nucleophosmin were treated with APE1-III inhibitor and MX as single agents, to determine the role of NPM1 expression in sensitivity to APE1 inhibition (Vascotto *et al.*, 2013). $NPM1^{-/-}$ cells were significantly more sensitive to both inhibitors, compared to $NPM1^{+/+}$ cells. Similarly, APE1-III demonstrated promising results in a synthetic lethality screen when used to treat PTEN deficient melanoma cell lines (Abbotts *et al.*, 2014). PTEN deficient cells were hypersensitive to APE1 inhibition using APE1-III and other APE1 inhibitors (Abbotts *et al.*, 2014). Moreover, APE1-III significantly increased the accumulation of uncleaved AP sites, γH2A foci phosphorylation, and induced apoptosis.

Other Inhibitors

CRT00044876 or 7-nitro-indol-2-carboxylic acid (NCA) (Figure 1.5) is a first generation APE1 endonuclease specific inhibitor. It was described by Madhusudan *et al.,* (2005) as an inhibitor of APE1 endonuclease activity and shown to potentiate the cytotoxicity of a number of DNA damaging agents including methyl methanesulfonate, temozolomide, H_2O_2 and Zeocin at a non-cytotoxic dose (200 μM), when administered as a single agent. Additionally, the authors reported that CRT0044876 did not alter the cytotoxicity of agents that induce DNA lesions not commonly repaired by BER, suggesting that CRT0044876 acts specifically through inhibition of APE1 endonuclease function. More recent studies demonstrate phosphorylation of H2AX and accumulation of AP sites following APE1 inhibition using CRT00044876 (Hong *et al.*, 2016). However, several studies failed to demonstrate APE1 inhibition using this compound, raising questions about the effectiveness of this compound (Fishel and Kelley, 2007; Simeonov *et al.*, 2009; Naidu *et al.*, 2010).

Lucanthone was first recognised as an anti-Schistosoma treatment and also as a topoisomerase II inhibitor (Fishel and Kelley, 2007). The molecular structure of luchanthone is more complex than MX. It has also been found to inhibit RNA synthesis as well as APE1 DNA endonuclease activity (Naidu *et al.*, 2011). Recent studies have demonstrated that lucanthone cleaves and degrades APE1 at low μM concentrations (50 – 100 μM) (Naidu *et al.*, 2011). Breast cancer cell lines treated with lucanthone exhibited significant AP site accumulation in a dose dependant manner, suggesting inhibition of the DNA repair function of APE1 (Mendez *et al.*, 2002). Another study found that lucanthone inhibits DNA repair activity and potentiates the cytotoxicity of alkylating agents TMZ and MMS in the MDA-MB231 breast cancer cell line (Luo and Kelley, 2004). However, it remains to be determined whether these observations are because of anti-APE1 activity or anti-topoisomerase activity. Therefore, more studies are required in order to elucidate the exact mechanism by which lucanthone acts on cancer cells.

There is considerable effort being applied to the search for APE1 inhibitors capable of targeting specific APE1 functions without off-target effects at low μM concentrations. Recent reports have revealed a new novel APE1 inhibitor, ML199, which was found to specifically inhibit APE1 in the low μM dose range (Raia *et al.*, 2013). This agent also potentiates MMS at non-toxic doses in HeLa cells (Raia *et al.*, 2013). However, there are other APE1 inhibitors reported in the literature, including reactive blue 2, myricetin, arylstibonic acid, aurintricarboxylic acid and other molecules (Wilson and Simeonov, 2010; Mohammed *et al.*,

2011; Al-Safi *et al.*, 2012; Srinivasan *et al.*, 2012; Feng *et al.*, 2015). Further work is required to characterise these agents and the mechanisms of their interaction with APE1.

1.2.1.4.2. Redox function Inhibitors

E3330

APE1 redox inhibitors are limited, and are available for preclinical use only. Efforts aimed at the identification of redox inhibitors are restricted by the lack of appropriate high throughput screening approaches to identify molecules that inhibit the redox function of APE1 (Li and Wilson, 2014). The first APE1 redox inhibitor, E3330, was identified as an inhibitor for TNF-α secretion from monocytes and macrophages, and exhibited anti-inflammatory properties when used to treat hepatitis (Miyamoto *et al.*, 1992; Nagakawa *et al.*, 1992; Goto *et al.*, 1996).

E-3-[2-(5,6-dimethoxy-3-methyl-1,4-benzoquinonyl)]-2-nonylpropenoic acid (E3330) (Figure 1.5) is a potential specific APE1 redox activity inhibitor. It interferes with the interaction between APE1 and target transcription factors involved in cancer promotion, such as NF-κB, AP-1 (Fos/Jun), HIF-1α and other downstream transcription factors (Kelley *et al.*, 2012). E3330 also blocks retinal angiogenesis *in vivo* as well as functioning *in vitro* by blocking APE1 redox transcriptional activity (Jiang *et al.*, 2011). Additionally, E3330 has been found to inhibit macrophage-mediated inflammatory responses through inhibition of APE1, which consequently suppresses the transcriptional activity of NF-κB and AP-1 (Jedinak *et al.*, 2011). Moreover, E3330 demonstrated inhibition of STAT3 as well as APE1 transcriptional activity in pancreatic cancer cells which inhibited their proliferation and migration (Cardoso *et al.*, 2012). Recent studies have also revealed that high concentrations of E3330 (> 100 μM) also inhibit the DNA endonuclease activity of APE1 (Zhang *et al.*, 2013), suggesting that this agent is not a specific inhibitor of APE1 redox function. The same authors also derived a novel compound from this inhibitor by replacing the carboxyl group on E3330 with an amide group, resulting in a compound (E3330 amide) capable of specifically inhibiting APE1 redox function at lower concentration relative to E3330 (Zhang *et al.*, 2013). Recently, several analogues of E3330 (RN8-51, RN10-52, and RN7-60) were identified using global mass spectrometric analysis with lower IC_{50} against ovarian cell lines relative to E3330.

1.2.1.4.3. Naturally occurring inhibitors

There are a number of naturally occurring compounds reported to inhibit APE1 redox function, including soy isoflavons, gossypol, resveratrol, curcumin and ascorbate (Raffoul *et al.*, 2012; Qian *et al.*, 2014). Soy isoflavons inhibit APE1 endonuclease and redox functions, and sensitise prostate cancer cells and non-small-cell lung cancer cells to radiation, in a dose and time dependant manner (Raffoul *et al.*, 2007; Singh-Gupta *et al.*, 2011). The mechanism by which soy isoflavons inhibit APE1 is still largely unknown, but it is thought that soy isoflavons prevent APE1 from reducing NF-κB, leading to increased cellular sensitivity to radiation (Kelley *et al.*, 2012).

Gossypol is a naturally occurring BCL-2 homology 3–mimetic compound extracted from cotton seeds and tropical trees. It inhibits the BCL-2 (B-cell lymphoma 2) anti-apoptotic protein and interacts with caspases released from mitochondria during apoptosis induction (Qian *et al.*, 2014). The inhibitory effect of gossypol on APE1 was identified following reports that BCL-2 directly interacts with APE1 through BCL-2 homology 3 domain (Zhao *et al.*, 2008). Gossypol has been shown to inhibit APE1 redox and endonuclease function, and demonstrated antitumor activity in both *in vivo* and xenograft models (Qian *et al.*, 2014). Phase III clinical trials are now ongoing to determine if gossypol could improve docetaxel and cisplatin in patients with non–small cell lung carcinoma with high levels of APE1 expression (clinicaltrials.gov website).

Resveratrol is another potential APE1 inhibitor, found in grapes, mulberries and other plants (Raffoul *et al.*, 2012). Several epidemiology studies have reported that consumption of this agent protects against a number of cancers, including skin cancer, liver cancer, prostate cancer and others (Raffoul *et al.*, 2012). It has been shown that resveratrol inhibits APE1 binding to AP1 in melanoma cells which sensitised to the alkylating agent dacarbazine. However, a recent study revealed that resveratrol protected rat brain neural cells from inflammation by increasing APE1 expression (Zaky *et al.*, 2013). One explanation of this finding is that neural cells recognise APE1 inhibition caused by this compound and compensate for the deficiency by increasing transcription levels to maintain APE1 protein level.

1.2.1.5. APE1 as a therapeutic target in AML

APE1 is the key protein in the BER pathway and down-regulation/inhibition of this protein reduces DNA damage repair capacity and potentiate the cytotoxicity of alkylating agents. Overexpression of APE1 has also been reported in several malignancies, such as multiple myeloma, ovarian cancer, osteosarcoma and AML (Wang *et al.*, 2004; Casorelli *et al.*, 2006;

Abbotts and Madhusudan, 2010; Al-Attar *et al.*, 2010; Xie *et al.*, 2010). Although APE1 targeting has been investigated extensively in other malignancies, the value of this approach has not yet been well established for AML. Nevertheless, the high levels of ROS reported in AML, and particularly poor prognosis AML, suggest that targeting APE1 could be of therapeutic value, owing to the involvement of APE1 in DNA repair and maintenance of genome stability. Consistent with this model, targeting the redox function of APE1 could also prove of therapeutic value in AML maintaining a balanced redox system, which is important for cellular homeostasis.

It is becoming clear that epigenetic changes and chromosomal translocations in AML activate several signalling pathway, such as STAT3, PI3K and FLT3, which lead to increase ROS production and induce DNA damage response. Thus, activation of these pathways can induce a mutator phenotype leading to the acquisition of further mutations that can drive disease progression. ROS is normally generated in haematopoietic stem cell (HSC), predominantly as superoxide, to regulate its quiescence state (Hole *et al.*, 2011). The level of ROS production and elimination is tightly regulated via complex pathways including oxidation/reduction reactions and enzymatic activities (Hole *et al.*, 2011). Nevertheless, evidence of overproduction of ROS has been demonstrated in several pathological and cancer conditions such as atherosclerosis, rheumatoid arthritis, diabetes and leukaemia (Sallmyr *et al.*, 2008b; Uttara *et al.*, 2009).

Focusing on AML, increased cellular stress caused by ROS production leads to the induction of oxidative DNA damage, double strand breaks, and triggering DNA repair. Any defect in the DNA repair pathways, particularly the BER and NHEJ pathways, leads to increased genomic instability and chromosomal translocations/deletions (Rassool *et al.*, 2007; Sallmyr *et al.*, 2008b; Esposito and So, 2014). Furthermore, ROS can modulate BER directly and indirectly, through oxidation/reduction of BER enzymes and through transcriptional dysregulation of genes that encode BER enzymes, respectively (Luo *et al.*, 2010). This evidence suggests that targeting ROS and/or its regulatory mechanisms might be of therapeutic value in AML.

This led to hypothesis that APE1 would be a potential target in AML treatment, owing to its function to maintain genome stability through DNA repair and redox regulation. This hypothesis is founded on two critical observations (Figure 1.7). AML cells rely on APE1 DNA repair function, which repairs DNA damage caused by oxidative stress and chemotherapeutic agents. Therefore, APE1 inhibition can lead to accumulation of DNA damage and may trigger apoptosis to cancer cells. Secondly, APE1 inhibition would disrupt

transcription factors by which AML cells coordinate their survival prior to and following treatment.

Some studies have begun to address the potential of APE1 targeting in human leukaemia. The use of methoxyamine in conjunction with fludarabine or manumycin was found to enhance cytotoxicity of these drugs in leukaemia cells (She *et al.*, 2005; Bulgar *et al.*, 2010). The authors demonstrated that methoxyamine inhibits APE1 endonuclease repair function and enhances the cytotoxicity of fludarabine by three fold compared to fludarabine alone in HL60 AML cells and primary CLL leukaemia cells *in vitro* and in a mouse xenograft model (Bulgar *et al.*, 2010). Furthermore, disruption of BER by methoxyamine enhances manumycin-induced apoptosis in HL60 and U937 AML cell lines (She *et al.*, 2005). Most recent studies have revealed that histone deacetylase inhibitors downregulate APE1. For example, vorinostat (a histone deacetylase inhibitor) downregulates APE1 expression in kasumi-1 AML cells and the authors concluded that this suggests a new strategy for APE1 inhibition (Petruccelli *et al.*, 2013). This inhibition might be explained by another study which showed that another histone deacetylase inhibitor (trichostatin) induces APE1 extracellular secretion from HEK293 cells (Choi *et al.*, 2013), which could lead to decreased intracellular APE1 level. Although these studies clearly demonstrate the potential of APE1 inhibition as a therapeutic strategy in AML, more work is required to elucidate the role of this molecule in AML and further validate it as a therapeutic target.

There are limited data regarding the use of the redox function inhibitor, such as E3330, in the context of leukaemia. Fishel et al (2010) investigated the effects of E3330 on retinoic acid-induced differentiation in HL60 and PLB acute myeloid leukaemia cells. They found that combining E3330 with retinoic acid inhibited NF-κB reduction by APE1 and blocked DNA binding of APE1 to retinoic acid receptors, leading to increased apoptosis and growth arrest. They also found that single agent E3330 induced growth inhibition of HL60. However, further work is required to establish the inhibitory effects of this agent on leukaemia cells.

However, it is essential to consider the importance of APE1 for normal cellular functions, and targeting this protein may trigger backup mechanisms to compensate for its deficiency and maintain cell viability. For example, targeting APE1 using E3330 redox function conferred protection phenotype driven by upregulation of NRF2 expression, which paly essential role in cellular protection against high oxidative stress (Fishel *et al.*, 2015).

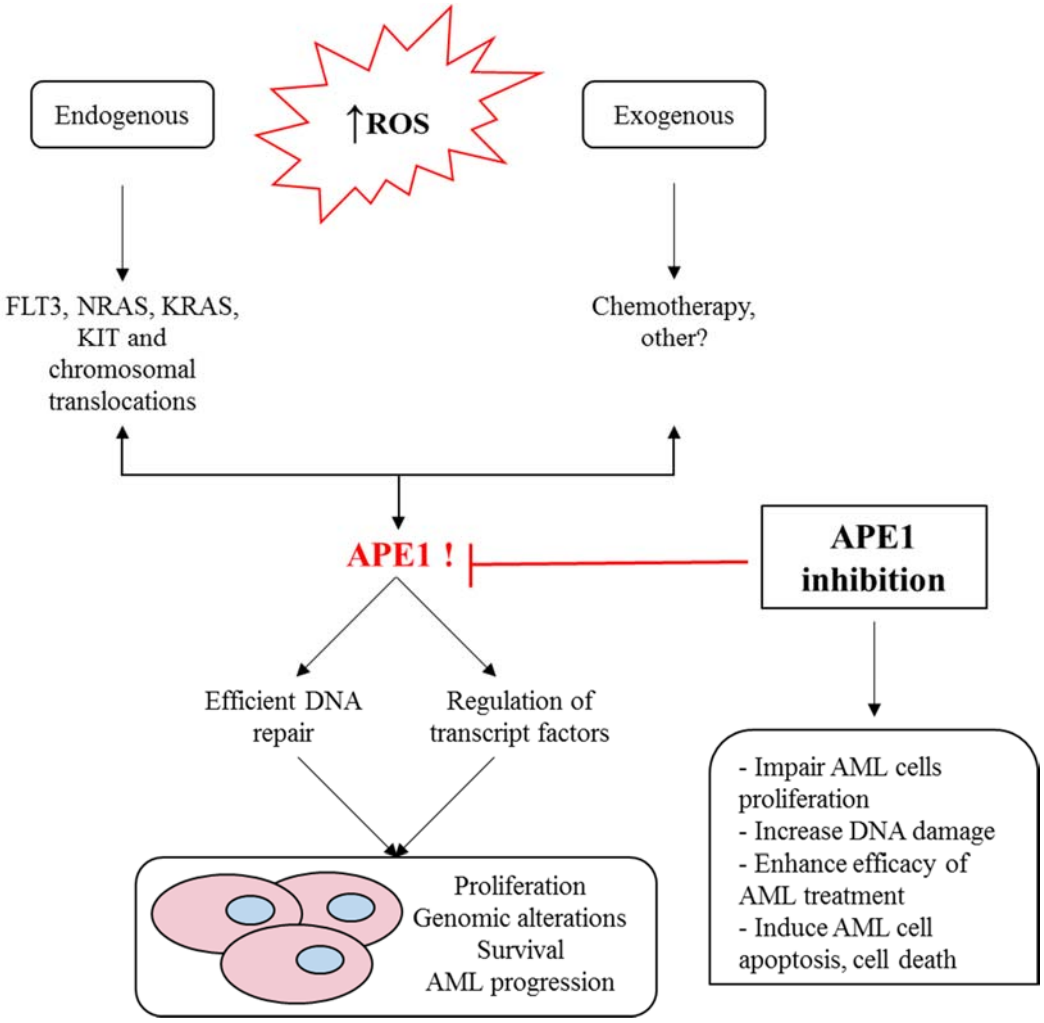


Figure 1.7: Proposed model of APE1 involvement in AML.

AML cells are characterised by elevated reactive oxygen species (ROS) that modulate their differentiation, proliferation and self-renewal advantages. This occur via modulation oncogenes, transcription factors redox status an enzymes. APE1 may play an essential role in leukaemogenesis through preserving genome stability under oxidative stress and via redox regulation of transcript factors that enhance leukaemia progression. Therefore, targeting APE1 functions could increase the efficacy of AML treatment by increasing DNA damage, reducing cells proliferation and induction of apoptosis.

1.2.1.6.Synthetic lethality studies

Cancer cells are dependent on multiple DNA repair pathways to maintain their survival and any deficiency in one repair pathway can trigger activation of alternative pathways to compensate for this deficiency and maintain cell survival. However, targeting activated alternative DNA repair pathways can lead to inhibition of cancer cells viability and sensitises them to apoptosis; this is what referred to synthetic lethality (Figure 1.8A). The concept of synthetic lethality in targeting DNA repair pathways was exploited following successful sensitisation of breast and ovarian cancers with mutations in *BRCA1* or *BRCA2* by inhibition of PARP-1. In this model, homologous recombination DNA repair is compromised in some breast and ovarian cancers due to mutation in *BRCA1* or *BRCA2*. BRCA-deficient cells are dependent on PARP-1 which coordinates single strand break repair operated by BER pathway (Shaheen *et al.*, 2011; Curtin and Szabo, 2013). PARP-1 inhibition selectively induces cell killing in BRCA-deficient cells through accumulation of double strand breaks resulting from unrepaired single strand breaks, which consequently causes collapse of replication forks during DNA synthesis (Figure 1.8B).

The promising results with PARP inhibition in ovarian and breast cancer cells harbouring BRCA1/2 mutations led to attempts to exploit this approach in other cancers with defective DNA repair. APE1 is absolutely essential in BER and likely to be promising alternative target for synthetic lethality in cancer. APE1 inhibition was synthetic lethal in cancer cells harbouring mutation/deficiency in BRCA, ATM (ataxia telangiectasia mutated) and PTEN (phosphatase and tensin homolog) (Sultana *et al.*, 2012; Abbotts *et al.*, 2014). BRCA/ATM-deficient Chinese hamster cell lines were more susceptible to APE1 inhibition compared to BRCA/ATM proficient cells (Sultana *et al.*, 2012). Notably, APE1 inhibition led to significant accumulation of AP sites and double strand breaks, and induced cell cycle arrest at the G2/M phase. This evidence indicates that APE1 inhibition is responsible for synthetic lethality, similar to PARP inhibition, in a BRCA/ATM deficient background. However, further characterisation of APE1 synthetic lethality in other cancer models is required to confirm these findings.

The same research team have conducted further investigation to explore the potential of APE1 synthetic lethality in different cancer models (Abbotts *et al.*, 2014). Firstly, it was noted that melanoma patients showing low PTEN and high APE1 mRNA expression have poor overall survival and relapse free survival. This led to questioning the relationship between PTEN and APE1. PTEN is transcriptionally regulated by APE1 redox function via EGR-1, and is involved in maintenance of genomic stability through regulation of RAD51 (Shen *et al.*,

2007; Fantini *et al.*, 2008). In addition, PTEN is important negative regulator of anti-apoptotic PI3K/AKT pathway. This study illustrated the utility of synthetic lethality approaches in melanoma treatment through inhibition of APE1, which may also apply in other cancer models.

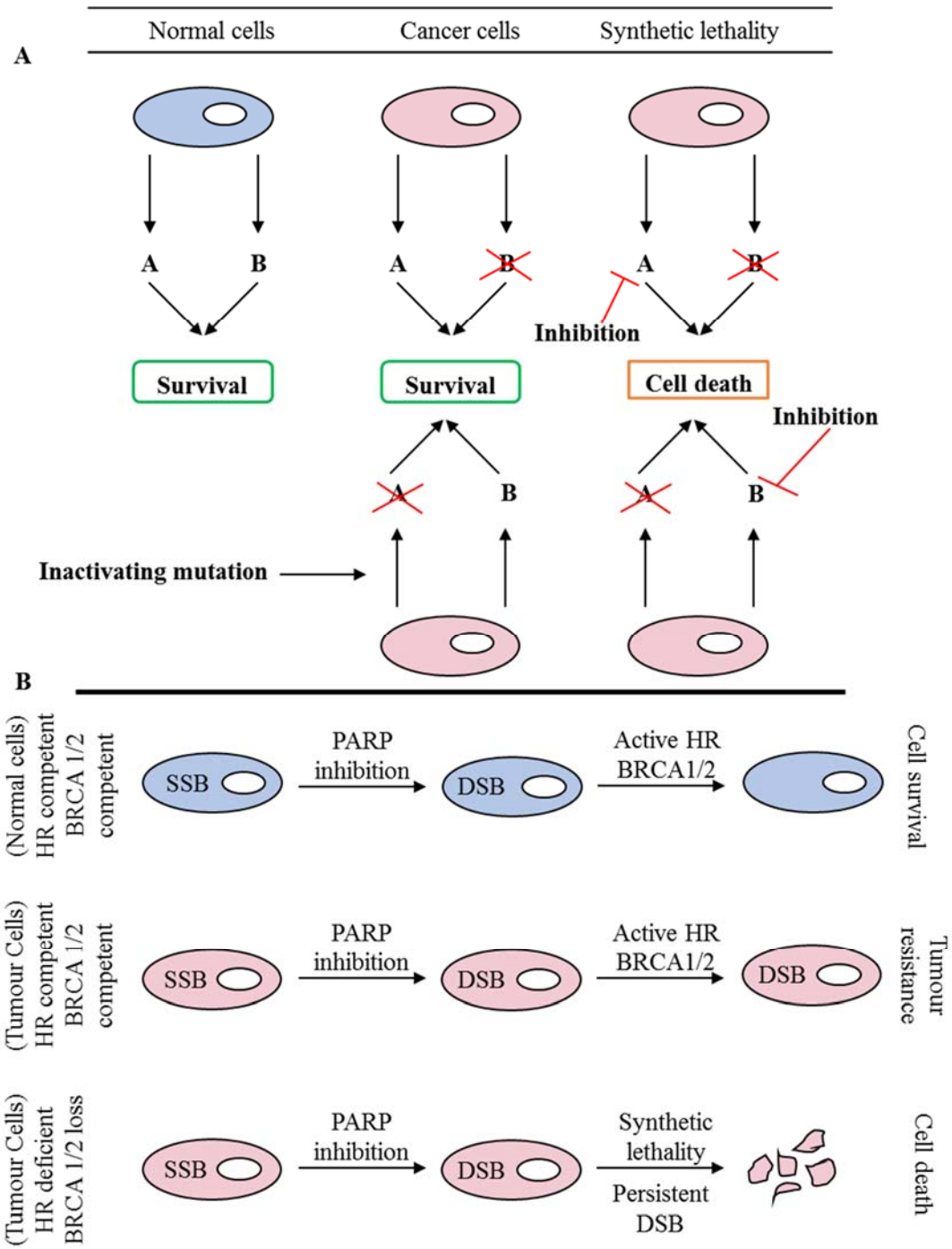


Figure 1.8: Schematic representation of synthetic lethality principle in cancer.

(A) Normal cells rely on multiple mechanisms/pathways (gene A and gene B) to maintain cells survival and preserve genome stability. In cancer cells, one of the pathways is inactive due to mutation (either gene A or gene B) and cells rely on the alternative pathway to support their survival. Exploiting this advantage in cancer cells by inhibition of the activated pathway can selectively drive cancer cells to apoptosis. **(B)** PARP-1 inhibition selectively induces cell killing in breast and ovarian cancer cells with BRCA1/2 germline mutation through accumulation of double strand breaks resulting from unrepaired single strand breaks. SSB; single strand breaks, DSB; double strand breaks.

1.2.2. Targeting 8-oxoguanine DNA glycosylase (OGG1) as a therapeutic target in AML

1.2.2.1. OGG1 overview

Targeting other BER components were explored for therapeutic purposes, specifically DNA glycosylases (Speina *et al.*, 2005; Huang *et al.*, 2009; Jacobs *et al.*, 2013; Donley *et al.*, 2015). DNA glycosylases play essential role in early steps in BER by excising wide range of DNA lesions. OGG1 is a critical DNA glycosylase involved in the BER pathway (Figure 1.4– long patch pathway) where it catalyses removal of 8-hydroxyguanine (8-OHG), which is a pro-mutagenic damaged DNA base induced by oxidative stress. Unrepaired 8-OHG can lead to transversion of G:C to T:A by DNA polymerases during DNA replication (Boiteux and Radicella, 2000). OGG1 also catalyses removal of formamidopyrimidine DNA adducts generated following exposure to ionising radiation or free radicals (Hu *et al.*, 2005a). Therefore, OGG1 is involved in preserving genome integrity and supressing tumorigenesis. Human *OGG1* gene is located on chromosome 3p25-26, which is frequently lost or deleted in several tumours including lung, breast, colon, prostate tumours; that suggest loss of OGG1 function and possible contribution to cancer progression (Boiteux and Radicella, 2000; Hardie *et al.*, 2000).

There are two major isoforms of OGG1 mRNA splice variants, including type 1 and type 2, based on their last exons (Boiteux and Radicella, 2000). OGG1 type 1 with exon 7 (variants 1a and 1b), and OGG1 type 2 with exon 8 (variants 2a to 2e). Variants 1a and 2a are predominant in human cells and encode OGG1 1a and OGG1 2a proteins. Both OGG1 protein variants share the same N terminal structure of the protein, but however, OGG1 1a protein has a unique nuclear localisation signal on its C terminal structure, hence actively localised to the nucleus (Nishioka *et al.*, 1999; Boiteux and Radicella, 2000). In contrast, OGG1 2a has mitochondrial localisation signal on the C terminal of the protein and exclusively localised to mitochondria (Boiteux and Radicella, 2000).

OGG1 is bifunctional glycosylase enzyme; meaning that it excises specific damaged DNA base (referred as mono-functional activity) and also has the ability to create an incision 3' to the AP site on the phosphodiester backbone of the DNA. OGG1 has AP lyase activity on 8-oxoG:C and AP:C DNA (Bjoras *et al.*, 1997). However, an AP site is created in the DNA backbone following removal of the damaged base. The AP site is either recognised and processed by the endonuclease function of APE1 to generate 5'-dRP and 3'-OH, or processed by the AP lyase of OGG1. AP lyase activity creates a polymerase blocking 3'-phospho-α-β-unsaturated aldehyde at and a 5' phosphate (Hill *et al.*, 2001). The resultant product processed by the 3'-phosphodiesterase activity of APE1, followed by engagement of polymerase β to

incorporate the correct base occurs via the short patch BER pathway. The AP lyase activity is regulated and enhanced by APE1 (Saitoh *et al.*, 2001; Sidorenko *et al.*, 2007).

Since BER consists of two distinct sub-pathways; short patch and long patch pathways (section 1.2.1.1), it is not yet known how or whether OGG1 activity influences the pathway by which BER is executed. However, XRCC1 is thought to modulate OGG1 activity by influencing it to perform as a mono-functional glycosylase then stabilising it following removal of damaged bases at AP sites until recruitment of APE1 (Melissa *et al.*, 2013).

1.2.2.2. OGG1 polymorphism

Single nucleotide polymorphisms and mutations in *OGG1* have been reported in a variety of cancer cases and linked to increased cancer risk, although their role has not yet been fully elucidated. The S326C variant (ref SNP ID: rs1052134) is one of the most commonly studied polymorphisms in human OGG1. It is caused by transversion of cytosine to guanine at nucleotide 1245, which results in substitution of serine with cysteine at codon 326 located in the C terminal domain of the protein (Simonelli *et al.*, 2013). This variant has been linked to impaired OGG1 DNA repair activity (Kershaw and Hodges, 2012).

A reported point mutation in *OGG1* leads to functional loss of enzymatic activity in the KG-1 AML cell line (Hyun *et al.*, 2000). This mutation, encoded by a CGA to CAA base mutation, leads to substitution of arginine with glutamine at position 229. More recently, a polymorphism was reported in KG-1 which results from substitution of arginine with glutamine at position 229 (R229Q) (ref SNP ID: rs1805373) (Hill and Evans, 2007). Earlier studies reported that KG-1 AML cells harbouring Arg229Glu variant were more sensitive to radiation due to reduced OGG1 activity, accumulation of 8-oxoG lesions and activation of apoptosis (Hyun *et al.*, 2002). However, it is important to highlight that possibly multiple factors (many of which are cancer-type dependent) can influence the sensitivity of cells to either DNA damaging agents or ionising radiation.

Reports of statistical association between SNPs in *OGG1* and leukaemia risk are inconsistent and inconclusive. For example, a study was performed on 99 AML patients to evaluate the influence of polymorphic variants in BER genes, found that *OGG1* S326C variant had no significant correlation with cytogenetic group (Saitoh *et al.*, 2013), although this study is statistically underpowered. Another study demonstrated that the *OGG1* S326C variant may contribute to susceptibility to paediatric acute lymphocytic leukaemia (ALL) (Stanczyk *et al.*,

2011). Presence of this particular variant in advanced myelodysplastic syndrome (MDS) was thought to contribute accumulation of 8-OxoG lesions and disease development (Jankowska *et al.*, 2008).

1.2.2.3. Role of OGG1 in leukaemia

OGG1 expression is constitutively downregulated by the AML1-ETO fusion oncoprotein encoded by the t(8;21) chromosomal translocation in haematopoietic cells in AML patients (Liddiard *et al.*, 2010; Forster *et al.*, 2016). OGG1 expression down regulation in this AML sub-group may contribute to leukaemogenesis via reduction of DNA repair capacity and acquisition of cooperating mutations (Liddiard *et al.*, 2010; Forster *et al.*, 2016). A recent study confirmed that AML1-ETO fusion oncoprotein binds to the *OGG1* gene promoter and negatively regulates transcription (Forster *et al.*, 2016). This, leads to loss of OGG1 expression and the acquisition of a mutator phenotype via the accumulation of G:C to T:A transversion mutation.

Gene expression data from 174 patients representing the full range of FAB AML groups (except M3) recruited to MRC AML trials 10-15 was analysed to explore the prognostic value of OGG1 expression in AML (Liddiard *et al.*, 2010). This study found that OGG1 was downregulated in AML patients with core binding factor (CBF) mutations, including inv(16) and t(8;21). Furthermore, high OGG1 expression was correlated with adverse cytogenetic patients group, who had significantly shorter overall survival with a higher risk of relapse. This study highlighted the importance of OGG1 as a valuable prognostic factor in a particular subset of AML patient, who may fail to respond to conventional chemotherapy. Hence, because high expression of OGG1 has prognostic value, it is possible that targeting OGG1 could have clinical utility.

1.2.2.4. OGG1 inhibitors

Despite the fact that OGG1 is an essential enzyme for the maintenance of genome stability, and its dysregulation is associated with a number of cancers, there are few reports about specific small molecules inhibitors. Initial attempts to develop inhibitors to glycosylases involved in BER pathway, including OGG1, NEIL1 and NTH1, were disappointing (Jacobs *et al.*, 2013). A high throughput screen that included 400,000 molecular from the Molecular Libraries Small Molecule Repository identified 4 potential purine analogues that exhibited

anti-glycosylases activity toward NEIL1, OGG1, and NTH1 with relatively equivalent potencies (Jacobs *et al.*, 2013). However, the identified molecules were unselective and inhibit multiple DNA glycosylases with equivalent potencies. This is because several DNA lesions can be excised by more than one DNA glycosylase. Such redundancy may hamper efforts to find clinically efficacious inhibitors.

Donley and colleagues recently identified 5 small hydrazide molecules that exhibit anti-OGG1 activity. However, this study did not characterise the exact mechanism by which OGG1 glycosylase/lyase activity is inhibited, and this need further investigation. Nevertheless, the identified molecules did not show any inhibitory effect on OGG1 substrate interaction and had limited reactivity with DNA. The prevailing evidence suggests that these molecules function through inhibition of Schiff base formation during OGG1 catalysis (Donley *et al.*, 2015). Schiff base is an important intermediate, and transient complex formed between glycosylases and AP site following removal of damaged base, and function as stabiliser for the enzyme until APE1 recruitment (Hill *et al.*, 2001).

1.3. Hypothesis and Aims

1.3.2. Hypothesis

Inhibition of the repair and/or redox functions of APE1 or the glycosylase activity of OGG1 will sensitise acute myeloid leukaemia cells to standard induction chemotherapy, improving the efficacy of these agents.

1.3.3. Aims

1. Generate AML cell lines with stable expression of shRNA constructs specifically targeting APE1/OGG1 using lentiviral transduction system.
2. Determine the effect of APE1/OGG1 shRNA targeting on cell phenotype, including growth characteristics, clonogenicity and cell cycle.
3. Determine the effects of APE1/OGG1 shRNA targeting on cellular sensitivity to standard induction chemotherapy used in AML, including temozolomide daunorubicin, cytarabine, etoposide, clofarabine and fludarabine.
4. Determine the effect of APE1 small molecule inhibitors on AML cell phenotype as single agents, including growth characteristics, clonogenicity, cell cycle, AP site accumulation and gene expression.
5. Determine the effect of APE1 inhibition using small molecule inhibitors on cellular sensitivity to standard induction chemotherapy used in AML, including temozolomide, daunorubicin, cytarabine, etoposide, clofarabine and fludarabine.

Chapter 2. Materials and Methods

2.1. AML cell lines

All acute myeloid leukaemia (AML) cell lines used in this work are detailed in

Table **2.1**, along with their corresponding media and morphology. All AML cell lines used in this project were originally purchase from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, (DSMZ, Germany) and kept in liquid nitrogen. Specific features of each cell line are described in detail in the relevant results chapters.

2.2. Chemicals and Reagents

All chemical and reagents used were Analar grade and were purchased from Sigma Aldrich Co LTD (Dorset – UK) unless otherwise stated. Phosphate buffered saline (PBS) was prepared from PBS tablets (Life technologies – Paisley – UK). Each tablet was dissolved in 500 ml of deionised distilled water and autoclaved prior to use. All other chemicals and reagents prepared for use in specific experiments are described in this chapter in relevant sections below.

2.3. General Cell Culture Methods

2.3.1. Routine Cell Culture

Fetal bovine serum (FBS) supplement for cell culture media was purchased from Gibco, Thermo Fisher scientific. Tissue culture RPMI media (Roswell Park Memorial Institute 1640) and reagents were purchased from Sigma Aldrich Co Ltd (Dorset – UK). Tissue culture plasticware was from Corning and Costar (VWR International Ltd., Leicestershire, UK). All tissue culture work was performed in a class II microbiological safety cabinet (BIOMAT-2, Medical Air Technology Ltd., Oldham, UK). All cell lines were routinely maintained in suspension in 10 ml of RPMI media supplemented with 10-20 % (V/V) of FBS, 50 µg/ml Penicillin/streptomycin in T25 sterile tissue culture flasks. Cultures were incubated at 37 °C in a humidified 5% CO_2 incubator (Heraeus Equipment Ltd., Essex, UK).

Cell line	Description	TP53 and MGMT status
HL-60	Acute Promyelocytic leukaemia cells (M2 FAB classification).	- Proficient MGMT gene expression * - TP53 gene deletion (Wolf and Rotter, 1985)
U937	Promonocytic leukaemia M4	- Absent MGMT gene expression * - TP53 deletion (46 base deletion ending exactly at the 3' end of exon 5) (Sugimoto et al., 1992)
THP-1	AML M5 with t(9;11)(p21;q23) and MLL-AF9 fusion gene.	- TP53 deletion (Sugimoto et al., 1992)
NB4	AML M3 with t(15;17)(q22;q11) and PML-RARα fusion gene	- Proficient *MGMT* gene expression * - *TP53* missense mutation (Kojima et al., 2005)
MV4-11	AML M5 with t(4;11)(q21;q23) and MLL-AF4 fusion gene	- *TP53* point mutations at codon 344 exon 9 (Fleckenstein et al., 2002)
OCI-AML2	AML M4	- Proficient MGMT gene expression *
OCI-AML3	AML M4 with NPM1 mutation and hemizygous for RB1.	- Proficient MGMT gene expression * - *TP53* wildtype (Kojima et al., 2005)
Kasumi1	AML with t(8;21) and AML1-ETO fusion protein	

Table 2.1: List of AML cell used in this project.

* Gene expression was determined by RT² PCR Array – Personal communication – Professor James Allan – Newcastle University.

2.3.2. Cell Counting and determination of cell density

All cell counting was performed using either a Neubauer haemocytometer (VWR international Ltd) and/or a TC20 automated cell counter (Bio-Rad Laboratories Ltd., Hertfordshire, UK). TC20 automated cell counter results were validated by comparing cell counts using a Neubauer haemocytometer prior to routine use. For quality purposes, a verification slide system was used to validate the cell counter every time the cell counter was turned on and prior to taking results. The verification slide system contains two sides (A and B) with a well-defined number of micro wells that could be recognised and counted by the cell counter. To perform a cell count, an aliquot of cell suspension was mixed with an equal volume of 0.4% Trypan blue solution (Sigma Aldrich Co Ltd. Dorset – UK) and 10 µl of the mixture was loaded onto a haemocytometer or a TC20 cell counter counting slide.

2.3.3. Cryopreservation of Cells in Liquid Nitrogen

After determination of cell count, an appropriate volume of cell suspension containing $5x10^6$ cells was dispensed into a sterile BD Falcon tube and centrifuged at 300 g for 5 minutes at room temperature. The supernatant was discarded and cells pellets were resuspended in 1 ml of freezing media consisting of FBS supplemented with 10% v/v Dimethyl sulfoxide (DMSO). Cells suspensions were transferred into sterile cell culture cryogenic tubes (Thermo scientific) and frozen slowly in Mr. Frosty™ Freezing Container (Thermo scientific) in a -80 °C freezer, then transferred into liquid nitrogen for long term storage.

2.3.4. Resuscitation of Frozen Cells

Deeply frozen cryogenic tubes were brought out of liquid nitrogen and thawed quickly by incubation in warm water. Cells suspensions were transferred into sterile Falcon tubes containing 5 ml of pre-warmed culture media and centrifuged at 300 g for 5 minutes. The supernatant was completely removed and cells pellets were re-suspended in 5 ml of appropriate cell culture media, after which cell suspensions were transferred to T25 sterile culture flask and incubated at 37 °C in 5% CO_2. Cell cultures were monitored daily for growth and were not used in any experiment until cells achieved exponential growth kinetics.

2.4. Gene Expression Analysis Using Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

2.4.1. Preparation of Cell Pellets

Five to seven million cells were dispensed into a sterile Falcon tube and centrifuged at room temperature for 5 minutes at 300 g. The supernatant was discarded and the cell pellet was washed in 5 ml of cold PBS. Cells were centrifuged at 300 g at room temperature for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml of cold PBS and transferred to a sterile 1.5 ml eppendorf tube and centrifuged at 300 g at room temperature for 5 minutes. Supernatant was discarded and cell pellets were kept at -80 °C until required.

2.4.2. RNA Extraction and Quantitation

Total RNA was extracted from frozen cell pellets consisting of $7x10^6$ cells using the RNeasy Mini Kit (Qiagen, Crawley, UK). Cells pellets were thawed on ice and RNA extracted following protocols provided by the manufacturer. Briefly, cells were lysed in lysis buffer supplemented by the manufacturer and homogenised using a 21 gauge needle. Lysed cells were transferred into an RNeasy mini spin column containing a silica gel membrane where RNA binds to the membrane and contaminants are removed using washing buffers provided in the kit. RNA was eluted in 50 µl nuclease-free deionised water and quantified using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, DE, USA). NanoDrop® measures the absorbance at 260 nm, and performs the necessary calculations according to the Beer Lambert Law to provide an estimate of the RNA concentration. RNA samples were adjusted to a concentration of 200 ng/µl in 15 µl of nuclease-free distilled H_2O and used in the next step of the reaction (section 2.4.3). Any remaining RNA was stored at -80 °C.

2.4.3. Reverse Transcription of RNA into complementary DNA (cDNA)

A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) was used to prepare cDNA. This kit utilises the random primer method for initiating reverse transcriptase mediated cDNA synthesis of RNA molecules present in the sample. Reverse transcriptase master mix was prepared according to manufacturer's protocol as shown in Table 2.2 and 10 µl of the prepared master mix was dispensed into appropriate number of 0.2 ml MicroAmp® Reaction Tubes (Applied Biosystems). In each well, 10 µl of 200 ng/µl total

RNA was added. A negative control was prepared using nuclease free water instead of RNA, to confirm that reagents were not contaminated. Reaction tubes were covered with a thick adhesive film to prevent overheating and evaporation of the reaction mixture. A GeneAmp® PCR System 9700 (Applied Biosystems) was used to perform single cycle cDNA amplification as follows:

Step 1	25°C	10 min
Step 2	37°C	120 min
Step 3	85°C	5 seconds
Step 4	4 °C	Hold

This reaction yield 100 ng/µl cDNA which was then diluted to 5 ng/µl using nuclease free distilled H_2O and stored at 4 °C until required.

Reagent name	**Required volume**
RT Buffer (10x)	2 µl
dNTP mix (100 mM)	0.8 µl
RT Random Primers (10x)	2 µl
MultiScribe® Reverse Transcriptase	1 µl
RNase inhibitor	1 µl
Nuclease-free H_2O	3.2 µl
Total volume per reaction	10 µl

Table 2.2: Components of reverse transcriptase master mix used for cDNA synthesis.

2.4.4. Real-Time PCR Setup

Inventoried and validated TaqMan® Gene Expression Assays for human APE1, OGG1 and β-ACTIN were purchased form Applied Biosystems. Each assay contains oligonucleotide probe with fluorophore (FAM = 6-carboxyfluorescein) covalently attached to the 5' end and a quencher (NFQ/MGB = non-fluorescent quencher/Minor groove binder) at the 3' end. Assays were received as 20x concentrated stock, which was aliquoted to minimize freeze-thaw cycles and stored at -20 °C.

An appropriate amount of TaqMan® Gene Expression Assay was mixed with TaqMan® Universal PCR Master Mix (Applied Biosystems), according to manufacturer protocols. Eleven microlitres of each master mix was dispensed into an appropriate number of wells of a 96-well Optical PCR Plate (Applied Biosystems). Nine microlitres of 5 ng/µl cDNA was added to each well. A negative control was prepared using the negative control prepared in the previous section (2.4.3) to ensure that reagents used are not contaminated. Q-PCR reactions for each gene for each cell line and controls were set-up in quadruplicate. PCR plates were sealed appropriately, mixed gently using a plate shaker and placed in a 7300 Real time PCR system (Applied Biosystems).

The RT-PCR system was programmed to cycle as follows:

- 50 °C 2 minutes
- 95 °C 10 minutes
- 95 °C 15 second
- 60 °C 1 minutes

} 40 cycles

2.4.5. Data Analysis

The $2^{-\Delta\Delta Ct}$ method was used to quantify relative gene expression as described in Livak and Schmittgen, 2001. Briefly, cycle threshold (Ct) values for each reaction were obtained following fluorescence detection by the RT-PCR system and saved in a file compatible with SDS version 1.4 (Applied Biosystems) software. Ct values refer to the number of cycles required for the fluorescent signal to exceed background level. Data were copied into an excel data sheet and all calculations after this step were performed Microsoft excel. The means of quadruplicate Ct values were calculated for each gene in each cell line and used for quantification of gene expression. *APE1* and *OGG1* gene expression in each cell line was normalised to the β-ACTIN housekeeping gene. Normalised expression levels were compared

to expression of *APE1* and *OGG1* in NB4 cell line. This result generated values representing fold change in expression for *APE1* and *OGG1*. Gene expression data are displayed on a bar chart showing the mean fold change in gene expression for each AML cell line relative to expression in NB4 cells, and error bars shown are the standard error of the mean. Two independent experiments using 2 independent RNA/cDNA samples were performed to generated gene expression data.

2.5. Generation of Stable APE1 Deficient AML Cell Lines Using Short Hairpin RNA (shRNA) Mediated Gene Knockdown

Stable APE1 and OGG1 deficient subclones of AML cell lines were generated using short hairpin RNA (shRNA) mediated gene knockdown. This method uses lentiviral particle to carry shRNA constructs (Figure 2.1) into cells for incorporation into the host genome. A permanent blockage of the expression of the target genes occurs through expression of integrated constructs by RNA polymerase III which result in constitutive production of a shRNA molecule. ShRNA molecules are cleaved by DICER to generate a small interfering RNA (siRNA), which then becomes integrated in the active RNA Interference Specificity Complex (RISC). Integrated siRNA guides RISC to bind and degrade mRNA of the target gene.

2.5.1. *shRNA constructs, Control and Reagents*

Verified and prepacked MISSION® shRNA lentiviral constructs targeting APE1 (Table 2.3), OGG1 (Table 2.4) and empty pLKO.1 plasmid vector (off target control) were purchased from Sigma-Aldrich, UK. All lentiviral particles were received as frozen stocks, and aliquoted to avoid freezing/thaw cycles that may reduce functional viral titre. Hexadimethrine bromide stock was prepared by dissolving 800 μg hexadimethrine bromide in 1 ml sterile distilled H_2O to yield 800 μg/ml stock. This stock solution was then sterilised by passage through a 0.2 μm filter (VWR International Ltd.) and stored at 4°C.

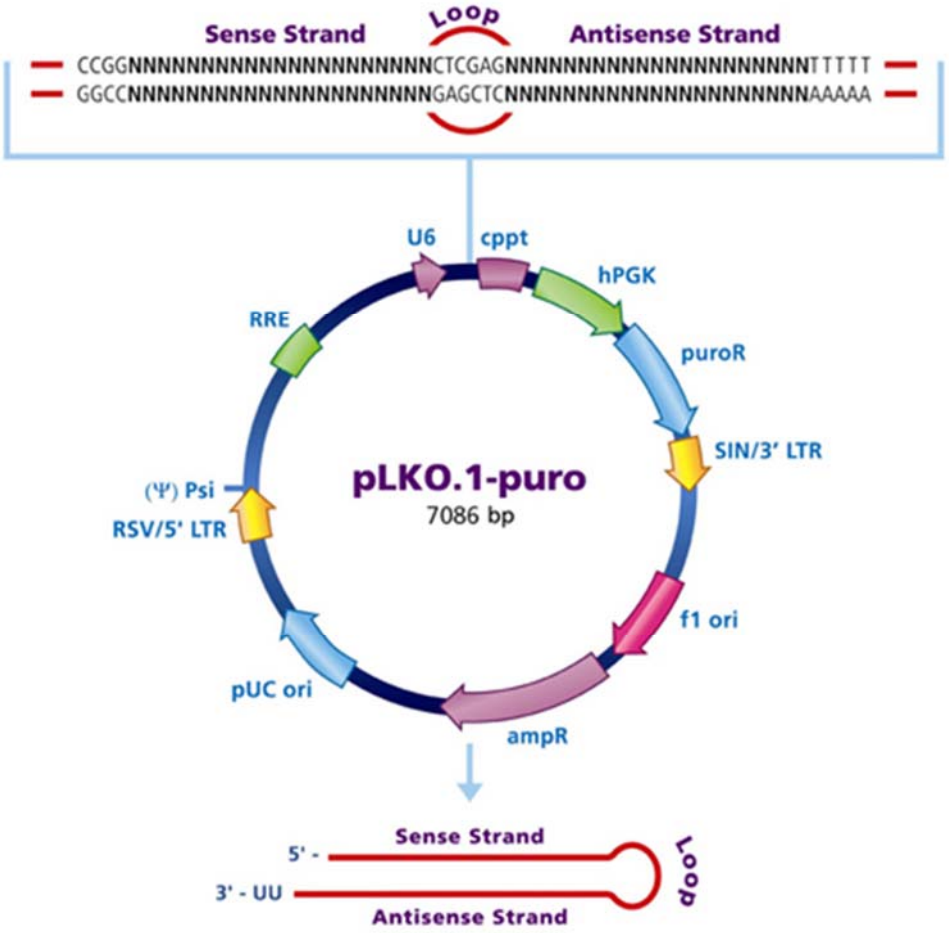


Figure 2.1: pLKO.1-puro Lentiviral TRC1.5 Vector Map

PLKO.1-puro vector allow for transduction of the shRNA as packaged in lentiviral particle. The vector length is 7,086 bp including shRNA insert, and 7,052 bp without an shRNA insert. The pLKO.1 puro plasmid vectors carry a puromycin resistance (puroR) cassette which can be used as selection marker and to enable stable gene silencing. Figure adapted from www.sigmaaldrich.com

Cppt: Central polypurine tract
hPGK: Human phosphoglycerate kinase eukaryotic promoter
puroR: Puromycin resistance gene for mammalian selection
SIN/LTR: 3' self inactivating long terminal repeat
f1 ori: f1 origin of replication
ampR: Ampicillin resistance gene for bacterial selection
pUC ori: pUC origin of replication
5' LTR: 5' long terminal repeat
Psi: RNA packaging signal
RRE: Rev response element

Clone ID	Clone Name	Vector	Match Region	Target Sequence
TRCN0000007958 (C1)	NM_080649.1-1305s1c1	pLKO.1	3UTR	CAGAGAAATCTGCATTCTATT
TRCN0000007959 (C2)	NM_080649.1-609s1c1	pLKO.1	CDS	GCCTGGACTCTCTCATCAATA
TRCN0000007961 (C3)	NM_080649.1-497s1c1	pLKO.1	CDS	CCTGGATTAAGAAGAAAGGAT
TRCN0000011214 (C4)	NM_080649.1-740s1c1	pLKO.1	CDS	GCCGGGTGATTGTGGCTGAAT
TRCN0000342686 (C5)	NM_080649.1-609s21c1	pLKO_TRC005	CDS	GCCTGGACTCTCTCATCAATA

Table 2.3: list of shRNA construct used to knockdown APE1 in AML cells.

Clone ID	Clone Name	Vector	Match Region	Target Sequence
TRCN0000004913 (G1)	NM_002542.4-1555s1c1	pLKO.1	CDS	CGCAAGTACTTCCAGCTAGAT
TRCN0000004915 (G2)	NM_002542.4-1771s1c1	pLKO.1	CDS	CGGCTCATCCAGCTTGATGAT
TRCN0000004916 (G3)	NM_002542.4-1445s1c1	pLKO.1	CDS	CGGATCAAGTATGGACACTGA
TRCN0000314672 (G4)	NM_002542.5-1135s21c1	pLKO_TRC005	CDS	TGTGCCCGTGGATGTCCATAT
TRCN0000314740 (G5)	NM_002542.5-848s21c1	pLKO_TRC005	CDS	CGGCTCATCCAGCTTGATGAT

Table 2.4: list of shRNA construct used to knockdown OGG1 in AML cells.

2.5.2. *Assessment of Puromycin Sensitivity and Hexadimethrine Bromide*

Puromycin is an antibiotic that inhibits protein synthesis and is toxic to eukaryotic cells. The pLKO.1 puro/ pLKO_TRC005 plasmid vectors used in this project carry a puromycin resistance cassette which can be used as selection marker; cells successfully transduced with shRNA are insensitive to puromycin. Assessment of puromycin sensitivity was performed to determine the minimum concentration of puromycin required to kill all non-transduced cells.

Briefly, a cell suspension at a density of $2x10^4$ cells/ml and in a final volume of 2 ml in 6 wells plate was established. Cells were treated with escalating doses of puromycin including 0, 2, 4, 6, 8 and 10 µg/ml. Cells were monitored microscopically and counted every 24 hours for 3 days.

All cell lines tested in this project were dead after 3 days of treatment with 2 µg/ml puromycin. This was confirmed by morphological examination following microscopic examination and trypan blue exclusion. To confirm this as the optimal concentration, the assay was repeated but with lower puromycin; i.e. 0, 1 and 2 µg/ml puromycin. It was observed that 1 µg/ml puromycin reduced the viability of cells up to approximately 89%, but did not induce 100% cytotoxicity. Therefore, 2 µg/ml puromycin concentration was used as standard concentration for all shRNA selection media.

2.5.3. *Assessment of Hexadimethrine Bromide sensitivity*

Hexadimethrine bromide is used to enhance the transduction efficiency of mammalian cell lines. Some cells are sensitive to cytotoxicity induced by hexadimethrine bromide and it is important to examine this prior performing transduction. Sigma-Aldrich, the manufacturer of transduction shRNA constructs, recommended supplementing the transduction media with 8 µg/ml hexadimethrine bromide.

To assess the sensitivity of AML cells to hexadimethrine bromide, a cell suspension at a density of $2x10^4$ cells/ml and in a final volume of 2 ml in 6 wells plate was established. Cells were treated with 8 µg/ml hexadimethrine bromide. Untreated control were setup in each plate and plates were incubated at 37 °C / 5% CO_2 for 3 days.

After 3 days, cell growth and viability were assessed microscopically and by counting cells using a haemocytometer and trypan blue dye exclusion. If cell growth and viability were not affected by 8 µg/ml of hexadimethrine bromide compared to untreated control, this concentration of hexadimethrine bromide was used in transductions.

2.5.4. *Lentiviral Transduction*

Exponentially growing cells were counted using a TC20 cell counter and seeded at a final density of $5x10^4$ cell/ml in 10 ml of full media, supplemented with 8 µg/ml hexadimethrine bromide. A 1 ml aliquot of cell suspension was transferred into a sterile 15 ml Falcon tube and an appropriate volume of thawed shRNA lentiviral particles was added directly to the cell suspension. The required volume of lentiviral was calculated prior to use to give the required MOI as follows:

$$Volume\ required\ (ml) = \frac{required\ MOI\ x\ total\ number\ of\ cells}{viral\ titer\ (TU/ml)}$$

Viral titer is the concentration of viruses and expressed as titer unit/ml (TU/ml). Viral titer was supplied by the manufacturer for each lentiviral particles. MOI used = 2 in all shRNA knockdown experiments performed during this project, to minimise multiple integration of shRNA into the cell genome, and to reduce the probability of insertional mutagenesis.

After the addition of the shRNA lentiviral particles, cells were centrifuged for 30 minutes at 800 g at 32°C. Following centrifugation, the supernatant was carefully discarded and cells were resuspended in 2 ml of full media and transferred to a 6 well plate. Plates were incubated for 4 days in a humidified, 5% CO_2 incubator at 37°C. Parallel with this experiment, the same procedure was performed for controls, but without adding lentiviral particles.

2.5.5. *Puromycin Selection of Transduced Cells*

After 4 days incubation, all lentiviral transduced cells and control cells were transferred into sterile universal tubes and centrifuged at 300 g to remove media. Cells were resuspended in fresh full media supplemented with 2 µg/ml puromycin, transferred to new 6 well plates and incubated for 3 days in humidified, 5% CO_2 incubator at 37°C. Cells were monitored every day and media replaced every 3 days until they resumed exponential growth, which usually takes up to 3 weeks post-transduction. At this stage, all cell populations consist only of cells with shRNA construct integrated into their genome, while non-transduced cells were killed by puromycin selecting media.

Following expansion of transduced populations, single cell subclones with stable target protein knockdown were generated by plating at low density in semi-solid soft agar (described later in section 2.8.2).

2.5.6. *Assessment of Knockdown Efficiency*

The efficiency of APE1 and OGG1 knockdown was assessed by determining cellular protein levels using western blotting (described in section 2.6).

2.5.7. *Routine culture of knockdown cell lines*

All transduced cells were maintained in full media and passaged regularly as described in section 2.3. All transduced cells were passaged as required in full media supplemented with 2 μg/ml puromycin.

2.6. Western blotting

Western blot analysis was performed to determine protein expression of APE1 and OGG1 and the expression of housekeeping genes α-tubulin and/or GAPDH. This technique involves a number of steps including extraction of cytosolic protein, separation of proteins depending on their size using electrophoresis, transfer of separated proteins onto a membrane and visualisation of proteins using specific antibodies. All reagents and solutions used in western blotting are described in detail in Table 2.5.

2.6.1. Preparation of cell lysate

A previously prepared frozen cell pellet (described in section 2.4.1) containing 5 million cells was thawed on ice and resuspended in 150 μl sodium dodecyl sulphate (SDS) sample buffer. The cells suspension was homogenised using a 21 gauge needle attached to a 1 ml syringe to disrupt and lyse the cells. The cell lysate was heated on at 100 °C for 5 minutes and centrifuged at 14,000 g for 10 minutes to precipitate cells debris. Protein concentration was determined using the Pierce BCA assay kit (described in section 2.6.2).

After determination of protein concentration, this was adjusted to 1 mg/ml in a final volume of 0.5 ml. Briefly, 500 μg of protein was transferred to a 1.5 ml eppendorf tube and made up to 450 μl using SDS sample buffer. Then, β-mercaptoethanol was added to a final concentration of 20% v/v and bromophenol blue was added to a final concentration of 0.01% v/v. The resultant protein extract was aliquoted and stored at -20°C until required.

Reagent	Description	
SDS sample buffer	Tris-HCl SDS glycerol pH 6.8	62.5 M 2% (w/v) 20% (v/v)
SDS electrode buffer	Tris-HCl Glycine SDS pH 6.8	41.2 mM 192 mM 0.1% (w/v)
transfer buffer	CAPS-NaOH methanol pH 11	10 mM 10% (v/v)
TBS/Tween (washing buffer)	Tris-HCl NaCl, Tween-20 pH 7.5	0.01 M 0.1 M 0.05% (v/v)
Blocking buffer	TBS-Tween Non-fat skimmed milk powder	5% (w/v)

Table 2.5: Buffer solutions used in western blotting.

2.6.2. Determination of protein concentration by Pierce BCA assay

Protein concentration was determined using the Pierce® BCA Protein Assay Kit manufactured by Fisher Scientific UK Ltd Leicestershire, UK. The principle of this assay is based on colorimetric detection following reduction of copper cations (Cu^{+2} to Cu^{+1}) by protein with bicinchoninic acid (BCA) in an alkaline medium. This results in the generation of a purple coloured complex with absorbance that can be measured at 562 nm and which is linearly related to protein concentration. The assay was performed according to manufacturer instructions which are summarised as follows:

Samples were diluted in distilled water, i.e. 10 µl of each protein extract was diluted in 90 µl of distilled H_2O. This step is crucial to bring the protein concentrations within the detection range of 0.2 to 1.2 mg/ml. Protein standards were prepared by diluting 2 mg/ml albumin standard provided with the kit using distilled H_2O to a final concentration of 1.2, 1.0, 0.8, 0.6, 0.4 and 0.2 mg/ml. For each standard and diluted extract, 10 µl was dispensed into a 96 well plate (VWR International Ltd) in quadruplicate. For blank wells, 10 µl of distilled H_2O was added. Then, 190 µls of BCA working reagent was dispensed quickly into each well using a multichannel pipette and mixed gently using a plate shaker. The plate was then incubated at 37°C for 30 minutes.

Immediately after incubation, a Spectromax® 250 Microplate Spectrophotometer System (Molecular Devices Corporation, Crawley, UK) was used to read the absorbance at 570 nm. Protein concentrations were calculated from corrected mean absorbance values using Microsoft Excel, via generation of a standard curve using absorbance values of albumin standards. The standard curve was used to calculate protein concentration in each protein extract.

2.6.3. SDS PAGE and electrophoretic transfer

Separation of proteins was performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Bio-Rad 4–20% Mini-PROTEAN® TGX™ precast protein gels (Bio-Rad Laboratories Ltd., Hertfordshire, UK) were used for separation of proteins and were placed in a Mini-PROTEAN® Vertical Electrophoresis Cell system (Bio-Rad Laboratories Ltd., Hertfordshire, UK) filled with SDS electrode buffer. For each cell line, 15 µl of pre-warmed protein extract (15 µg of protein) was loaded into each well. For every gel, 5 µl of

PageRuler™ pre-stained protein ladder was loaded into the last lane. Using a constant voltage at 150V, samples were electrophoresed for approximately 45 minutes.

After electrophoresis, separated proteins were transferred from the gel onto a polyvinylidenefluoride (PVDF) membrane (GE Healthcare Ltd, Buckinghamshire, UK) by electrophoresis. PVDF membranes were soaked in 100% ethanol prior to use and placed in transfer buffer. Also, 2 transfer sponges and 2x 3 mM Whatman® chromatography paper (supplied by VWR International Ltd) were pre-soaked in transfer buffer prior to use. Gels were transferred into transfer cassettes with a PVDF membrane inserted between Whatman papers and transfer sponges. Transfer cassettes were placed in the electrophoresis system filled with transfer buffer and electrophoresed for 45 minutes at 100v. Ice pack was placed in the transfer tank to prevent overheating.

2.6.4. Antibody detection and visualisation of bound proteins

After transferring proteins onto PVDF membranes, the membranes were removed from the transfer cassette and soaked in 5% blocking buffer (Table 2.5) for 1 hour at room temperature on a roller mixer. PVDF membranes were then transferred into a 50 ml BD Falcon™ tube containing 5 ml blocking buffer with primary antibody (Table 2.6) and incubated overnight at 4°C on a roller mixer. Following incubation with primary antibodies, PVDF membranes were washed with 10 ml TBS/Tween 3 times at room temperature for 10 minutes each. Membranes were transferred into a 50 ml BD Falcon™ tube containing 5 ml of appropriate secondary antibody conjugated to horseradish peroxidase and incubated for 1 hour at room temperature on a roller mixer. Membranes were then transferred into new Falcon tubes and washed with 10 ml TBS/Tween for 10 minutes 3 times at room temperature.

Bound antibodies were detected by Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Ltd, Buckinghamshire, UK) according to the manufacturer instructions. Chemiluminescence signals emitted by bounded antibodies were visualised by exposing the membrane to Carestream® Kodak® BioMax® light film (Sigma Aldrich Co Ltd, Dorset, UK). Exposure times varied form 2 seconds to 10 minutes depending on target protein. Films were developed using Mediphot 937 X-Ray Filmprocessor (Colenta Lobortechnik, Austria).

APE1 and OGG1 antibodies were examined prior to use in further experiments to identify optimal concentration and to determine their specificity for APE1 and OGG1 by using knockdown cells (Appendix A).

In order to quantify western blot protein bands, developed films were analysed by FujiFilm Intelligent Dark Box, LAS-3000, (Luminescent Image Analyser System, USA). The generated data was analysed by the LAS 3000 Image Reader software and Microsoft Excel software. To determine the extent of knockdown, proteins were quantified and normalised firstly to protein level in the loading control and then normalised to protein level of parental control cells.

Antibody		Source and type	Supplier	catalogue number	Dilution
Primary antibodies	**APE1**	Mouse , Monoclonal	Abcam plc	AB194	1:2500
	OGG1	Rabbit, polyclonal	Abcam plc	AB91421	1:10000
	α-tubulin	Mouse , Monoclonal	Sigma-Aldrich	T6074	1:80000
	P21	Mouse , Monoclonal	Calbiochem	OP64	1:100
	PAX5	Mouse , Monoclonal	Santa Cruz Biotechnology	SC55515	1:400
	GAPDH	Rabbit, polyclonal	Santa Cruz Biotechnology	SC25778	1:400
Secondary antibodies	**Anti-mouse Ab**	Goat, Polyclonal	Dako	P0447	1:5000
	Anti-rabbit Ab	Goat, Polyclonal	Dako	P0448	1:5000

Table 2.6: Primary and secondary antibodies used in western blotting.

2.7.RNA sequencing

RNA was extracted from APE1 and control shRNA transduced cell clones as described in section 2.4.2. RNA integrity number (RIN) was determined by using the Agilent RNA 6000 Nano Kit (Agilent Technologies, UK) on the Agilent 2100 Bioanalyzer.

The RIN was determined according to the manufacturer protocol. Briefly, 65 µl of pre-filtered gel was mixed with 1 µl RNA 6000 Nano dye concentrate and vortexed, and centrifuged at 13,000 g for 10 minutes at room temperature. The gel/dye mixture was loaded into wells marked G in the RNA 6000 Nano chip and compressed using a plunger, followed by adding 5 µl of RNA 6000 Nano marker to all wells. All RNA samples were denatured by incubation at 70°C for 2 minutes prior to loading 1 µl into each well. RNA was loaded into its corresponding well. The Nano chip was vortexed at 2400 rpm for 1 minute on IKA mixer and placed into Agilent 2100 Bioanalyzer analysed by the Eukaryote Total RNA Nano series II programme. Samples with a RIN of 7 or higher were considered suitable for RNA sequencing.

RNA samples were sequenced using the Illumina HiSeq 2500 platform by AROS Applied Biotechnology, Denmark. RNA sequencing data was analysed by DESeq2 software V 3.2.

2.8. Cytotoxicity assessment assays

The purpose of cytotoxicity assays was to assess the sensitivity of knockdown cells to different chemotherapeutic agents compared to control parental cells. These assays were also used to investigate the efficacy of APE1 inhibitors as a single agent and in combination with other chemotherapeutic agents. Two methods were used to determine cell cytotoxicity in response to anti-leukaemia treatment including growth inhibition assay using trypan blue dye exclusion with cell counting, and the colony formation assay.

2.8.1. Growth inhibition assay using trypan blue and cell counter

2.8.1.1. Drugs and exposure

Chemotherapeutic agents and APE1 inhibitors used throughout this project are listed in Table 2.7. Drugs were dissolved using an appropriate solvent, aliquoted and stored in the dark at -20°C (or at -80°C for the E3330 APE1 inhibitor). Working solutions were prepared shortly prior to use by diluting concentrated drug stocks using FBS-free RPMI culture medium.

Drug name	Provider	Solvent	Stock concentration	Dose range used
Cytarabin (Ara-C)	Sigma-Aldrich	DMSO	50 mM	5 – 40 nM
Daunorubicin	Sigma-Aldrich	DMSO	10 mM	4 – 16 nM
Etoposide	Sigma-Aldrich	DMSO	50 mM	50 – 600 nM
Temozolomide	Sigma-Aldrich	DMSO	100 mM	5 – 300 nM
Clofarabine	Sigma-Aldrich	DMSO	50 mM	2.5 – 100 nM
Fludarabine	Sigma-Aldrich	DMSO	50 mM	0.1 – 50 μM
Methoxyamine	Sigma-Aldrich	PBS	0.5 M	0.5 – 18 mM
E3330	Novus Biologicals	DMSO	40 mM	5 – 60 μM
APE1 inhibitor III	Calbiochem	DMSO	10 mM	0.2 – 10 μM

Table 2.7: Cytotoxic agents and APE1 inhibitors used in growth inhibition and cytotoxicity assays.

2.8.1.2. Growth inhibition assay setup, cell counting and data analysis

To test drug sensitivity, exponentially growing cells were counted using the TC20 cell counter and seeded at a final density of $2x10^4$ cells/ml in an appropriate volume of culture media. For each drug dose, a 5 ml aliquot of cell suspension was transferred to a 6 well plate (or a T25 culture flask for methoxyamine treatment), and supplemented with an appropriate volume of drug to give the required final dose. A vehicle control was performed with appropriate solvent (mainly DMSO) but without drug. Plates/Flasks were incubated for 4 days in a humidified CO_2 incubator at 37°C.

After 4 days incubation, the number viable cells in each culture was counted using the TC20 cell counter and trypan blue dye exclusion. For shRNA knockdown cells or single drug treatment, the percentage of viable cells for each treated cell culture was determined by calculating the percentage of viable cells compared to the vehicle control of the same cell line/subclone. For cells treated with a combination of chemotherapy and APE1 inhibitor, the percentage of viable cells for each treated cell culture was determined by calculating the percentage of viable cells in treated cultures to viable cells treated with APE1 inhibitor as a single agent. Microsoft excel was used to perform all calculations. Data were processed using GraphPad Prism V6 software to generate cytotoxicity curve. Error bars on kill curves represent the standard error of three independent experiments.

2.8.2. Colony formation assay

Colony formation assay (CFA) was performed to determine the effect of inhibition or knockdown of APE1 or OGG1 on cloning efficiency and the ability of AML cells to form colonies from single cells in semi-solid soft agar. It was also used to investigate the sensitivity of AML cells to chemotherapy alone or in combination with APE1 inhibitors or after APE1 knockdown using shRNA. Furthermore, CFA was performed to generated single cell clones from shRNA transduced cell populations to generate sub-clones with stable knockdown of the gene of interest.

2.8.2.1. Drugs and exposure

Drugs used in the CFA are described in section 2.8.1.1. In order to study the effect of APE1 inhibitors as a single agent or in combination with chemotherapy, exponentially growing cells were counted using a TC20 cell counter and seeded to a final density of $1x10^5$ cells/ml in an appropriate amount of complete culture media. Cell suspensions were incubated for 24 hours before drug addition to allow exit out of lag phase and the establishment of exponential growth. After 24 hours, cell suspensions were aliquoted into 5 ml into T25 culture flasks, and supplemented with an appropriate volume of drug to give the required final dose. A vehicle control was performed with appropriate solvent but without drug. Each experiment was performed on 3 independent occasions. Cells were exposed to drug for 24 hours and incubated in a humidified 5% CO_2 incubator at 37°C. For generating sub-clones from shRNA transduced cells, the same process was performed except that cells were not treated with drug.

2.8.2.2. Cell preparation after treatment

Following 24 hours incubation all treated and control cells were transferred into sterile universal tubes (VWR International Ltd) and centrifuged at 250 g at room temperature to remove culture media. After centrifugation, supernatant was discarded and cell pellets were resuspended in sterile pre-warmed PBS and centrifuged to remove any remaining drug. Cells were then resuspended in full culture media and cell counts were performed using the TC20 cell counter. Cells were counted in triplicate for accurate estimation of cell density and transferred to a new flacon tube at a density of $1x10^4$ cell/ml. Serial dilution was performed to give final density of 40 cells/ml for drug sensitivity experiment or 10 cells/ml for generating subclones from shRNA transduced populations. Following serial dilution, 5 ml or 1 ml of diluted cells were transferred into 10 cm petri dishes or 6 well plates prior to the addition of soft agar.

2.8.2.3. Preparation of semi-solid soft agar

Semi-solid soft agar was prepared by adding 5 ml of sterile PBS to 200 mg of SeaKem ME Agarose (Lonza – supplied by VWR International Ltd) in a Falcon tube. Agarose was dissolved by microwaving to give a final agarose concentration of 40 mg/ml. An appropriate volume of molten agarose was quickly added to an appropriate volume of pre-warmed full media to give 4 mg/ml agarose. Then, 5 ml or 1 ml of prepared agarose media was transferred

to a 10 cm petri dishes or 6 well plate and mixed gently to give 2 mg/ml (or 0.2 %) semi-solid-soft agar. This protocol results in 200 cell/plate in 10 cm dishes and 10 cells/well in 6 wells plates. Plates were incubated in humidified incubator at 37°C with 5% CO_2 for 2-3 weeks depending on cell line.

2.8.2.4. Colony expansion, visualisation, counting and data analysis

For generating sub-clones from shRNA transduced cells population, single colonies were picked using a pipette and transferred to 0.5 ml of complete media in 24 well plates and incubated for 2-3 days. Cells were then expanded and used in several experiments. For drug sensitivity and cloning efficiency experiments, colonies were counted after visualised using 0.05% thiazolyl blue tetrazolium bromide (MTT) stain.

For shRNA knockdown cells or single drug treatments, the percentage of viable cells for each treated cell culture was determined by calculating the percentage of colonies in treated cultures compared to vehicle control treated cells of the same line or clone. For cells treated with a combination of chemotherapy and APE1 inhibitors, cloning efficiency for each treated cell culture was determined by calculating the percentage of colonies in treated cultures compared to colonies treated with APE1 inhibitor as a single agent. Microsoft excel was used to perform all calculations. Data were processed using GraphPad Prism V6 software to generate cytotoxicity curve. Error bars on kill curves represent the standard error of three independent experiments.

2.9. Cell cycle analysis using flow cytometer

Propidium Iodide (PI) stain was used to determine the cell cycle distribution of cells after APE1 or OGG1 knockdown and/or after treatment with APE1 inhibitors. PI is a fluorescent dye, which can bind to both DNA and RNA and can be detected in either the FL-2 or FL-3 channels on a FACS Calibur instrument. Viable cells do not take up PI and need to be artificially permeabilised to allow PI entry, which can be achieved by fixation in 70% ethanol. Cell cycle analysis depends on measuring the amount of DNA in each single cell. Cells going through S phase undergo DNA replication and have more DNA compared to cells in G0/G1 (2N), therefore take up more PI and have a stronger fluorescent signal. Cells in G2/M phase have double the DNA content (4N) compared to cells in G0/G1, until they undergo mitosis.

2.9.1. Cell preparation, fixation and staining with propidium iodide (PI)

To study the effects of APE1 inhibitors on cell cycle, cells were counted using a TC20 cell counter and seeded to a final density of $1x10^5$ cells/ml and treated with APE1 inhibitors. Cells were incubated for 24, 48 or 72 hours and harvested for cell cycle analysis. After treatment, cells were counted using a TC20 cell counter and $5x10^5$ cells were washed as described in section 2.4.1. Cells then were fixed using 70% ethanol and vortexed to prevent cell clumping and kept at -20°C prior to PI staining. The same process was performed to prepare APE1/OGG1 shRNA transduced cells for cell cycle analysis but without treatment.

Ethanol was removed by centrifugation at 300 g for 15 minutes at room temperature and cell pellets were resuspended in 100 µl of citrate buffer (0.25 M Sucrose, 40 mM sodium citrate, pH 7.6) and transferred into FACS tubes. Cells were then stained with 400 µl PI (20 µg/ml propidium iodide, 0.5% NP-40, 0.5 mM EDTA) and treated with 1 µl of RNase A to eliminate RNA. Tubes were kept at 4°C protected from light for 1 hour prior to cytometric analysis.

After incubation, cell cycle analysis was performed on a FACSCalibur flow cytometer with BD Cell Quest Pro software (BD Biosciences) and PI detected in the FL-2 channel. Instrument settings were optimised for each cell line using control untreated cells/shRNA cells. A dot plot of FL-2 area versus FL-2 width was created to identify target cells and gating was setup around G0/G1/G2/M populations to exclude cell aggregates and doublets. Gated cells were plotted on a histogram of event count versus linear FL-2 area. G_0/G_1 peak was adjusted to 200 on the FL-2 area linear axis and 10,000 events were collected for each sample.

2.9.2. Data analysis

Data was analysed using Cell Quest Pro software (BD Biosciences) to determine the percentage of cells in each cell cycle phase. Data in histograms generated during data collection (described in previous section 2.9.1) were used to determine the proportion of cells in each cell cycle phase. Different phases in the cell cycle were marked and a table of statistics identifying the percentage of cells in each phase was generated from the histogram.

2.10. Abasic (AP) site quantification

Abasic sites or Apurinic/Apyrimidinic (AP) sites are one of the most frequent spontaneous DNA lesions and it is estimated that high ROS could generate about 50,000 to 200,000 AP site per cell per day in the genome and that brain cells contain the most AP sites (Martin et al 2008). AP sites are potentially mutagenic and also potentially lethal via the ability to block DNA replication. Primarily, the majority of AP sites are recognised and repaired by the BER system and any malfunction in BER components would lead an accumulation of unrepaired AP sites. AP site accumulation was determined, after APE1 inhibition or knockdown using shRNA, by a colorimetric assay kit supplied by Abcam, UK (cat# ab65353). In this assay, Aldehyde reactive probe (ARP) reacts specifically with an aldehyde group on the open ring form of AP sites. After ARP and AP site reaction, the resulting compound can be tagged with biotin, which can then be quantified via the avidin-biotin assay followed by colourimetric detection of peroxidase conjugated to avidin.

2.10.1. Cells treatment and extraction of genomic DNA

For shRNA transduced cells, cells were treated with 50 μM H_2O_2 for 1 hour and washed with pre-warmed sterile PBS and transferred to complete media. For cells to be treated with APE1 inhibitors, cells were treated with 50 μM H_2O_2 for 1 hour and washed with pre-warmed PBS and centrifuged at 300 g for 5 minutes at room temperature. Supernatant was discarded and cells were transferred to complete RPMI media supplemented with the required dose of APE1 inhibitor. Cell pellets were prepared prior to treatment and after 2, 4 and 8 hours of H_2O_2 treatment (as described in section 2.4.1) and kept at -80°C until required.

Genomic DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Crawley, UK) according to manufacturer protocols. Briefly, cells were lysed and homogenised using the provided buffers, then dispensed into spin column containing a QIAamp® silica gel membrane. DNA binds to the silica membrane and other contaminants were removed by washing with provided buffers. DNA was eluted in 200μl buffer AE (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) and quantified using a NanoDrop® ND-1000 spectrophotometer. Extracted DNA was aliquoted and stored at -20°C until required.

2.10.2. Quantification of AP site using the Aldehyde Reactive Probe base kit

AP site quantification was performed according to manufacturer protocols. Briefly, extracted DNA was diluted to 0.1 µg/µl in TE buffer and then tagged with ARP. Tagged DNA was precipitated by centrifugation at 14000 g and washed 3 times with 70% ethanol. TE buffer was added to ARP tagged DNA. ARP-DNA standards were prepared by diluting the ARP-DNA standard (containing 40 ARP sites per 10^5 base pair (bp)) provided with the kit to generate standards of 0, 8, 16, 24, 32 and 40 ARP sites per 10^5 bp. For each ARP standard and test sample, 60 µl was dispensed in triplicate into a 96 well plate and 100 µl of DNA binding solution was added to each well. Plates were sealed to avoid any evaporation and incubated at room temperature overnight to allow DNA to bind to the plate surface. After incubation, DNA binding solution was discarded and plates were washed 5 times with 250 µl washing buffer. HRP-Streptavidin solution was added to each well and incubated at room temperature for 1 hour with gentle agitation. HRP-Streptavidin solution was discarded and plates were washed 5 times with 250 µl washing buffer. After washing, HRP Developer was added to each well, and incubated at 37°C for 1 hour. Following incubation, absorbance was determined at 650 nm using a Spectromax® 250 Microplate Spectrophotometer System.

Absorbance values of ARP-DNA standards were used to generate a standard curve and to generate an equation used to calculate AP sites in each sample. All calculations were performed using Microsoft excel and data are shown in graph bars.

2.11. TARDIS assay

The trapped in agarose DNA immunostaining (TARDIS) assay was originally developed to detect and quantify melphalan and cisplatin DNA adducts, but later adapted to quantify topoisomerase-DNA complexes (Tilby *et al.*, 1987; Cowell *et al.*, 2011). This assay was performed to assess the ability of APE1 to remove TOP2 α and β complexes after treatment with etoposide.

2.11.1. Cell treatment and staining

The TARDIS assay was performed as previously described (Mariani et al., 2015). Briefly, TOP2 DNA adducts were generated by treating exponentially growing cells with 100 µM

etoposide for 1 hour. Following treatment, $5x10^5$ cells were washed twice with cold PBS, re-suspended in 50 µl of cold PBS, then pre-warmed at 37 °C for 30 seconds and embedded in an equal volume of pre-warmed molten 2% W/V agarose in PBS (SeaPrep ultra low gelling agaros - Lonza, US). The mixture was spread consistently on microscopic slides pre-coated with 0.5% agarose, and placed on a cold surface to solidify the cell-agarose mixture. Slides were then dipped for 30 minutes at room temperature in lysis buffer [1% SDS, 20 mM Na phosphate pH 6.5 and 10 mM EDTA] in the presence of protease inhibitors [1% Benzamidine, 1% phenylmethanesulfonyl fluoride, 0.1% Leupeptin, 0.1% Pepstatin and 0.1% DTT] obtained from Sigma-Aldrich UK. Following cell lysis, slides were incubated for 30 minutes in 1 M NaCl + protease inhibitors followed by washing with PBS+ protease inhibitors 3 times (5 minutes per wash) at room temperature. After washing with PBS and protease inhibitors, slides were exposed to primary specific antibodies directed against TOP2A and TOP2B for 1.5 hours at room temperature. Primary antibodies used were rabbit anti-TOP2 polyclonal antibodies raised and purified in Professor Caroline Austin's laboratory at Newcastle University, and were used at 1:400 dilution in BSA-PBS-Tween [PBS, 0.1% (v/v) Tween-20 and 1% (w/v) bovine serum albumin (BSA)]. Following incubation with primary antibodies, slides were washed 3 times with PBST [PBS and 0.1% (v/v) Tween-20] + protease inhibitors, and incubated with secondary antibodies Alexa488 [1:250 dilution in BSA-PBS-Tween] (Life Technologies) for 1.5 hours protected from light. Slides were then washed as previously described and stained with 1 ml of 2 µg/ml Hoechst 33258 (Invitrogen) in PBS for 5 minutes. Finally, after staining, excess Hoechst was drained, mounting media Vectashield (without DAPI) was applied to the slide, and appropriate coverslips were placed and secured with a sealant. Slides were examined immediately or kept at 4 °C until required.

2.11.2. Microscopy and data analysis

Slides were examined using epifluorescence microscope Olympus IX-81 and images were recorded for each treatment and each cell line. Two different optical filters were used to separately capture the florescent signal emitted by Hoechst (exited at 352 nm and emit at 461 nm) and Alexa 488 (exited at 495 nm and emit at 519 nm). On average, 6 pairs of images were recorded for each slide to give approximately 300 cells/slide. Images were merged and their background was corrected and analysed using Volocity software version 1.0.0 (Perkin Elmer). Integrated fluorescence values were processed using GraphPad Prism to calculate mean values which were plotted on a volcano graph plot.

Chapter 3: The prognostic value of APE1 and OGG1 expression and Evaluation of their gene expression and protein levels in AML

3.1. Introduction

AML is heterogeneous disease characterised by high oxidative stress burden, DNA damage and genome instability, which is predicted to contribute to leukaemogenesis. It is important to stress that the DNA damage response mechanisms are essential in normal cells and their dysregulation may contribute to malignant transformation. Upregulation of DNA damage response pathways in AML cells may confer protection against DNA damaging agents, and consequently promote chemoresistance and cell survival. Accumulating evidence indicates that APE1 and OGG1 expression is upregulated in cancer and may be used as a prognostic marker for disease progression and chemotherapy resistance (Al-Attar *et al.*, 2010; Liddiard *et al.*, 2010; Abdel-Fatah *et al.*, 2014). However, the prognostic value of APE1 in AML has not yet been established. OGG1 has been suggested as a prognostic indicator in AML (Liddiard et al., 2010).

3.1.1. APE1 as a prognostic marker in AML

Although APE1 expression has been well characterised in several cancer types (Wang *et al.*, 2004; Di Maso *et al.*, 2007; Wang *et al.*, 2009a; Al-Attar *et al.*, 2010; Woo *et al.*, 2014), its expression and role in AML pathogenesis, prognosis and treatment response is still unknown. Published studies report heterogeneous *APE1* mRNA expression in AML, but have not characterised its prognostic value. High APE1 mRNA expression has been reported in a single case of acute promyelocytic leukaemia (APL) occurring as a second therapy-induced malignancy (Casorelli *et al.*, 2006). In contrast, another study showed that APL-associated fusion proteins PLZF-RARα and PML-RARα are linked to reduced APE1 mRNA and protein expression (Petruccelli *et al.*, 2013). Furthermore, this study demonstrated that PLZF-RARα and PML-RARα-expression cells are more sensitive to vorinostat histone deacetylase inhibitor compared to fusion negative cells, due to reduced BER capacity. Regardless of its role in determining sensitivity to chemotherapy, APE1 is essential component of BER pathway and it is important to maintain balanced expression. APE1 upregulation or downregulation may drive dysregulated activity of BER and contribute to genome instability.

3.1.2. OGG1 expression as prognostic marker in leukaemia

The OGG1 DNA glycosylase is an important enzyme that removes oxidised DNA lesions in DNA, including primarily 8-oxoguanine. Failure to remove such lesions can result in mutagenic transversion of G:C to T:A (Forster *et al.*, 2016). High expression of OGG1 is shown to confer poor prognosis in AML (Liddiard *et al.*, 2010). Specifically, patients with high OGG1 expression were more likely to relapse and have shorter overall survival and shorter relapse-free survival compared to patients with high OGG1 expression (Liddiard *et al.*, 2010). Moreover, high OGG1 expression is associated with adverse cytogenetics which is an independent marker of poor prognosis. Conversely, low OGG1 expression is reported in AML with the t(8;21) translocation and AML1/ETO fusion protein, which may contribute to the better outcome in this patient group.

3.1.3. APE1 and OGG1 are regulated by post-translational modification

Post-translational modifications are important mechanisms by which proteins are controlled and regulated in respect of their protein-protein interactions, protein function, cellular protein levels, enzymatic activities and cellular localisation. Several reports demonstrate that both APE1 and OGG1 are regulated by post-translational modification, via ubiquitination, phosphorylation and acetylation (Bhakat *et al.*, 2009; Hegde *et al.*, 2012; Carter and Parsons, 2016).

APE1 regulation by post-translational modifications

APE1 is regulated at the transcriptional and post-translational levels. It has been reported that mouse double minute 2 (MDM2) and Cyclin-dependent kinase 5 (Cdk5) modulates APE1 ubiquitination (Busso *et al.*, 2009; Busso *et al.*, 2011). APE1 ubiquitination occur at lysine residues K24, K25 and K27, which are located in the N-terminus. MDM2 inhibition using nutlin-3 (MDM2-P53 inhibitor) resulted in enhanced APE1 ubiquitination (Fantini *et al.*, 2010). Furthermore, mutant MDM2 was not able to ubiquitinate APE1 (Fantini *et al.*, 2010). Ubiquitin protein ligase E3 component N-recognin 3 (UBR3) also is also reported to ubiquitinate and regulate APE1 protein levels (Meisenberg *et al.*, 2012).

Several studies have reported APE1 phosphorylation and demonstrated effect on both DNA repair and redox functions (Yacoub *et al.*, 1997; Hsieh *et al.*, 2001; Huang *et al.*, 2010). Cdk5 and P35 complex interact with APE1 and induce phosphorylation at Thr232 in the C-terminus

of the protein (Huang *et al.*, 2010). Cdk5/P35 mediated phosphorylation resulted in a reduction of APE1 endonuclease activity and led to accumulation of AP sites and cell death in a neuronal cell model (Huang *et al.*, 2010). APE1 redox function has also been shown to be stimulated via protein kinase C (PKC) phosphorylation following treatment with DNA damaging agents (Hsieh *et al.*, 2001), although the mechanism remains unclear.

Acetylation of APE1 at lysine residues 6 and 7 by the transcriptional co-activator p300 enhances binding of APE1 to negative calcium response elements in response to elevated calcium level (Bhakat *et al.*, 2003). APE1 acetylation in this context acts as repressor for parathyroid hormone promoter. Acetylation of APE1 at lysine residues 6 and 7 was shown to enhance its binding to Y-box binding protein 1 (YB-1), which resulted in activation of the YB-1-dependent multidrug resistance gene 1 (MDR1) (Chattopadhyay *et al.*, 2008). Further investigation to delineate the mechanism by which APE1 acetylation controls YB-1-mediated activation of MDR1 gene found that APE1 is stably associated with RNA polymerase II transcription factor on MDR1 gene promoter (Sengupta *et al.*, 2011). Thus, APE1 acetylation is required to recruit and activate YB-1/p300 complex to enhance MDR1 expression. However, sirtuin1 (SIRT1) has been found to control the acetylation status of lysine residues 6 and 7 (Yamamori *et al.*, 2010). SIRT1 deacetylates these residues following DNA damage and promotes APE1 interaction with XRCC1 to induce BER. Furthermore, the APE1-NPM1 interaction is modulated by acetylation of lysine residues 27, 31, 32 and 35 which located in the N-terminal of APE1 (Fantini *et al.*, 2010).

OGG1 regulation by post-translational modifications

Like APE1, OGG1 also is regulated by post-translational modification as well as transcriptional regulation. OGG1 ubiquitination has only been reported in one limited study. Under hypothermic condition, OGG1 ubiquitination was shown to be catalysed by C-terminus of HSC70-interacting protein (CHIP) E3 ligase (Fantini *et al.*, 2013). Thermal inactivation of OGG1 resulted in loss of glycosylase function, followed by ubiquitination and proteasomal degradation (Fantini *et al.*, 2013). Furthermore, undegraded remnants of OGG1 translocate from the nucleus into the perinuclear region to further decrease OGG1 DNA repair activity (Fantini *et al.*, 2013).

OGG1 is phosphorylated *in vivo* by protein kinase c (PKC) (Dantzer *et al.*, 2002). In this study, OGG1 was bound to chromatin and phosphorylation reported at several serine residues of OGG1. Luna and colleagues studied the subcellular localisation and expression of OGG1

and its polymorphic variant OGG1- S326C, and reported that phosphorylation of serine residue 326 governs OGG1 translocation to the nucleus (Luna *et al.*, 2005). OGG1 phosphorylation can be mediated by two different tyrosine kinases c-Abl and cyclin dependant kinase 4 (Cdk4) (Hu *et al.*, 2005b). C-Abl mediated phosphorylation had no effect of OGG1 glycosylase activity, whereas Cdk4 mediated phosphorylation increased OGG1 glycosylase activity up to 2.5 fold (Hu *et al.*, 2005b).

P300 catalyses OGG1 acetylation at lysine 338 and 341, and enhances its glycosylase activity by reducing its affinity for AP sites generated during oxidative stress (Bhakat *et al.*, 2006). Furthermore, the OGG1 acetylation rate increased 2.5 fold following glucose oxidase-induced oxidative stress, which suggests DNA damage dependent activation of OGG1 repair activity (Bhakat *et al.*, 2006).

3.2. Aims of this chapter

The general and specific aims of the work in this chapter are as follow:

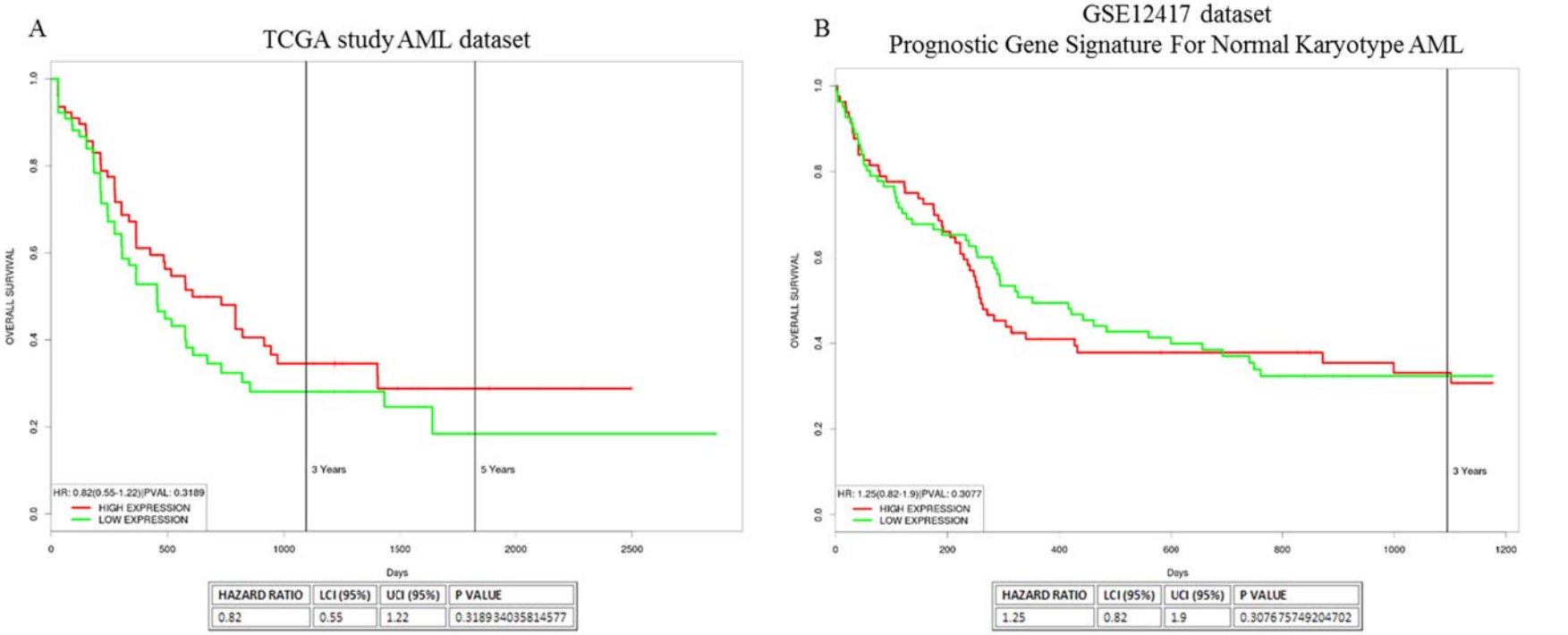
- To explore APE1 gene expression and protein expression in AML cells and relate this to disease progression and prognosis. To achieve this target:
    - Study prognostic impact of APE1 and OGG1 expression in AML using available data in the public domain.
    - Evaluate *APE1* and *OGG1* mRNA gene expression in AML cell lines using real time PCR.
    - Evaluate APE1 and OGG1 protein expression in AML cell lines using western blot assay.

3.3.Results

3.3.1. Prognostic impact of APE1 and OGG1 expression in AML

In order to evaluate the prognostic value of APE1 and OGG1 in AML patients, gene expression data from several public databases were evaluated. In particular, all data presented in this section was evaluated using PROGgeneV2 – the Pan Cancer Prognostics Database hosted by the Centre for Computational Biology and Bioinformatics at Indiana University and Purdue University in Indianapolis, USA (http://watson.compbio.iupui.edu/chirayu/proggene/database/index.php). This database provides comprehensive survival analysis based on mRNA expression, and presents data as a Kaplan Meier survival plot. Datasets used to evaluate the prognostic value of APE1 and OGG1 include The Cancer Genome Atlas (TCGA) acute myeloid leukaemia dataset (The Cancer Genome Atlas Research, 2013; Papaemmanuil *et al.*, 2016) (N=157) and the Prognostic gene signature for normal karyotype AML dataset (GSE12417) (N=163) (Metzeler *et al.*, 2008). Both APE1 and OGG1 gene expression was determined in all datasets using the Affymetrix U133 microarray. Data analysis was performed on gene level only, i.e. databases did not show information about probes of APE1 and OGG1 that has been used in the analysis.

Neither AML dataset provided evidence that *APE1* expression associates with overall survival in AML (Figure 3.1). Specifically, when categorised into two major groups by the median expression level, *APE1* did not significantly associate with overall survival in either the TCGA or GSE12417 datasets (P= 0.3 for both datasets). In the TCGA dataset, *OGG1* expression did not associate with survival in AML when categorised into two groups by the median (p=0.89) (Figure 3.2). However, in the GSE12417 dataset, low OGG1 expression was significantly associated with shorter overall survival (p=0.0049) (Figure 3.2).



HAZARD RATIO	LCI (95%)	UCI (95%)	P VALUE
0.82	0.55	1.22	0.318934035814577

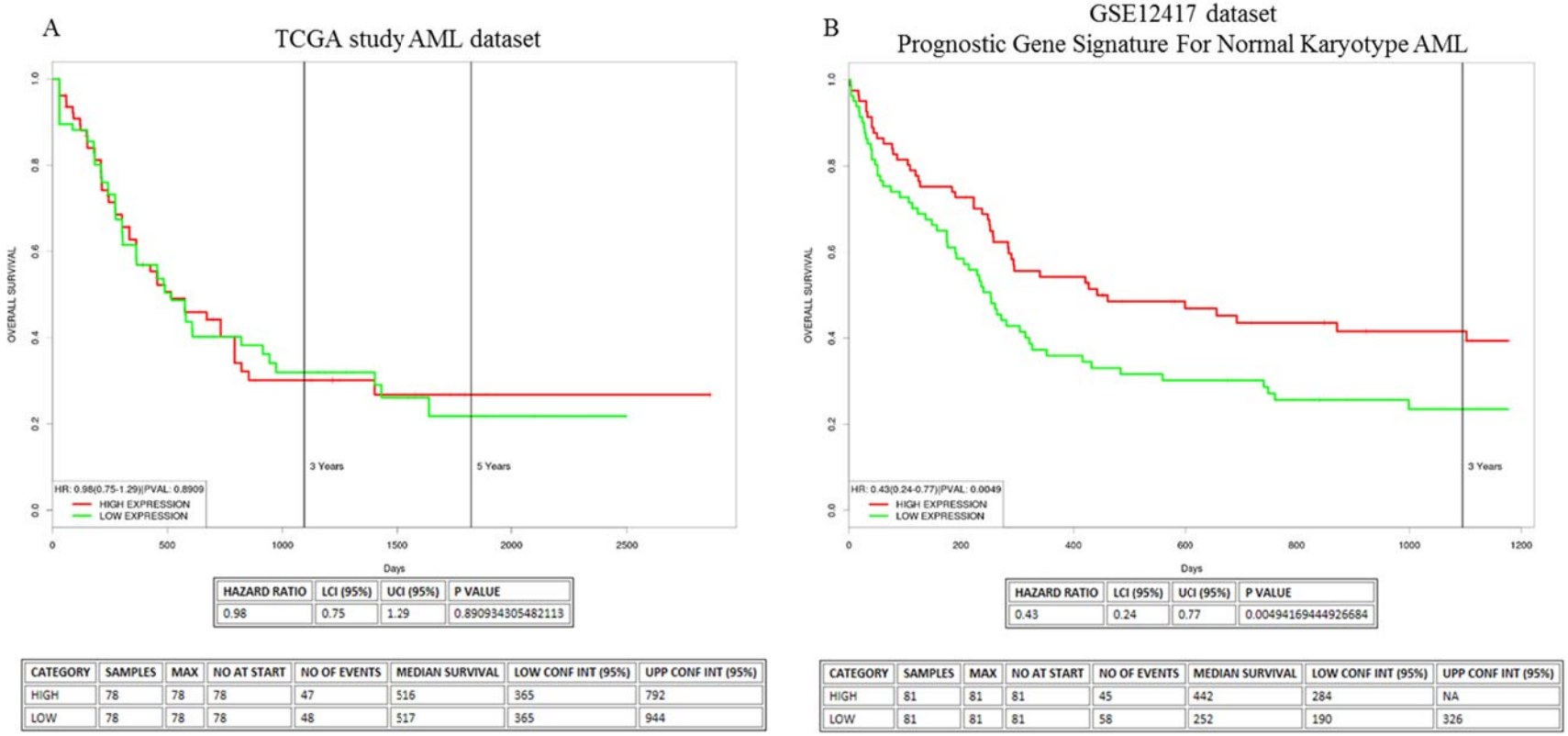
CATEGORY	SAMPLES	MAX	NO AT START	NO OF EVENTS	MEDIAN SURVIVAL	LOW CONF INT (95%)	UPP CONF INT (95%)
HIGH	78	78	78	45	607	426	972
LOW	78	78	78	50	456	305	671

HAZARD RATIO	LCI (95%)	UCI (95%)	P VALUE
1.25	0.82	1.9	0.307675749204702

CATEGORY	SAMPLES	MAX	NO AT START	NO OF EVENTS	MEDIAN SURVIVAL	LOW CONF INT (95%)	UPP CONF INT (95%)
HIGH	81	81	81	51	260	229	872
LOW	81	81	81	52	352	280	693

Figure 3.1: Correlation between APE1 gene expression and overall survival in AML patients.

Kaplan-Meier analysis for overall survival according to *APE1* expression in two publicly available datasets on (http://watson.compbio.iupui.edu/chirayu/proggene/database). **(A)** The Cancer Genome Atlas (TCGA) and **(B)** GSE12417 datasets utilised Affymetrix U133A microarray to determine *APE1* gene expression. APE1 expression appear to have no correlation with overall survival in AML patients.



HAZARD RATIO	LCI (95%)	UCI (95%)	P VALUE
0.98	0.75	1.29	0.890934305482113

CATEGORY	SAMPLES	MAX	NO AT START	NO OF EVENTS	MEDIAN SURVIVAL	LOW CONF INT (95%)	UPP CONF INT (95%)
HIGH	78	78	78	47	516	365	792
LOW	78	78	78	48	517	365	944

HAZARD RATIO	LCI (95%)	UCI (95%)	P VALUE
0.43	0.24	0.77	0.00494169444926684

CATEGORY	SAMPLES	MAX	NO AT START	NO OF EVENTS	MEDIAN SURVIVAL	LOW CONF INT (95%)	UPP CONF INT (95%)
HIGH	81	81	81	45	442	284	NA
LOW	81	81	81	58	252	190	326

Figure 3.2: Correlation between OGG1 gene expression and overall survival in AML patients.

Kaplan-Meier analysis for overall survival according to *OGG1* expression in two publicly available datasets at (http://watson.compbio.iupui.edu/chirayu/proggene/database). The Cancer Genome Atlas (TCGA) and GSE12417 datasets utilised Affymetrix U133A microarray to determine *OGG1* gene expression. **(A)** TCGA dataset demonstrated no difference between low and high *OGG1* expression groups in term of overall survival. **(B)** *OGG1* overexpression appear to have favourable effect, rather than poor effect, on overall survival, where low OGG1 expression patients group demonstrated low overall survival.

3.3.2. Determination of APE1 mRNA expression in AML cell lines by Quantitative RT-PCR

APE1 mRNA gene expression was assessed by real time polymerase chain reaction (RT-PCR) in 8 AML cell lines including HL-60, AML2, AML3, U937, MV4-11, NB4, Kasumi1 and THP-1. Data were expressed as fold change and were normalised to NB4 *APE1* gene expression. NB4 was selected because it represents M3 FAB classification AML, which is characterised by repressed DNA repair activity due to the presence of the PML-RARα fusion (Casorelli *et al.*, 2006; Esposito and So, 2014).

RT-PCR demonstrated a modest variation, but not significant according to p value calculated by an unpaired t-test, in *APE1* mRNA gene expression in the AML cell lines investigated (Figure 3.3A). HL-60 cells had the highest *APE1 mRNA* expression with mean of 1.33 fold compared to NB4 *APE1* mRNA transcripts. Other cell lines showed minimal variation in APE1 expression.

3.3.3. APE1 protein expression in AML cell lines determined by western immunoblotting

Western blotting was performed to investigate APE1 protein level in AML cell lines including HL-60, AML2, AML3, U937, MV4-11, NB4, Kasumi1 and THP-1. APE1 protein levels were consistently expressed in the majority of AML cell lines used (Figure 3.3B), demonstrating lack of correlation between APE1 mRNA and protein level and suggesting that APE1 protein levels are tightly regulated.

To further quantify APE1 protein levels, western blots were assessed by densitometry using Fuji Film Intelligent Dark Box, LAS-3000, Luminescent Image Analyser System and the data were analysed using LAS 3000 Image Reader software (Fujifilm Medical Systems U.S.A., Inc). APE1 protein levels were normalised to α-tubulin loading control, and then normalised to NB4 protein expression. Result showed consistent APE1 protein levels in the majority of investigated AML cell lines (Figure 3.3C). Highest APE1 protein expression was observed in MV4-11 (165%) and U937 (130%).

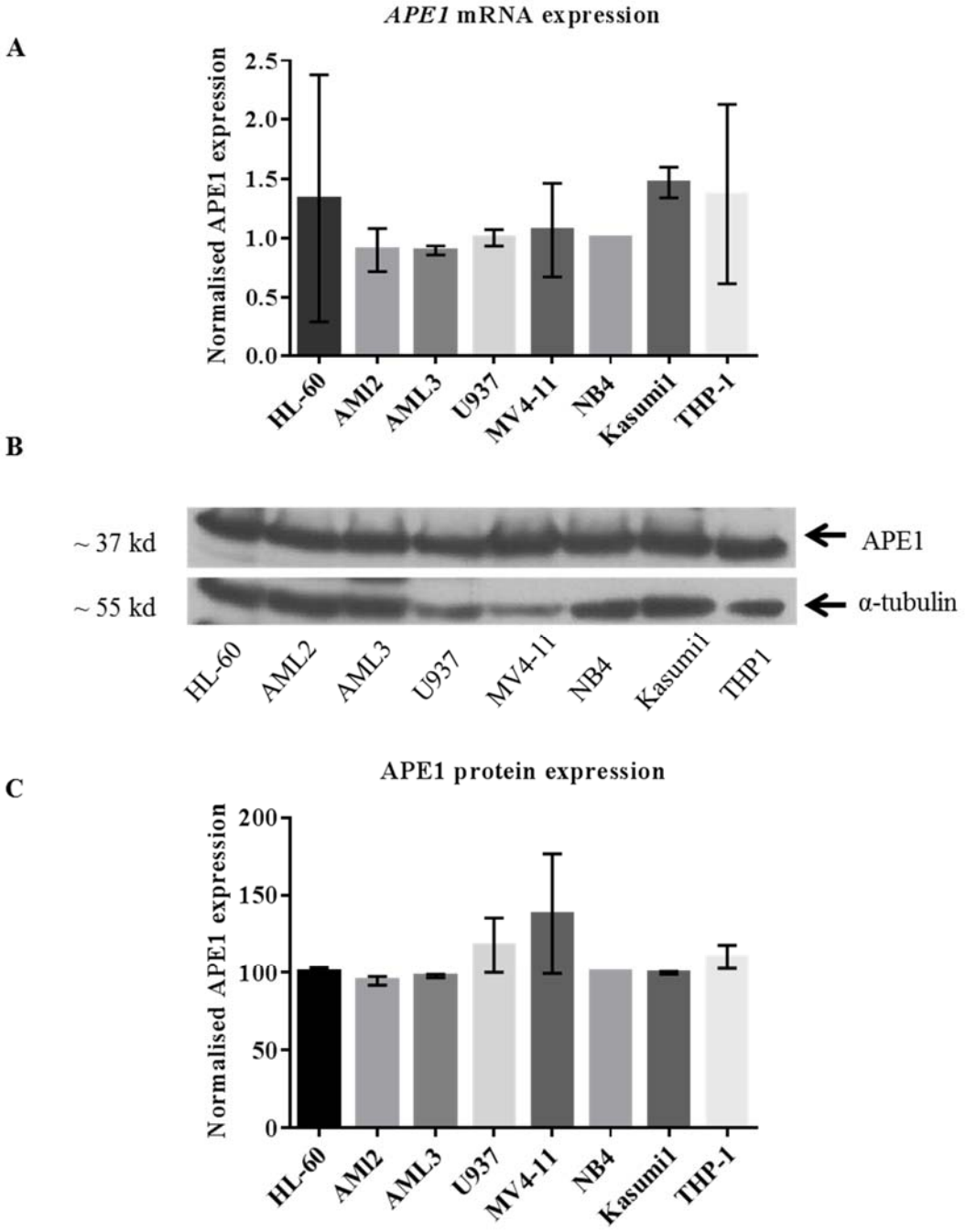


Figure 3.3: APE1 expression in AML cell lines.

(A) *APE1* mRNA expression determined in population of asynchronous AML cells using real time PCR. Data represent the mean of two independent experiments and error bars of standard deviation. There was no significant variation in *APE1* gene expression between cell lines according to the p values calculated by unpaired parametric t test.

(B) Western blot showing protein expression of APE1 in several AML cell lines.
(C) Representative quantification of APE1 protein from western blot, quantified by densitometer. There was no significant variation in APE1 protein expression between cell lines according to p values calculated by unpaired parametric t test.

3.3.4. OGG1 mRNA transcript expression in AML cell lines determined by Quantitative RT-PCR

RT-PCR was used to assess the expression of *OGG1* in AML cell lines including HL-60, AML2, AML3, U937, MV4-11, NB4, Kasumi1 and THP-1. *OGG1* mRNA gene expression data were expressed as fold change and normalised to NB4 *OGG1* gene expression.

As expected and consistent with previous reports, Kasumi-1 cells demonstrated the lowest *OGG1* mRNA expression, due to presence of the AML1-ETO1 t(8;21) chromosomal translocation, which represses OGG1 expression (Figure 3.4A) (Liddiard et al., 2010; Forster et al., 2016; Li et al., 2016). THP-1 cells demonstrated highest OGG1 expression compared to other cell lines, which was 3.57 fold higher than NB4 at the mRNA level. Other AML cell lines demonstrated comparable *OGG1* gene expression.

3.3.5. OGG1 protein expression in AML cell lines determined by western immunoblotting

Western blotting was performed to assess OGG1 protein expression in AML cells previously tested for mRNA expression. Surprisingly results showed stable OGG1 expression in all AML cell lines tested regardless of mRNA transcript levels (Figure 3.4B). In particular, Kasumi-1 cells were expected to have low OGG1 protein due to low mRNA transcript level. However, quantified OGG1 western blot result using densitometer showed slight consistency with mRNA levels, where kasumi1 cells expressed the lowest OGG1 protein (84% compared to NB4) (Figure 3.4C). Furthermore, THP1 and U937 demonstrated higher OGG1 protein expression compared to all other cell lines.

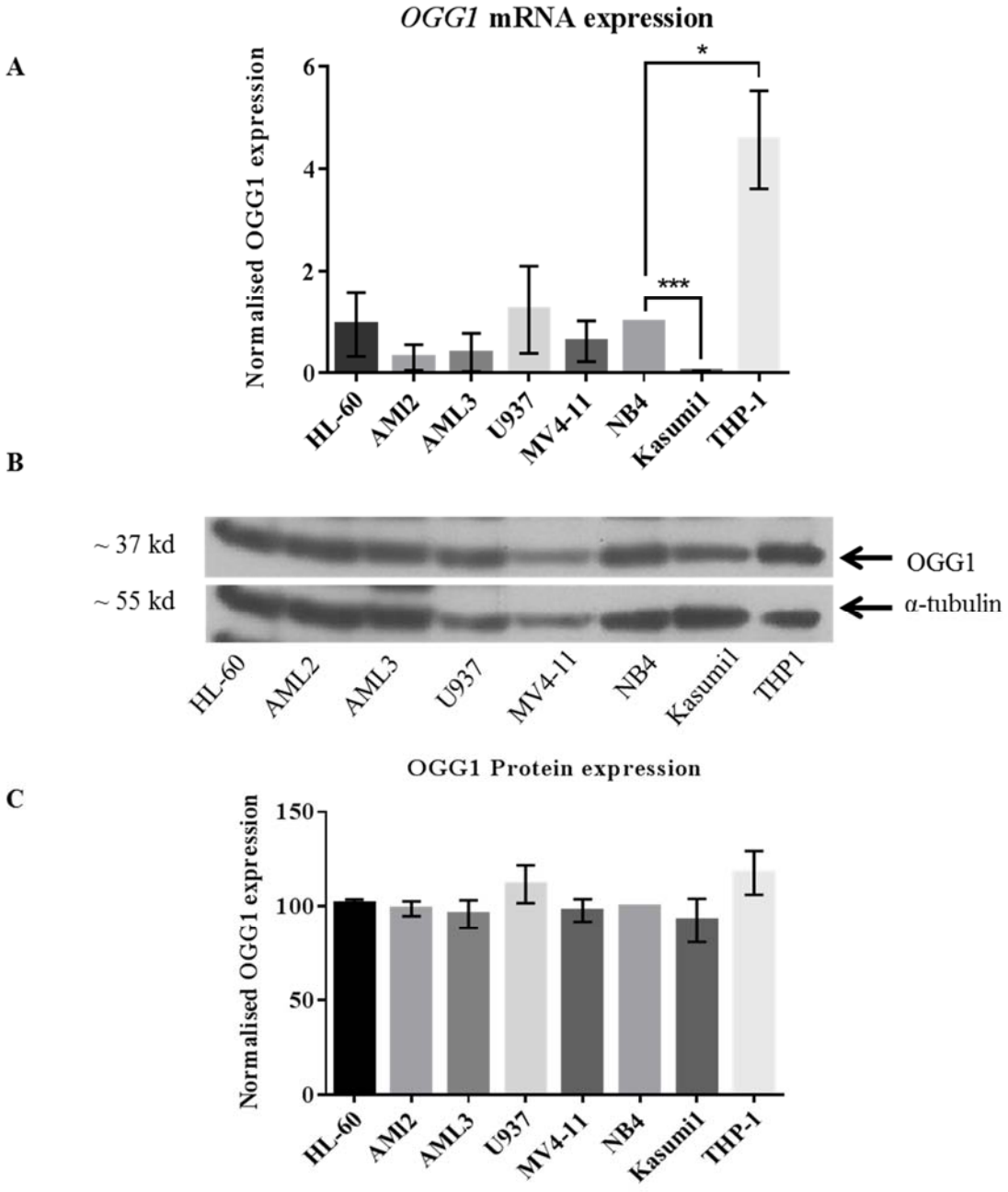


Figure 3.4: OGG1 expression in AML cell lines.

(A) *OGG1* mRNA expression determined in population of asynchronous AML cells using real time PCR. Data represent the mean of 2 independent experiments and error bars of standard deviation. Unpaired parametric t test was used to calculate p values. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

(B) Western blot showing protein expression of OGG1 in several AML cell lines.
(C) Representative quantification of OGG1 protein from western blot, quantified by densitometer. There was no significant variation in OGG1 protein expression between cell lines according to p values calculated by unpaired parametric t test.

3.4. Discussion

The current prognostication system for AML patients includes only cytogenetic abnormalities caused by chromosomal translocations/deletions and recurrent somatic mutations, but does not include any somatic alterations in DNA repair genes (Grimwade *et al.*, 2010). However, it is becoming clearer now how DNA repair dysregulation and genetic variation in DNA genes may influence AML prognosis/risk and determine treatment outcome following chemotherapy (Allan *et al.*, 2004; Kuptsova *et al.*, 2007; Saitoh *et al.*, 2013; Esposito and So, 2014). Despite these advances, relatively little is known about the utility of APE1 and OGG1 as prognostic markers in AML.

Although altered *APE1* expression correlates with prognosis and overall survival in several solid tumours (Wang *et al.*, 2009a; Al-Attar *et al.*, 2010; Abdel-Fatah *et al.*, 2014; Woo *et al.*, 2014), there was no association between *APE1* expression and overall survival in AML. In addition, OGG1 prognostic utility is inconclusive, despite previous report indicating its prognostic value in AML (Liddiard *et al.*, 2010). However, this is explained by extreme cytogenetic heterogeneity of AML and the presence of oncogenic fusion proteins that may limit any effect of altered *APE1*/*OGG1* expression. APE1 and OGG1 are sensitive biomarkers for oxidative stress induced DNA damage and their upregulation or downregulation is dependent on the type of DNA damage and possibly the molecular basis of AML (Li and Wilson, 2014; Thakur *et al.*, 2014). Accumulating evidence has associated oncogenic fusion proteins (such as AML1-ETO and PML-RARα) with defects in DNA repair, which result in further genomic instability that may drive disease progression (Alcalay *et al.*, 2003; Boehrer *et al.*, 2009; Petruccelli *et al.*, 2013; Cheng *et al.*, 2015; Forster *et al.*, 2016). On the other hand, high oxidative stress in AML may induce DNA damage and activate DNA damage response pathways (Seedhouse *et al.*, 2006; Cavelier *et al.*, 2009). Therefore, it is likely that the application of APE1 and OGG1 as independent prognostic markers in AML would be challenging, but could be feasible if combined with other AML prognostic markers such as FLT3, NPM1, N-RAS. Larger scale studies would be required to fully evaluate the prognostic value of APE1 and OGG1, taking into consideration other prognostic factors in AML and the extreme heterogeneity of the disease.

APE1 and *OGG1* gene expression was determined in several AML cell lines using RT-PCR and results were normalised to APE1 and OGG1 expression of NB4 cells. Although the expression of both genes in AML cells should be normalised to its relevant expression in normal haematopoietic cells such as +CD34 cells, this was difficult due to inability to obtain these cells. However, *APE1* and *OGG1* mRNA was differentially expressed in 8 AML cell

lines including HL-60, AML2, AML3, U937, MV4-11, NB4, Kasumi-1and THP-1. Notably, kasumi-1 cells showed highest *APE1* expression and lowest *OGG1* expression. APE1 and OGG1 protein was expressed in all AML cell lines investigated, but was not completely correlated with mRNA expression. The disparity between mRNA and protein expression of both APE1 and OGG1 suggests the presence of mechanisms that regulate protein levels, possibly through post-translational modifications, which can affect stability as well as function. Such mechanisms have been well demonstrated for both OGG1 and APE1 and include phosphorylation, acetylation and ubiquitination (Yacoub *et al.*, 1997; Dantzer *et al.*, 2002; Bhakat *et al.*, 2003; Bhakat *et al.*, 2006; Busso *et al.*, 2009) (see section 3.1.3 for more details about regulation of APE1 and OGG1 by post-translational modification).

OGG1 mRNA expression was consistent with its protein level in kasumi-1 cell line. Kasumi-1 cells carry the AML1-ETO fusion protein, which is known to negatively regulate OGG1 and suppress its transcription resulting in reduced protein levels (Alcalay *et al.*, 2003; Liddiard *et al.*, 2010; Forster *et al.*, 2016). Despite this, it is not clear whether this has biological effect on the protein function.

3.5. Summary of chapter

In summary, the results described in this chapter demonstrate:

- *APE1* expression in AML has no correlation with overall survival and may has no prognostic value.
- Prognostic impact of OGG1 is not conclusive in AML and require further study.
- *APE1* and *OGG1* mRNA expression vary between different cell lines
- Both APE1 and OGG1 are ubiquitously expressed on protein level in all investigated AML cell lines.

Chapter 4: Targeting APE1 in AML cells using short hairpin RNA (shRNA) interference.

4.1. Introduction

Treatment of AML is challenging and disease relapse is a major clinical problem, particularly in elderly patients and patients with unfavourable prognosis. Treatment outcome with current AML conventional chemotherapeutic regimens is still unsatisfactory, and finding new targeted therapies is certainly required. Identification of new targeted therapies essentially relies on exploring specific genetic alteration in AML cells that drive disease progression, cell survival and/or treatment resistance. Dysregulation of DNA repair pathways has been linked to AML promotion and treatment response (Rassool *et al.*, 2007; Fordham *et al.*, 2011; Esposito and So, 2014). Therefore, targeting dysregulated DNA repair pathways may be of therapeutic value for AML patients, and may increase efficacy of current treatments and minimise chemoresistance.

The value of targeting DNA repair components has been already demonstrated in preclinical and clinical studies. Focusing on the BER pathway, several components of this pathway have been extensively exploited for therapeutic purposes, including PARP-1, APE1, XRCC1 and polymerase β (Barakat *et al.*, 2012; Curtin and Szabo, 2013; Sultana *et al.*, 2013). However, only a few DNA repair inhibitors have been investigated in clinical trials, particularly PARP-1 inhibitors and methoxyamine, both inhibitors of the BER pathway. However, APE1 has been scrutinised as a therapeutic target in cancer. In particular, there is no known backup mechanism for its function in base excision repair as it is the only recognised protein responsible for cleaving abasic sites generated following removal damaged bases by DNA glycosylases.

APE1 is a key protein in the BER pathway and silencing/inhibition of this protein has been shown to reduce DNA damage repair capacity and potentiate the cytotoxicity of chemotherapeutic agents (Bapat *et al.*, 2010; Bulgar *et al.*, 2010; Fishel *et al.*, 2010; Mohammed *et al.*, 2011; Cun *et al.*, 2013). However, despite the promising reports that showed the utility of APE1 in cancer, only limited studies have attempted to characterise its therapeutic benefit in AML (She *et al.*, 2005; Bulgar *et al.*, 2010; Fishel *et al.*, 2010; Vascotto *et al.*, 2013). Nevertheless, these studies are interesting and should be confirmed using other techniques including siRNA/shRNA that would validate the findings. Furthermore, these studies did not demonstrate the effect of APE1 inhibition/knockdown on the cellular response to cytotoxic chemotherapy agents essential in AML treatment such as Ara-C, daunorubicin, clofarabine and etoposide.

Colleagues in the Institute for Cell and Molecular Biosciences at Newcastle University demonstrated that APE1 inhibition using CRT0044876 (Calbiochem, UK) led to an accumulation of alpha and beta topoisomerase 2 complexes (TOP2A and TOP2B) in K562 AML cell line. This was investigated using the Trapped in Agarose DNA Immunostaining (TARDIS) assay. Rescue experiments after APE1 inhibition were performed by addition of 25 unit of recombinant APE1 (New England Biolabs, UK) to K562 cells, which resulted in removal of approximately 30% of the TOP2A adducts and 15% of the TOP2B adducts (data not shown). However, this led to the hypothesis that APE1 knockdown may result in accumulation of TOP2A and TOP2B complexes in AML cells. Thus, APE1 knockdown cells generated in this work were used to investigate this hypothesis.

4.1.1. RNA interference and genome editing techniques as tool to silence gene expression

RNA interference (RNAi) refers to a process by which double stranded RNA (dsRNA) interferes and silences mRNA expression of a target gene. This is achieved by either degradation of mRNA of the target gene by siRNA (small interfering RNA) or shRNA (short hairpin RNA), or via suppression of translation of specific mRNA, which could be achieved by miRNA (microRNA). Furthermore, recent advances in CRISPR/cas9 genome editing have been developed providing a tool to knockout specific genes more efficiently. RNAi and genome editing techniques are an important tool in biomolecular studies which can be used to identify gene function in normal cells, and their role in disease phenotype.

4.1.1.1. siRNA

Small interference RNA (siRNA) is a commonly used RNAi technique to induce transient and short-term silencing of gene expression. It consists of double RNA strands, sense (passenger) and antisense (guide) strands which constitute 21-23 base pair nucleotides with a dinucleotide overhang at the 3' end (Whitehead *et al.*, 2009). This method utilises either electroporation by electrical pulse, or transfection using cationic liposomes or polymer based transfection method to deliver siRNA molecules to the cell (Whitehead *et al.*, 2009). Upon siRNA delivery to the cell cytoplasm, it is incorporated into the RNA induced silencing complex (RISC), which is consequently cleaved by Argonaute-2 to separate dsRNA. This results in activation of the RISC complex to guide the antisense siRNA strand to bind to its complementary mRNA and induce it degradation (Figure 4.1). Although this method provide

simple, fast and satisfactory knockdown of protein of interest, the disadvantage of transient knockdown and off-target issues limit the benefit of its use. Additionally, delivery methods of siRNA may have an effect on cellular phenotype.

4.1.1.2. shRNA

Unlike siRNA, shRNA induces long term, stable and more efficient knockdown of target gene mRNA expression. It consists of a 21-23 base double-stranded molecule with a loop region and dinucleotide overhang at the 3' end (Figure 4.1). However, shRNA expression can be driven from a vector following transduction into host cells using viral particles. Viral particles are able to penetrate the target cell and the shRNA expression vector is able to integrate into the host genome where it is constitutively transcribed to generate pre-shRNA. Pre-shRNA is then processed and cleaved by the dicer/TRBP (Tat–RNA-binding protein) complex to further generate mature shRNA. The resulting shRNA complex is exported to cell cytoplasm by exportin-5. The shRNA- dicer/TRBP complex associates with Argonaute-2-RISC complex generating siRNA molecules that cleave and degrade mRNA of the target gene (Rao *et al.*, 2009). A selection marker encoded into the shRNA allows for selection of cells that have been successfully transduced. Furthermore, recent developments in shRNA based technology have improved and modified this system to allow for transient or stable shRNA expression by a tetracycline inducible expression vector.

4.1.1.3. microRNA

MicroRNAs (miRNA) can be used to regulate mRNA gene expression, but which do not necessarily target and cleave mRNA unless there is a complete match of mRNA and miRNA. miRNA consists of single stranded 19-25 mer fragments which do not exactly match the specific target mRNA; hence could non-specifically targets several mRNAs. miRNAs are endogenously transcribed by the host cell genome as pre-miRNA, and are then exported to the cell cytoplasm where they become associated with an enzyme called Drosha. The dicer-RISK- Argonaute-2 complex then cleaves the miRNA molecule to induce transcriptional repression of protein production (Mack, 2007). Inhibition of translation occur when miRNA is partially complementary at the 3' UTR (untranslated region) region of the mRNA. However, cleavage and degradation of mRNA can occur if the miRNA is completely complementary to the target mRNA nucleotide sequence.

4.1.1.4. CRISPR/ cas9

Clustered regularly interspaced short palindrome repeats associated nuclease Cas9 (CRISPR/cas9) is a newly introduced technology that allows genome editing and knock-out of specific genes more efficiently compared to siRNA and shRNA. It consist mainly of two components; guide RNA (gRNA) and cas9 which has endonuclease activity. gRNA includes the sequence required for Cas9 binding and a sequence of approximately 20 nucleotides of target gene or spacer. Similar to shRNA, CRISPR/cas9 expression can be encoded into a vector and packaged inside viral particles for target cell transduction. Cas9 guided by gRNA induces DNA double strand breaks at specific genomic loci, which can be repaired by either non-homologous end joining (NHEJ) or homology direct repair systems. Both systems can imprecisely repair double strand breaks by introducing frameshift, insertion or deletion mutations at coding regions of target genes, which can result in a loss of function (Sander and Joung, 2014).

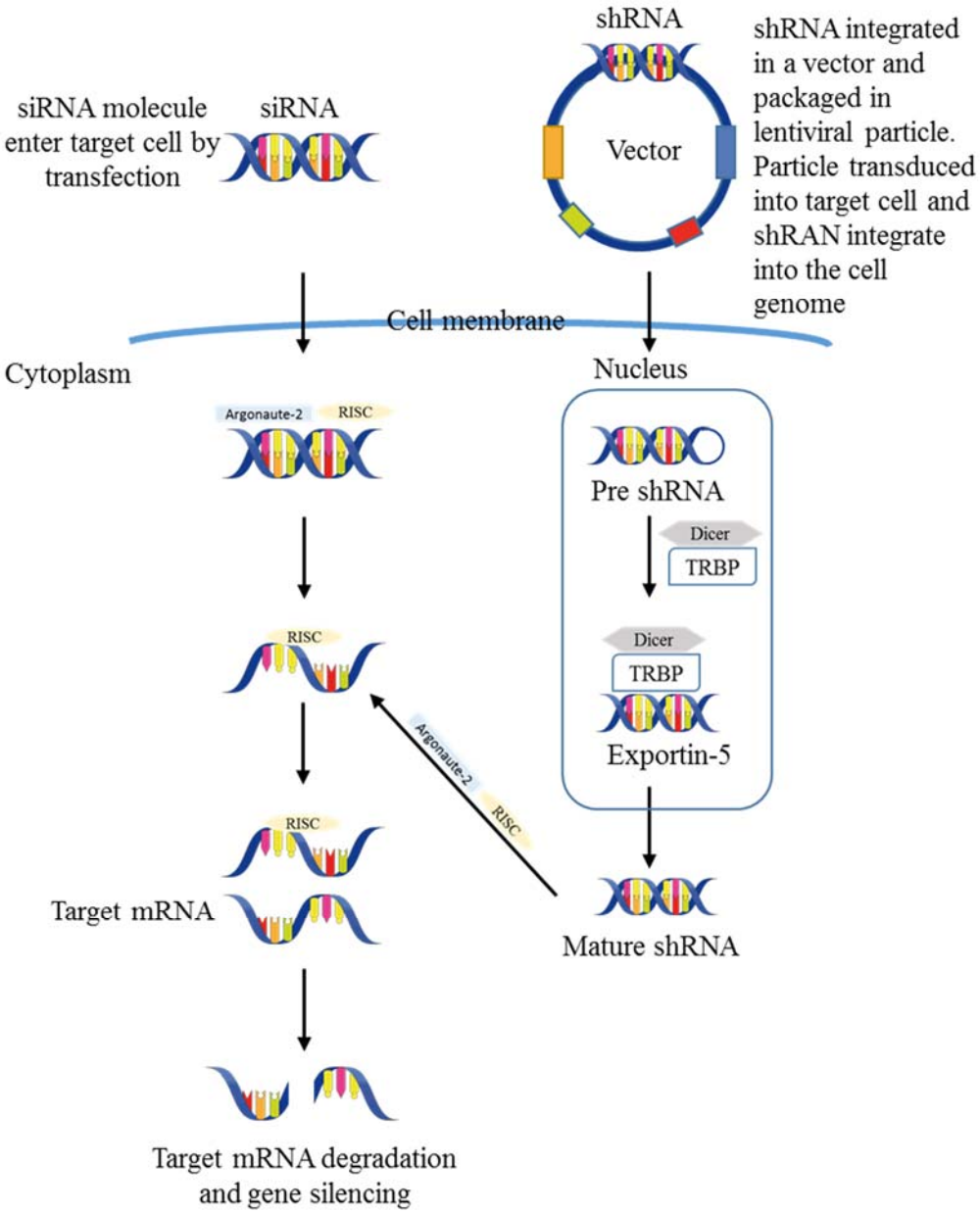


Figure 4.1: Schematic representation of gene silencing by siRNA and shRNA.

siRNA molecule is transfected into target cell using electroporation, or by transfection using cationic liposomes or polymer based transfection method. In the cytoplasm of target cell, siRNA incorporate into the RNA induced silencing complex (RISC) and cleaved by Argonaute-2 to separate double strand siRNA. This results in activation of the RISC complex to guide the antisense siRNA strand to bind to its complementary mRNA and induce its degradation. shRNA molecule is incorporated into a plasmid vector and packaged into viral particles. Viral particles are able to penetrate the target cell and the shRNA expression vector is able to integrate into the host genome where it is transcribed to pre-shRNA. Pre-shRNA is then processed and cleaved by the dicer/TRBP complex to mature shRNA. The shRNA- dicer/TRBP (Transactivating response –RNA-binding protein) complex associates with Argonaute-2-RISC complex generating siRNA molecules that cleave and degrade mRNA of the target gene.

4.2. Aims of this chapter

Despite the promising reports about targeting APE1 in cancer, this area of research is still unexplored in leukaemia, including AML. The aims of the studies described in this chapter were to determine whether APE1 silencing using shRNA sensitises AML cells to chemotherapy-induced cytotoxicity.

Specific experimental aims of this chapter were as follows:

- Generate stable APE1 deficient cells using shRNA and determine the phenotype effect of APE1 deficiency on AML cell proliferation and cloning efficiency.
- Determine the sensitivity of AML cells to different chemotherapeutic agents following APE1 knockdown.
- Determine AP site accumulation subsequent to APE1 silencing.
- Study the effect of APE1 knockdown on cell cycle kinetics.
- Determine accumulation of topoisomerase II α and β complexes following APE1 silencing.
- Study global gene expression changes following APE1 knockdown.

4.3. Results

4.3.1. TC20™ cell counter validation

The TC20™ cell counter (Bio-Rad – UK) is an automated cell counter used for cells counting during this project. Manual cell counting using the Neubauer haemocytometer is time consuming and cell counting is prone to user variability. In order to standardise cell counting and minimise variability, the TC20™ automated cell counter was used to provide fast, accurate and reproducible cell counts. Validation of the TC20™ automated cell counter was achieved by comparing manual cell counts using a Neubauer haemocytometer with the automated cell count for every cell line used in this project.

TC20™ cell counter generated reproducible cell counting with minimal variation compared to manual counting with haemocytometer (Figure and table in appendices B and C). Some variability in results was observed when cell counts fell below $1x10^5$ cell/ml, which is also a recognised problem with manual cell counting.

4.3.2. Generation of AML cell lines with stable APE1 knockdown using small hairpin RNA (shRNA)

Five different lentiviral particles carrying APE1 shRNA target sequence (Table 2.3) were used to knockdown APE1 in 3 AML cell lines including HL-60, AML3 and U937. Control shRNA, which contains non-human off -target shRNA sequence, was used alongside APE1 shRNA. AML3, U937 and HL-60 were selected as models for AML in experimental work during this project for general and specific reasons, as follows: these cell lines have a relatively short cell cycle (24-30 hours) and have good cloning efficiency in semi-solid soft agar. AML3 cells were specifically selected because it is the only AML cell line with a nucleophosmin-1 (*NPM1*) gene mutation, which is frequently mutated in AML, and its inclusion in this work may reflect the effect of APE1 inhibition on AML with *NPM1* mutation.

Several lentiviral shRNA constructs were able to efficiently silence APE1. Western blotting showed that shRNA constructs C1, C2 and C5 achieved 67% to 92% APE1 knockdown at the protein level (Figure 4.2), with the C5 construct the most efficient at inducing knockdown. As such, construct 5 (C5) was used in the work presented in this chapter. C5 was designed to target the GCCTGGACTCTCTCATCAATA sequence in of *APE1*. The C5 construct uses

pLKO_TRC005 backbone vector, which is a modified and improved version of pLKO.1 vector.

ShRNA knockdown work used a multiplicity of infection of 2 to minimise multiple integration events of the shRNA constructs into cell genome. Multiplicity of infection is the ratio of shRNA lentiviral particle to cells. Following shRNA transduction, transduced cell populations were exposed to puromycin to selectively kill non-transduced cells; cells carrying transduced puromycin resistance genes are not killed by puromycin. Transduced cells were then maintained indefinitely in puromycin selection RPMI media.

It was expected that cell populations knocked down for APE1 would express variable levels of APE1 protein on single cell level. Therefore, cells were seeded at low density and colony formation assay was performed to generate cell clones originated from single cell with stable APE1 knockdown. Cells were grown on semi-solid soft agar for 14 days, then isolated and expanded for further characterisation of APE1 expression.

APE1 protein level in AML cell clones was determined using western blotting and quantified using Fuji densitometry. As expected, APE1 protein expression varied between different clones and different cell lines (Figures 4.3 and 4.4). Some clones showed negligible protein levels. For example, AML3 clones C and D express very low APE1 protein level at 20% and 15% of APE1 protein, respectively, compared to normal control cells. However, some clones showed no reduction in APE1 protein level despite being puromycin resistant. This was possibly caused by incomplete integration of shRNA particle into the cell genome and failure to achieve sufficient knockdown. Complete knockdown of APE1 was not initially observed in AML3, U937 or HL-60 AML cell clones, but this was achieved in subsequent experiments.

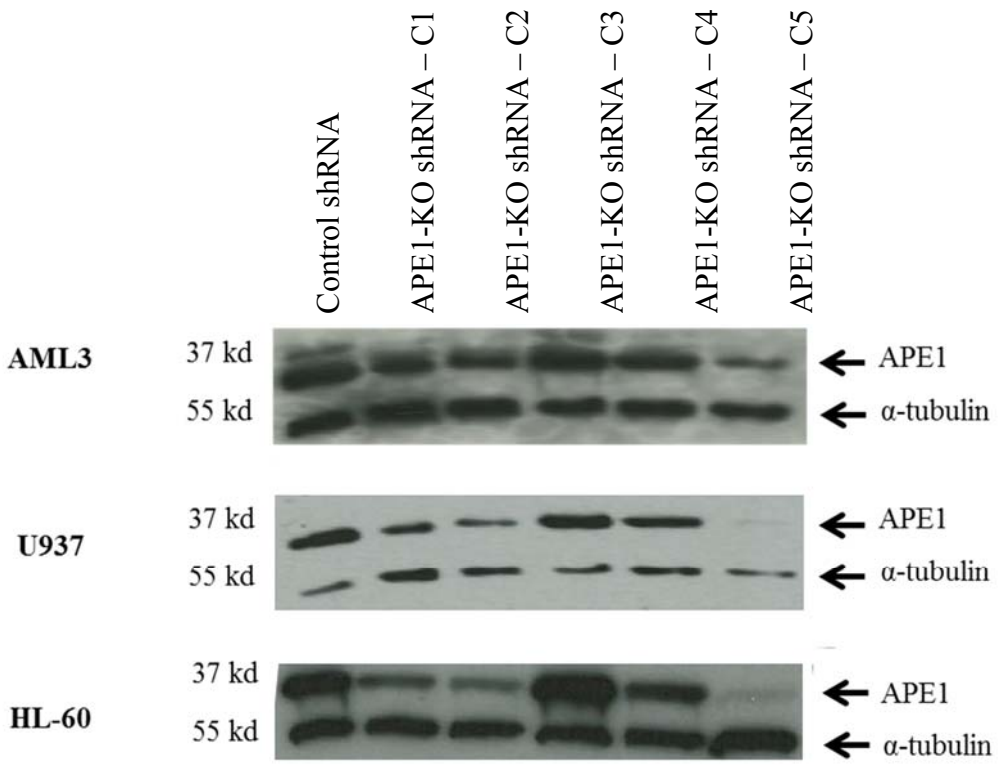


Figure 4.2: APE1 protein expression in AML cell lines following APE1 knockdown.

Five lentiviral shRNA constructs (C1 – C5) were used to knockdown APE1 in 3 AML cell lines; AML3, U937 and HL-60. APE1 knockdown was investigated using western blotting to determine APE1 protein level. Only constructs C1, C2 and C5 generated considerable APE1 knockdown in all cell lines. Alpha tubulin was used as a loading control. Each blot is representative of two independent experiments.

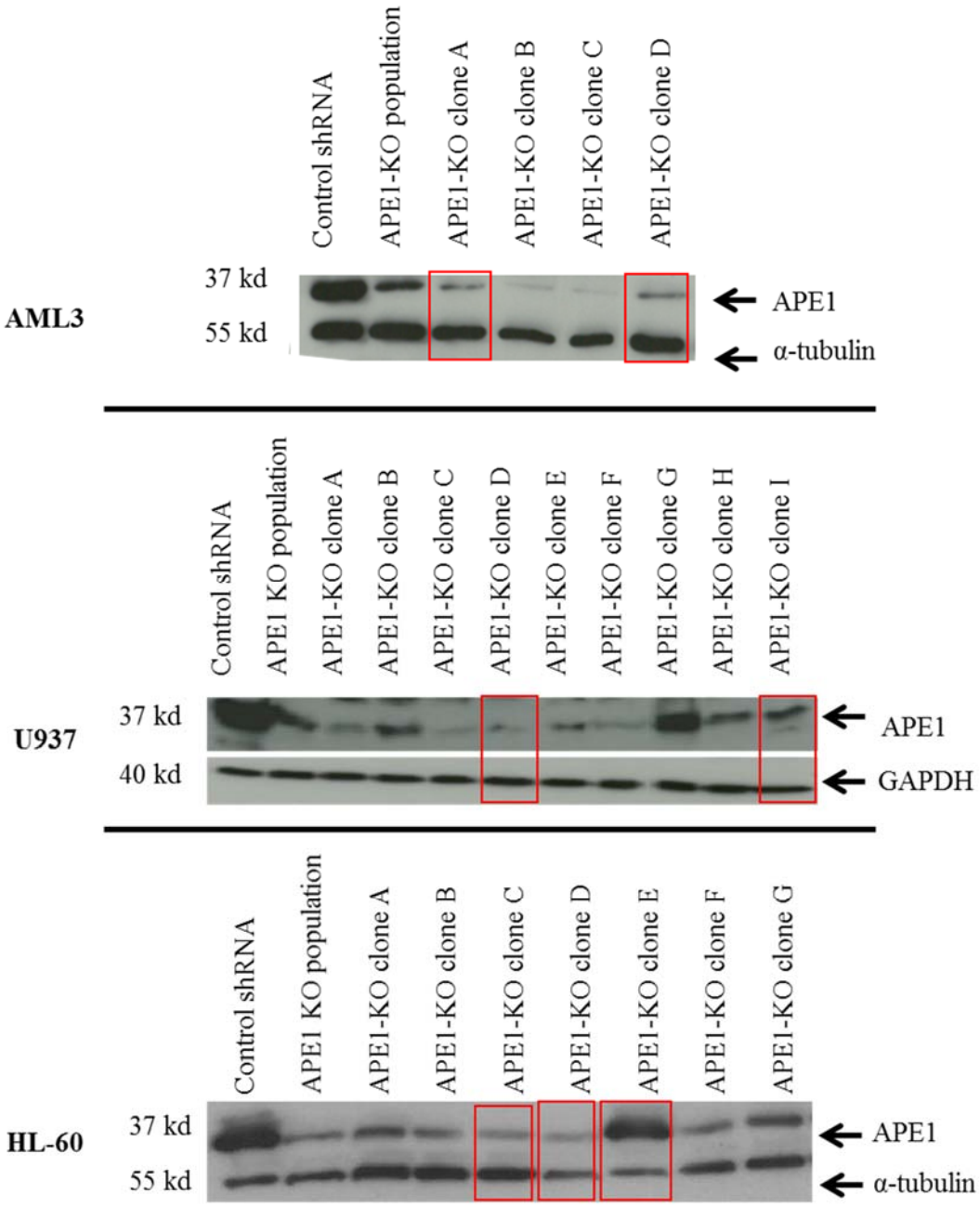


Figure 4.3: APE1 protein expression in AML3, U937 and HL-60 subclones generated following APE1 knockdown.

Cell clones were generated on semi-solid soft agar, from AML3, U937 and HL-60 cells populations transduced with shRNA C5. APE1 protein expression was determined by western blotting following APE1 knockdown. APE1 protein levels vary between different clones. Non-target shRNA (control shRNA) was used as control for effects of the transduction process on protein expression. Subclones highlighted in red box were used later in growth inhibition assay investigations. Alpha tubulin was used as a loading control for AML3 and HL-60 cell lines. GAPDH was used loading control for U937 cells.

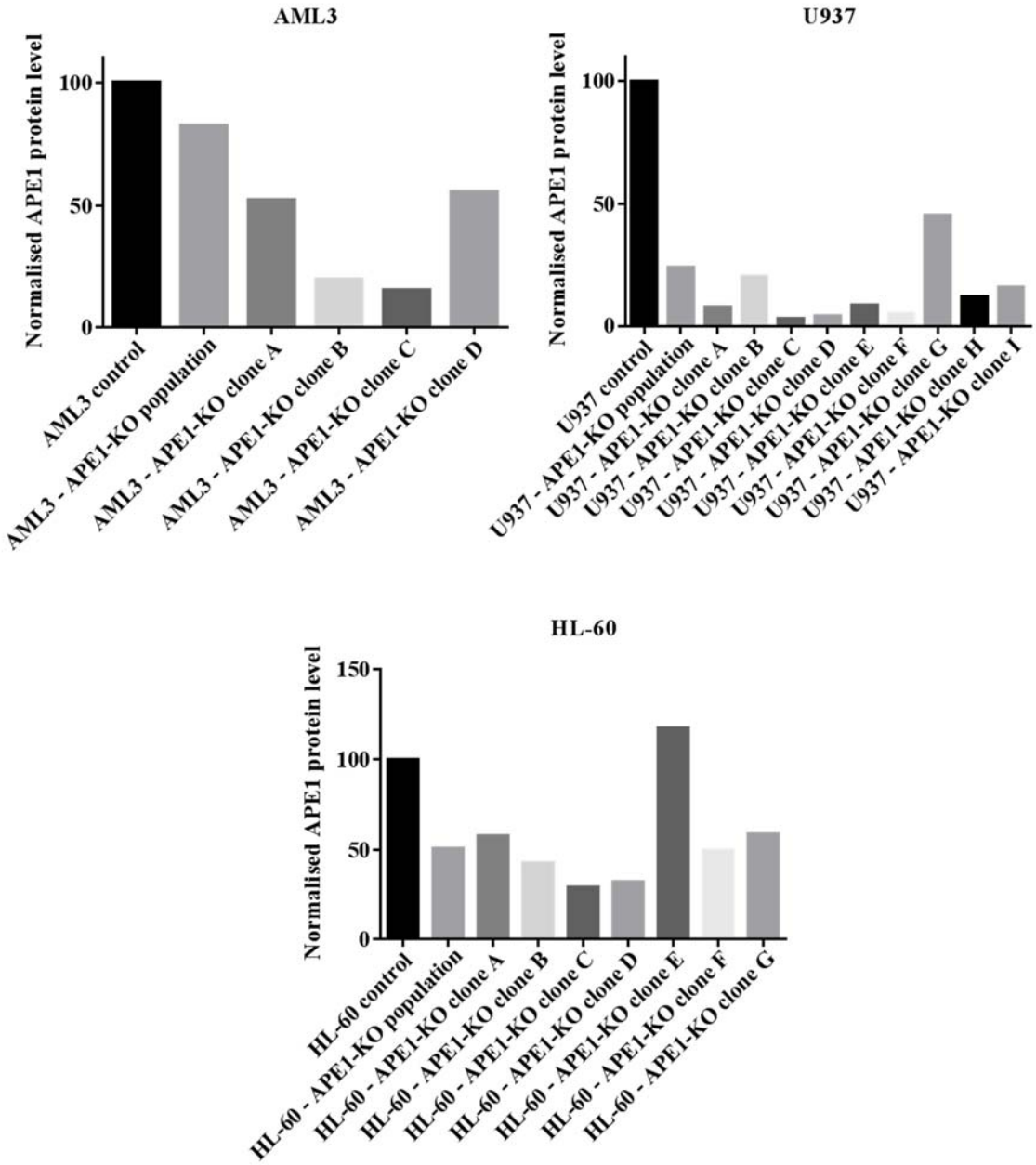


Figure 4.4: Quantification of APE1 protein after APE1 knockdown in AML cells.

APE1 protein bands and α-tubulin loading controls were quantified from western blots in figure 4.3 using Fuji densitometer. APE1 protein levels were firstly normalised to α-tubulin loading control for each cell line/clone. Normalised APE1 protein levels were relatively normalised to the level of APE1 protein in respective parental control cells. Data presented as the percentage of APE1 protein level relative to control cells.

4.3.3. Effects of APE1 knockdown on AML cell proliferation and cloning efficiency

Cell proliferation and colony formation assay survival experiments were performed to determine the effect of APE1 knockdown on AML cell growth and viability. Proliferation was determined using absolute cell count performed by the TC-20 cell counter and trypan blue exclusion dye. All AML cell lines were counted on the first day and seeded at an initial density of $5x10^4$ cells/ml and counted every 24 hours for 5 days.

ShRNA mediated APE1 silencing significantly slowed cell proliferation in HL-60, AML3 and U937, which was relatively correlated with APE1 knockdown level in the majority of cases (Figure 4.5). For example, HL-60 (clones C and D), U937 (clones C and D), and AML3 (clones B, C and D) had significantly slower proliferation rates compared to their respective controls and compared to clones with APE1 expression. HL-60 clone F, U937 clone B and AML3 clone A expressed relatively higher APE1 protein levels and proliferation was comparable to controls. These data are consistent with previous reports that APE1 is required for normal cell proliferation, and RNAi mediated silencing or inhibition of APE1 reduces cells proliferation (Bapat *et al.*, 2010; Zheng *et al.*, 2015).

Colony formation assays (CFA) were also performed to investigate the effect of APE1 knockdown on the ability of AML cells to survive in semi-solid soft agar.

APE1 silencing was associated with a significant reduction of cloning efficiency in AML3, U937 and HL-60 (Figure 4.6). In particular, AML3 cell line was particularly hypersensitive to APE1 knockdown. AML3 clone C were not able to form colonies and had a very slow cell proliferation rate.

To confirm whether APE1 protein level significantly correlate with proliferation and cloning efficiency, APE1 protein levels in AML cells (measured by densitometer in figure 4.4) were plotted against normalised proliferation rates and cloning efficiency for each cell line/clone to generate linear regression plot. Result showed significant correlation between APE1 protein level and cell proliferation and cloning efficiency in the majority of cases (Figure 4.7).

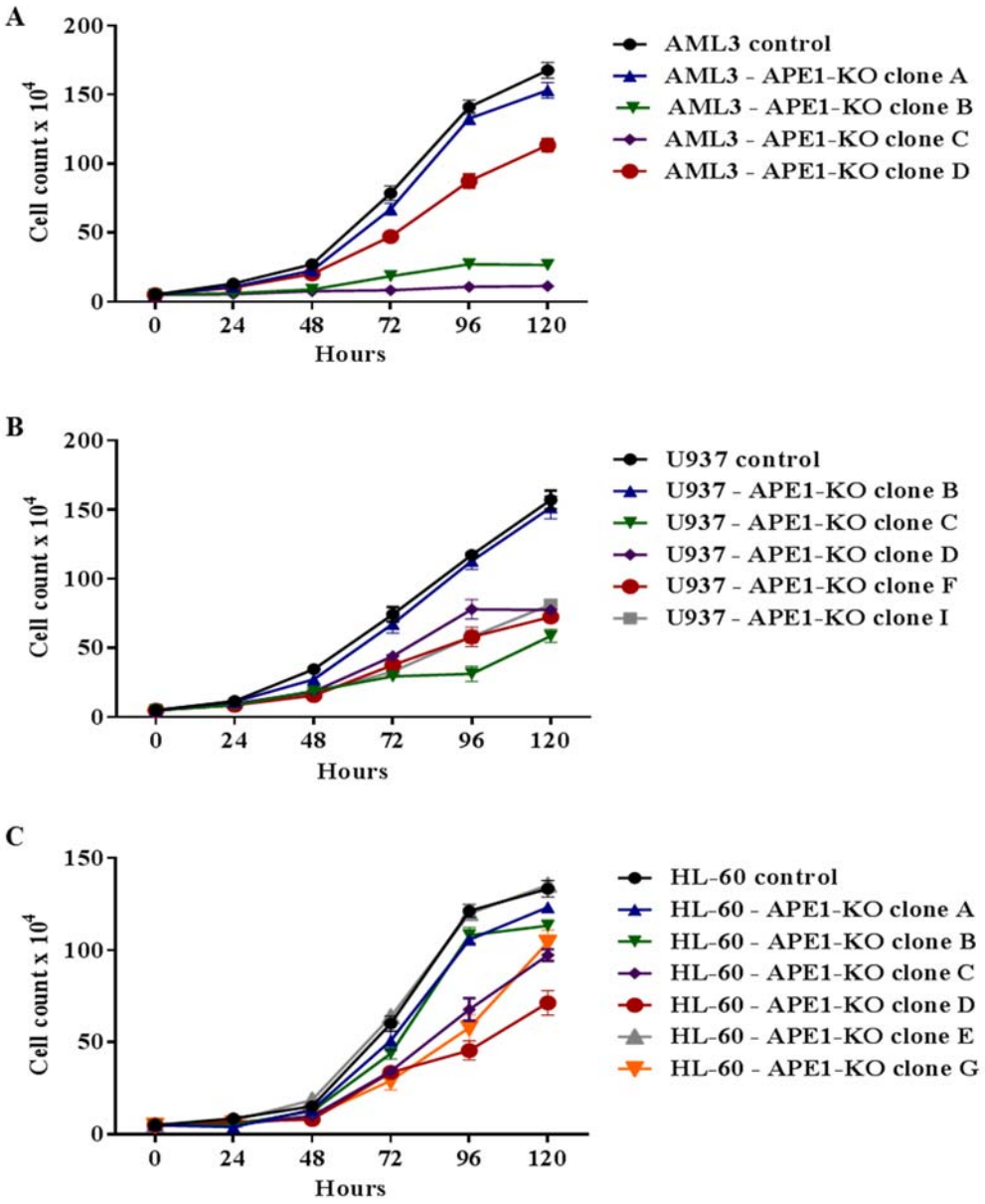


Figure 4.5: Effect of APE1 shRNA mediated knockdown on AML cells proliferation.

APE1 knocked down (APE1-KO) clones and off-target shRNA control cells were seeded at the density of $2x10^4$ cells/ml and cell count was performed every 24 hours for 5 days. APE1 knockdown significantly reduced cell proliferation of **(A)** AML3 **(B)** U937 and **(C)** HL-60 cells. Reduction of cell proliferation is consistent with APE1 knockdown level. All data presented are the mean and standard deviation of 3 independent experiments.

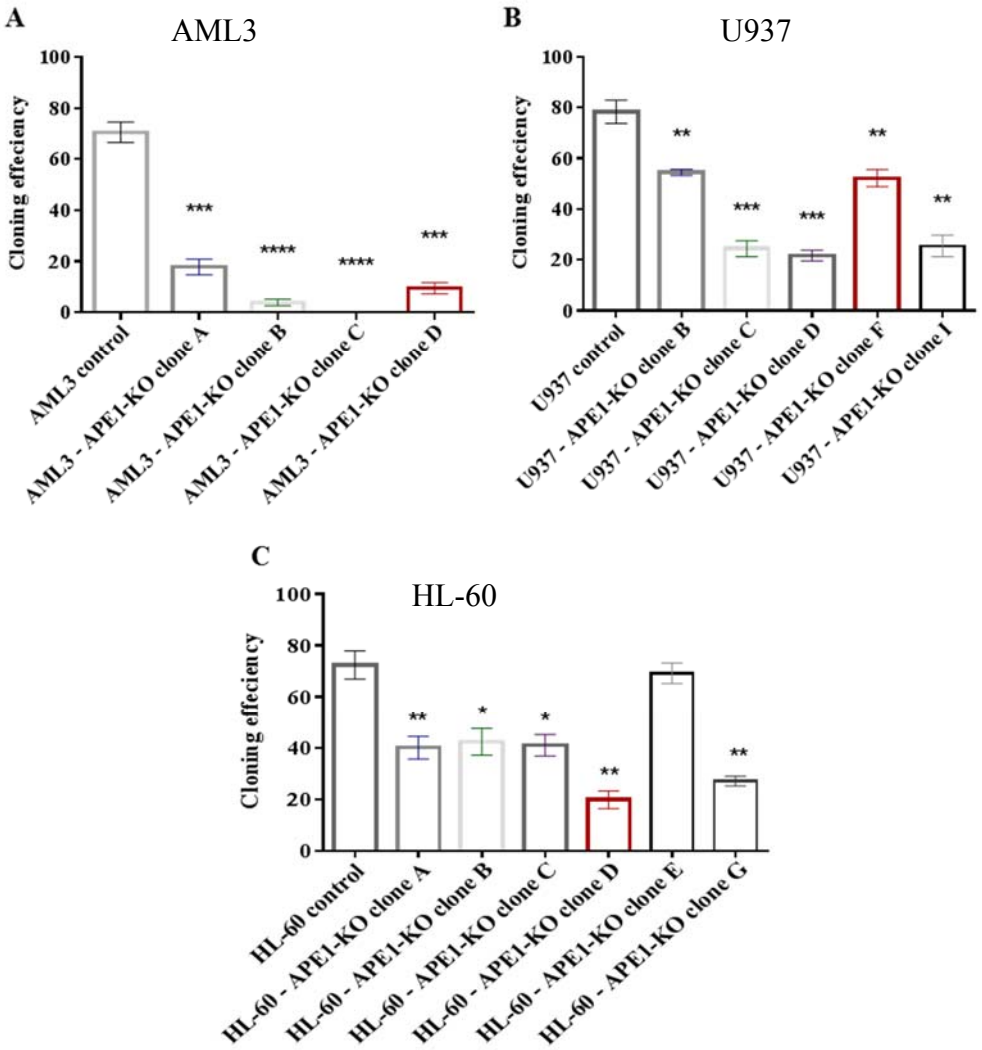


Figure 4.6: Effect of APE1 shRNA mediated knockdown on AML cells cloning efficiency.

APE knockdown in (A) AML3, (B) U937 and (C) HL-60 reduced their cloning efficiency. Data represent the mean of 3 independent experiment and error bar of standard deviation. Unpaired parametric t test was used to calculate p values. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

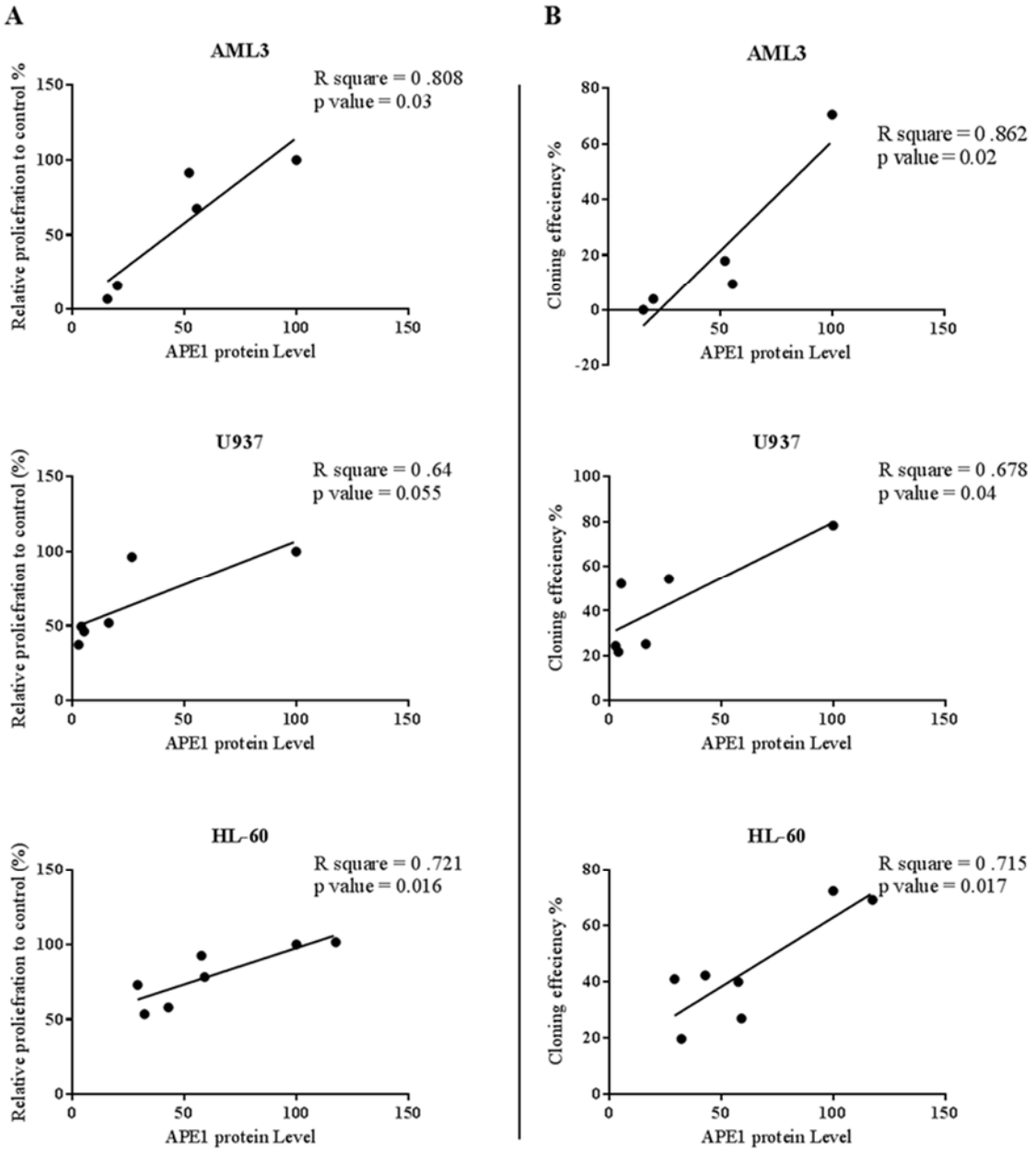


Figure 4.7: Correlation between APE1 protein level in AML cells, and proliferation rate and cloning efficiency.

APE1 protein levels were plotted against **(A)** proliferation rate (relative to controls) and **(B)** absolute cloning efficiency of AML3, U937 and HL-60 cells/clones. Linear regression plot was generated and analysis showed significant correlation between APE1 protein level and proliferation rate and cloning efficiency. P value < 0.05 were considered significant.

4.3.4. APE1 re-expression in AML enhanced cell proliferation

APE1 protein expression was assessed in APE1 knockdown clones at 4, 8 and 12 weeks of shRNA transduction by western blotting (APE1 knockdown levels after 4 weeks are shown in Figure 4.5). APE1 protein expression was stably knockdown in all investigated AML cells, however, it was re-expressed after 12 weeks in continuous culture for all three AML cell lines investigated. Furthermore, APE1 re-expression was associated with an increase in proliferation rate comparable to that seen in control cells (Figures 4.8 and 4.9). For example, AML3 APE1-KO clones B and C, which had a very low proliferation rate and low APE1 expression immediately post-transduction (Figure 4.5), retained their normal growth rate upon APE1 re-expression. This result provides further evidence that APE1 expression is directly associated with AML cell proliferation.

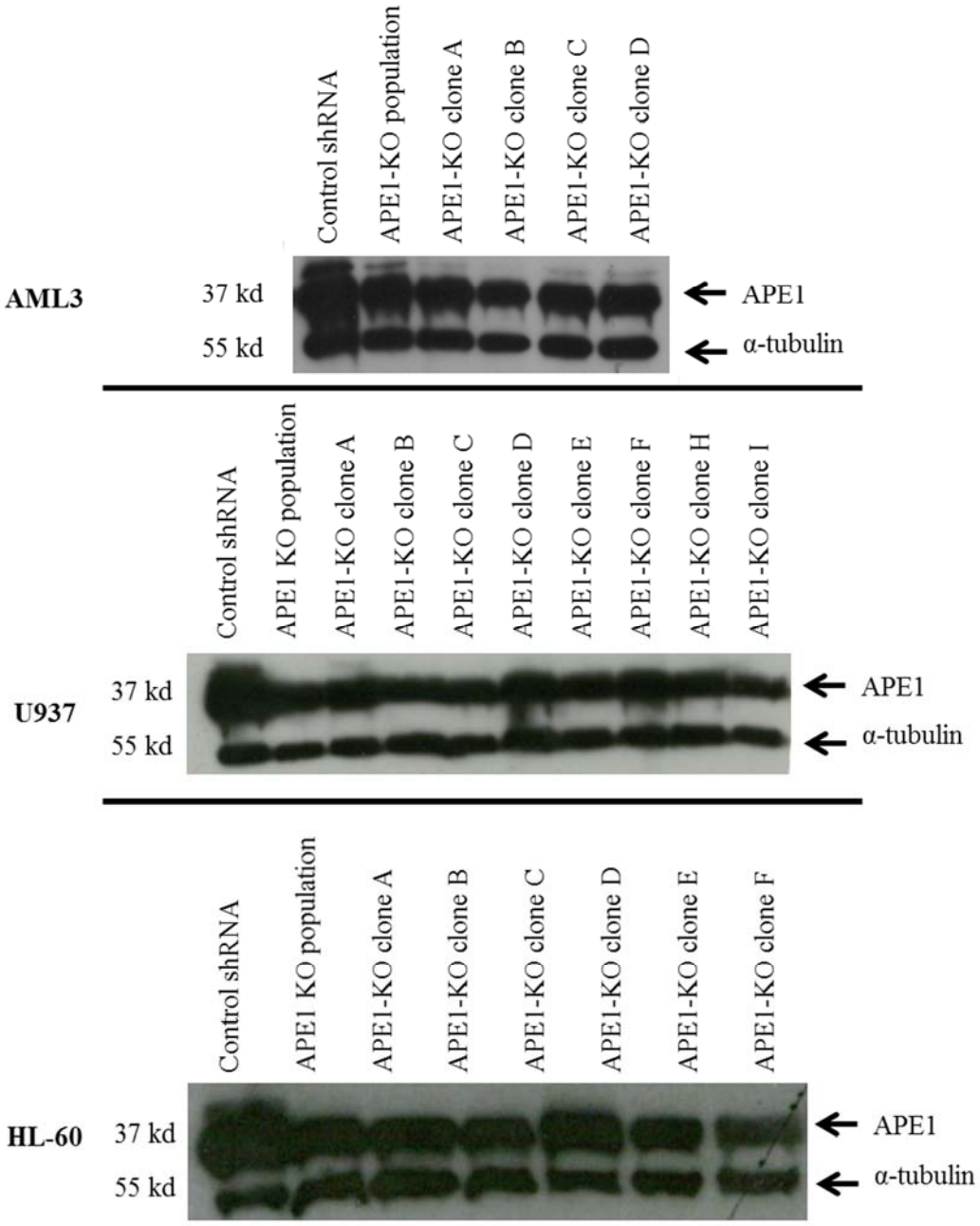


Figure 4.8: APE1 protein re-expression in APE1 knockdown cells after 12 week of shRNA transduction.

APE1 protein expression in APE1 knockdown cells was investigate every for 4 weeks after shRNA knockdown. APE1 protein was re-expressed after 12 weeks of knockdown.

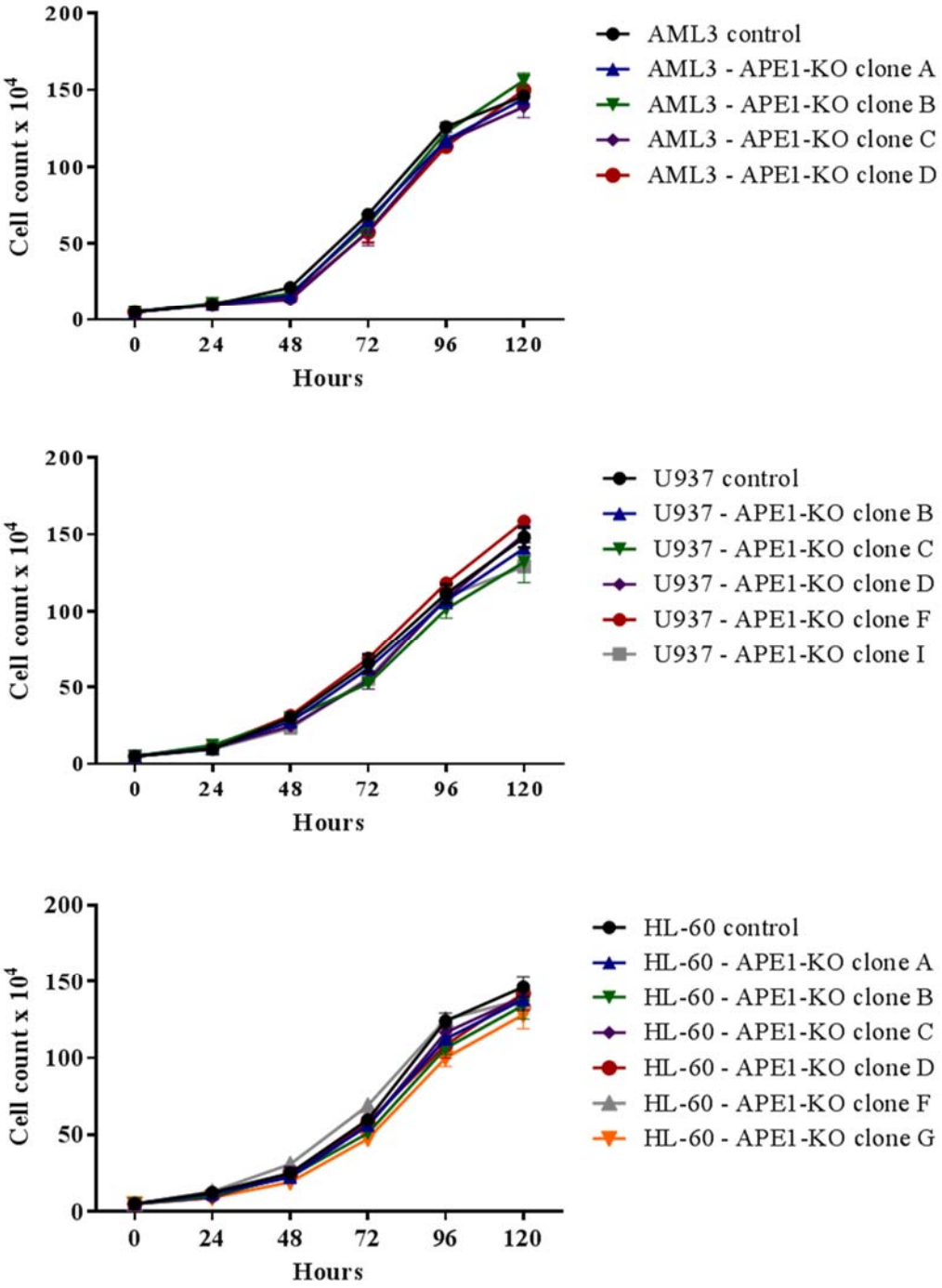


Figure 4.9: Effect of APE1 re-expression on cell proliferation of AML cells with APE1 knockdown.

AML cells with APE1 knockdown retain normal proliferation after simultaneous APE1 re-expression after 12 weeks of APE1 knockdown with shRNA.

4.3.5. Effect of APE1 knockdown on AML cell cycle

APE1 knockdown significantly reduced cell proliferation of AML cell lines AML, U937 and HL-60 as demonstrated in section 4.3.3. This may suggest a possible role for APE1 in the regulation of the cell cycle and induction of cell cycle arrest. To investigate this hypothesis, cell cycle distribution was investigated, and performed on unsynchronised APE1 knockdown cell clones and shRNA control cells. Propidium iodide (PI) was used to stain cellular DNA as described in section 2.9. Intriguingly, however, no alteration in cell cycle distribution was observed in APE1 knockdown cells (Figure 4.10). Further quantitation of cell cycle distribution using CellQuest software showed no significant alteration in APE1 knockdown cell clones compared to control clones (Table 4.1 and Figure 4.11).

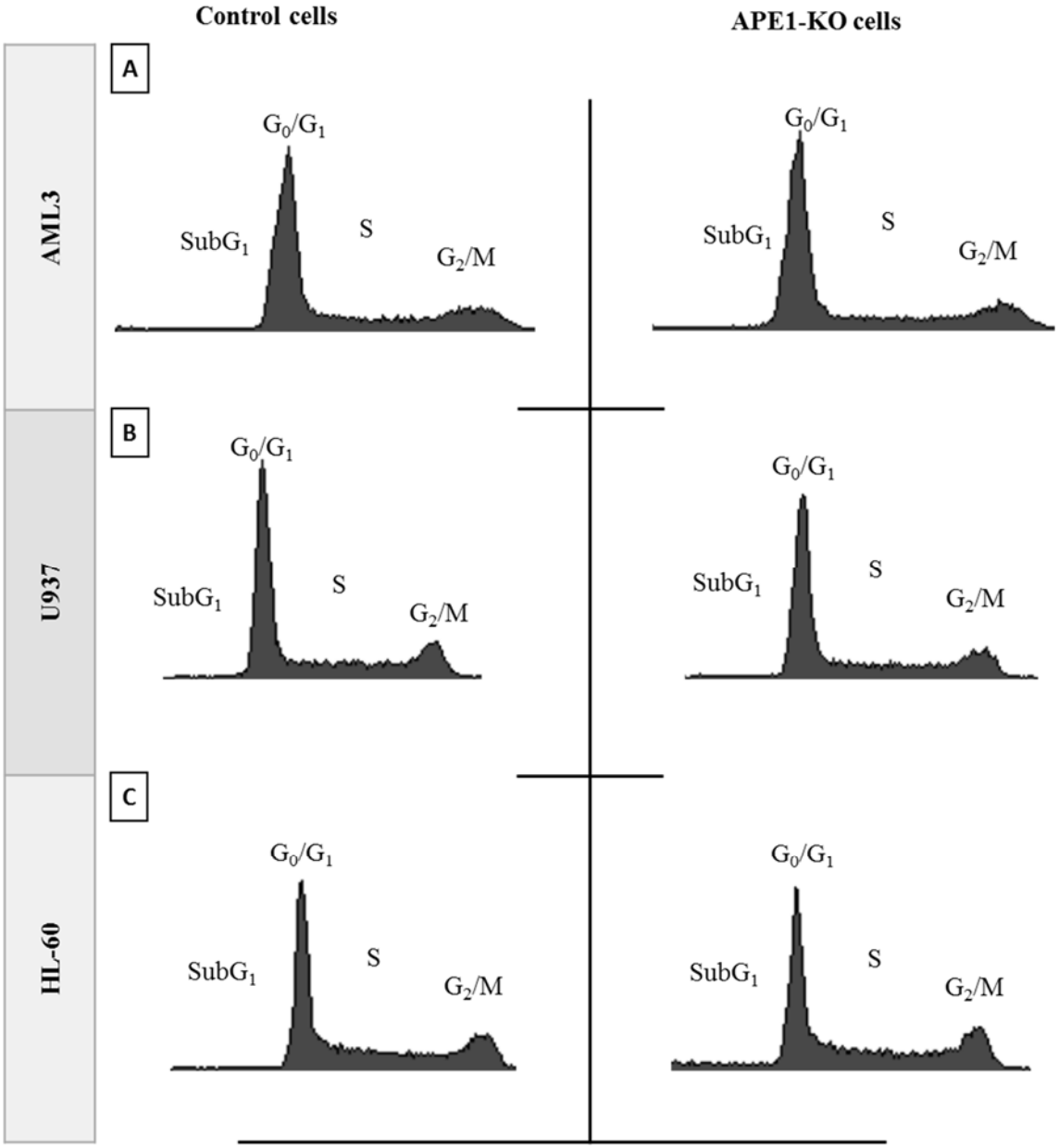


Figure 4.10: The effect of APE1 knockdown on AML cell cycle distribution.

Cell cycle analysis was performed on flow cytometer to investigate the effect of APE1 knockdown on cell cycle kinetics in **(A)** AML3, **(B)** U937 and **(C)** HL-60. Figures in the left panel represent shRNA off-target control cells and figures at the right panel represents APE1 knockdown (APE1-KO) cells. Cell cycle analysis was performed on 3 independent experiments. No alteration in cell cycle distribution was observed following APE1 knockdown in AML cells.

Cells	SubG1 %	G1/G0 %	S %	G2/M %
AML3 control	2.6	60.7	21.5	15.3
AML3 APE1-KO	2.5	59.7	23.4	14.5
U937 Control	0.4	58.9	29.7	11.8
U937 APE1-KO	4.7	54.5	26.6	14.0
HL-60 control	1.5	52.7	33.8	12.5
HL-60 APE1-KO	3.6	49.0	32.2	15.2

Table 4.1: Distribution of AML cells in different cell cycle phases after APE1 shRNA knockdown.

Cell cycle analysis was performed on unsynchronized APE1 knockdown (APE1-KO) cells and control cells. Quantification of cell cycle distribution was performed using CellQuest software. Values presented are the means 3 independent experiments. Percentage of cells in each phase of the cell cycle may not add up to exactly 100% in total for each sample.

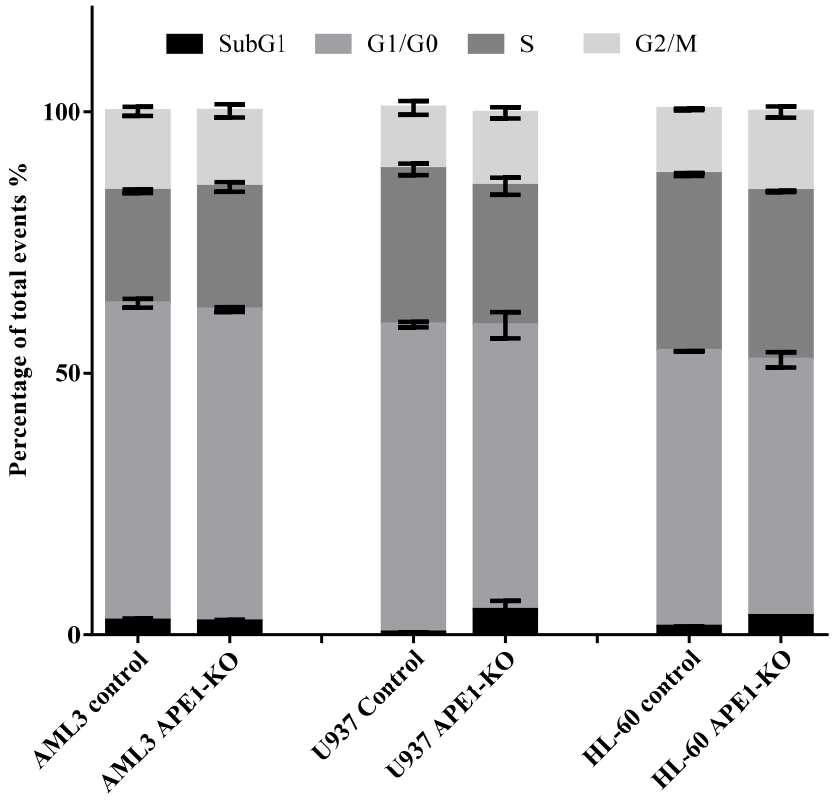


Figure 4.11: Distribution of AML cells in cell cycle phases after APE1 knockdown.

Cell cycle analysis was performed on flow cytometer to determine AML cell cycle kinetics after APE1 knockdown. Graphs shows that APE1 knockdown did not alter cell cycle kinetics. Some clones showed relatively less, but not significant, cells in s phase compared to control cells. Percentages of cells in each cell cycle phase are summarised in table 4.1. Data represent the mean of 3 independent experiments for 3 independent APE1-KO clones and the error bars of standard deviation.

4.3.6. Cytotoxicity in APE1 Knockdown Cell Lines Following Treatment with cytotoxic DNA damaging agents

It has been reported that APE1 silencing sensitises cancer cells to chemotherapy induced cytotoxicity (Wang *et al.*, 2004; Montaldi *et al.*, 2015; Zheng *et al.*, 2015). The aim of this section was to investigate the sensitivity of APE1 knockdown cells to chemotherapy induced cytotoxicity. This was achieved by two methods including growth inhibition assay using trypan blue exclusion dye and cell counter, and colony formation assay in semi-solid soft agar. Cytotoxic agents including temozolomide (TMZ), Ara-C, daunorubicin, etoposide, clofarabine and fludarabine, were used to assess sensitivity of AML cells to anti-leukaemic chemotherapy. These agents have different mechanisms of action and mainly operate in S phase of the cell cycle. TMZ was used as a positive control drug because it induces DNA damage repaired by BER, including N3-methyladenine (9%) and N7-methylguanine (70%) (Zhang *et al.*, 2012). Thus, APE1 knockdown is predicted to potentiate TMZ mediated cytotoxicity.

To investigate sensitivity of AML cells to APE1 knockdown using growth inhibition assay method, several independent APE1 deficient clones of AML3, U937 and HL-60 (APE1 protein knockdown represented in Figure 4.3) were treated with several cytotoxic chemotherapeutic agents. Results are presented as the percentage of viable cells in the treated cell suspension compared to viable cells in the vehicle control treated cell suspension.

APE1 knockdown AML cells showed either no or reduced sensitivity to all chemotherapy agents investigated, compared to their respective control cells. As such, APE1 knockdown was occasionally antagonistic. For example, AML3 APE1 knockdown clones were modestly sensitive to Ara-C, clofarabine and fludarabine, but sensitivity to TMZ, daunorubicin and etoposide was unaffected (Figure 4.12 left). U937 APE1 knockdown cell clones were not differentially sensitive to temozolomide, daunorubicin clofarabine or fludarabine, relative to their respective control (Figure 4.13 left). However, APE1 knockdown was associated with modest antagonism of Ara-C and etoposide-induced growth inhibition, and particularly so at high levels of growth inhibition. APE1 knockdown in HL-60 cells conferred slight sensitivity to etoposide, but antagonised fludarabine-induced growth inhibition (Figure 4.13 left).

Antagonism of chemotherapy-induced growth inhibition is likely to be influenced, at least, by slow proliferation of APE1 knockdown cells. To illustrate this point, growth inhibition curves were modified to show absolute cell count for each cell line/treatment. Consistent with the cell proliferation results described in section 4.3.3, APE1-deficient cells had significantly

slower proliferation compared to controls (Figures 4.12, 4.13 and 4.14 – right panel figures). Therefore, slow proliferation may reduce the efficacy of cytotoxic drugs that target rapidly proliferating cells.

It was noted that AML3 and HL-60 cell clones, irrespective of APE1 status, were relatively insensitive to TMZ-induced cytotoxicity (Figures 4.12A and 4.14A). This is potentially explained by high methylguanine DNA methyltransferase (MGMT) expression in these cell lines, which repairs TMZ induced DNA lesion O^6-methylguanine lesions. In contrast, U937 are MGMT-deficient and were hypersensitive to TMZ induced cytotoxicity. Moreover, U937 cells are proficient in DNA mismatch repair, which mediates cell death signalling in response to DNA methylating agents in cells with deficient MGMT expression (Horton *et al.*, 2009a).

Moreover, colony formation assay consistently confirmed the phenotype observed in the growth inhibition assay described above in this section. Two independent APE1 deficient HL-60, AML3 and U937 clones (Figure 4.15) and their counterpart control clones were exposed to TMZ, Ara-C or daunorubicin for 24 hours, prior to plating in soft agar for colony formation as described in section 2.8.2.

APE1 knockdown antagonised the inhibitory effects of TMZ, Ara-c and daunorubicin in all three cell lines tested, and particularly so for TMZ and daunorubicin (Figure 4.16).

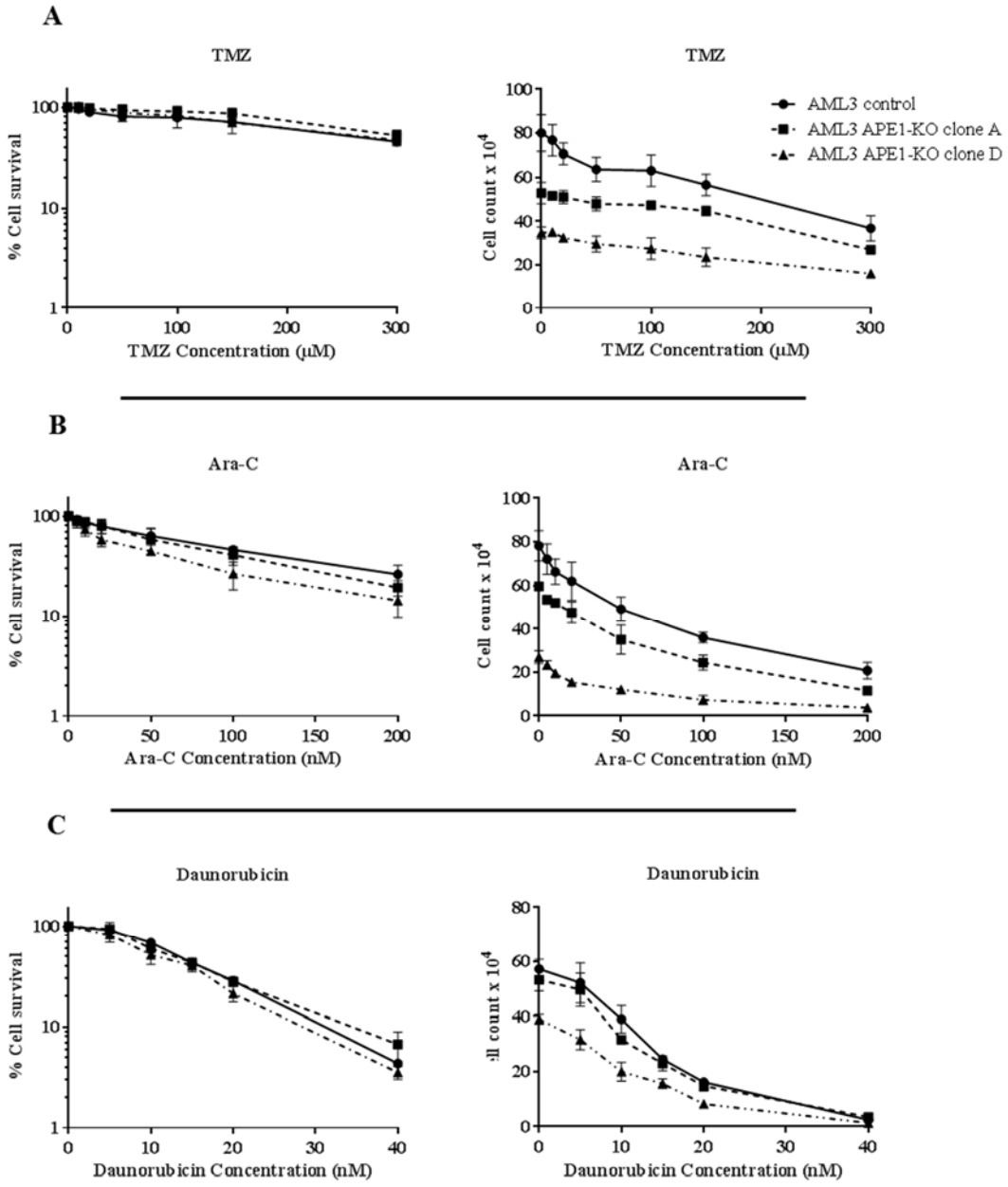


Figure 4.12: Growth inhibition in response to anti-AML treatment in APE1 knockdown AML3 cell line. (Continued on next page).

Cytotoxicity was assessed by growth inhibition assay. Two independent APE1 knockdown (APE1-KO) clones with off-target shRNA control cells were treated with temozolomide (TMZ), Ara-C and daunorubicin. Data in left panel figures represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. Right panel figures shows the absolute cell count for each cell line/drug dose. **(A)** Cytotoxicity in response to TMZ. **(B)** Cytotoxicity in response to Ara-C **(C)** Cytotoxicity in response to daunorubicin. In each case, results represent the mean and standard deviation of three independent experiments.

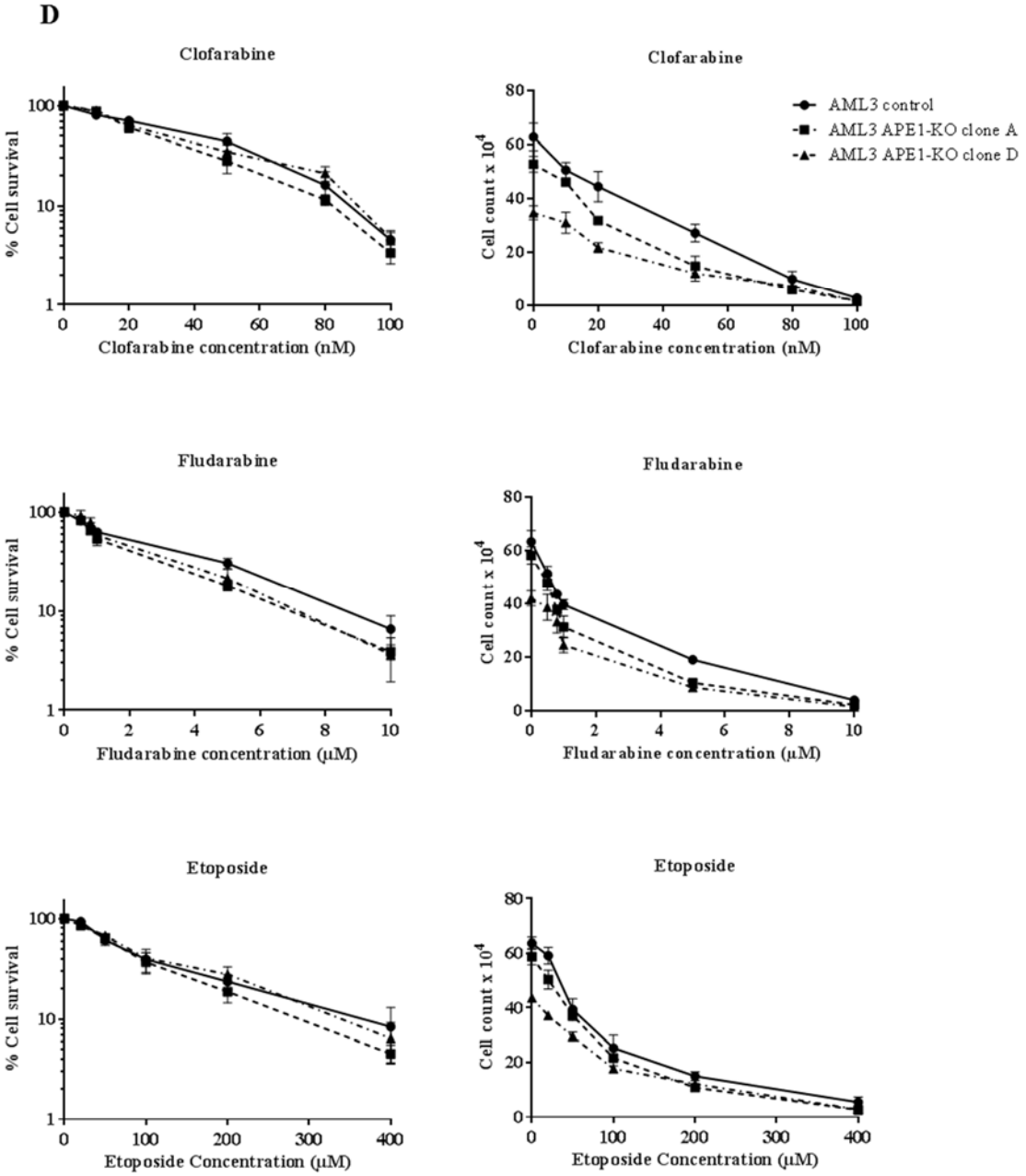


Figure 4.12: Growth inhibition in response to anti-AML treatment in APE1 knockdown AML3 cell line (Continued from previous page).

Cytotoxicity was assessed by growth inhibition assay. Two independent APE1 knockdown (APE1-KO) clones with off-target shRNA control cells were treated with clofarabine, fludarabine and etoposide. Data in left panel figures represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. Right panel figures shows the absolute cell count for each cell line/drug dose. **(D)** Cytotoxicity in response to clofarabine **(E)** Cytotoxicity in response to fludarabine and **(F)** Cytotoxicity in response to etoposide. In each case, results represent the mean and standard deviation of three independent experiments.

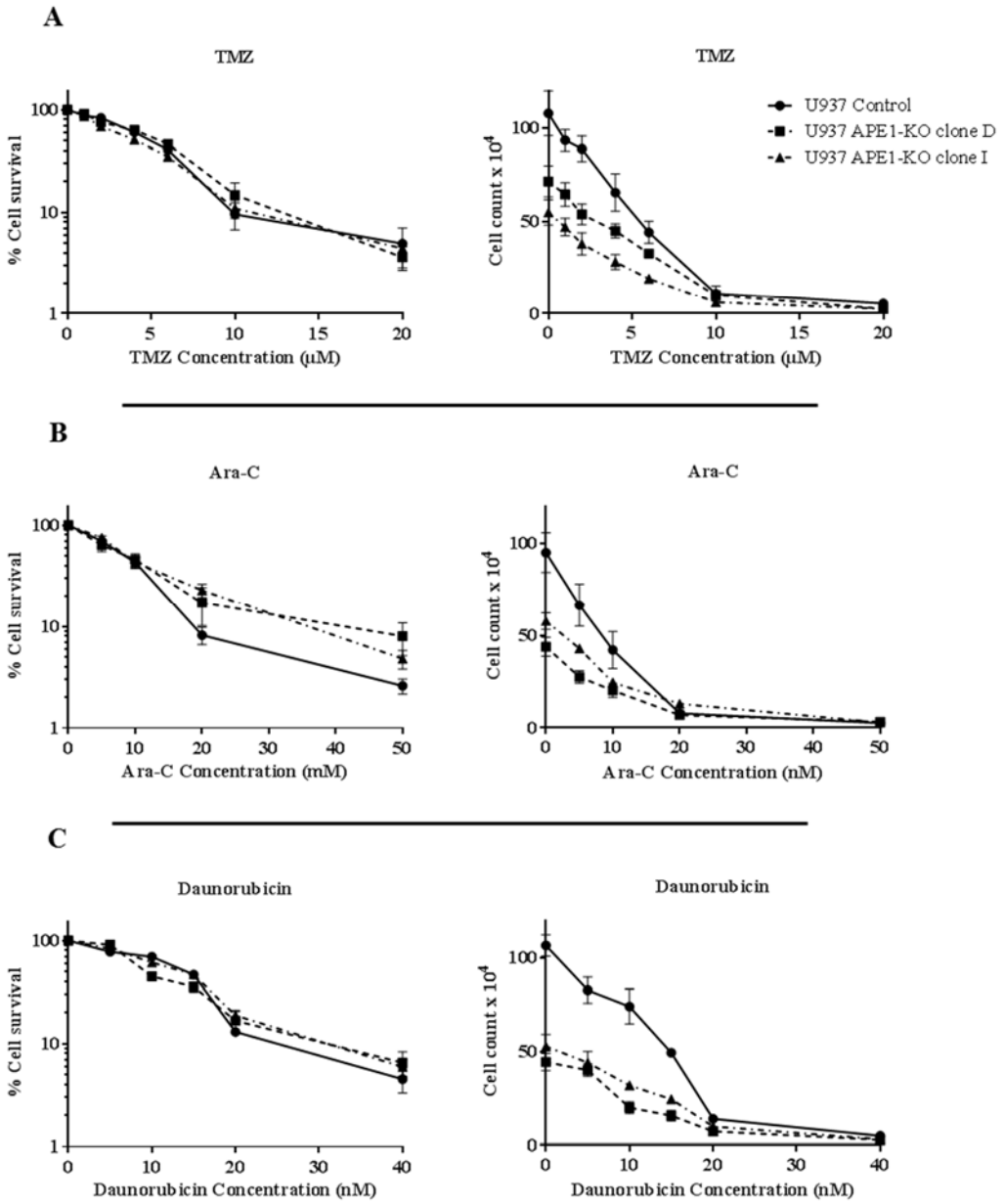


Figure 4.13: Growth inhibition in response to anti-AML treatment in APE1 knockdown U937 cell line. (Continued on next page).
Cytotoxicity was assessed by growth inhibition assay. Two independent APE1 knockdown (APE1-KO) clones with off-target shRNA control cells were treated with temozolomide (TMZ), Ara-C and daunorubicin. Data in left panel figures represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. Right panel figures shows the absolute cell count for each cell line/drug dose. **(A)** Cytotoxicity in response to TMZ. **(B)** Cytotoxicity in response to Ara-C **(C)** Cytotoxicity in response to daunorubicin. In each case, results represent the mean and standard deviation of three independent experiments.

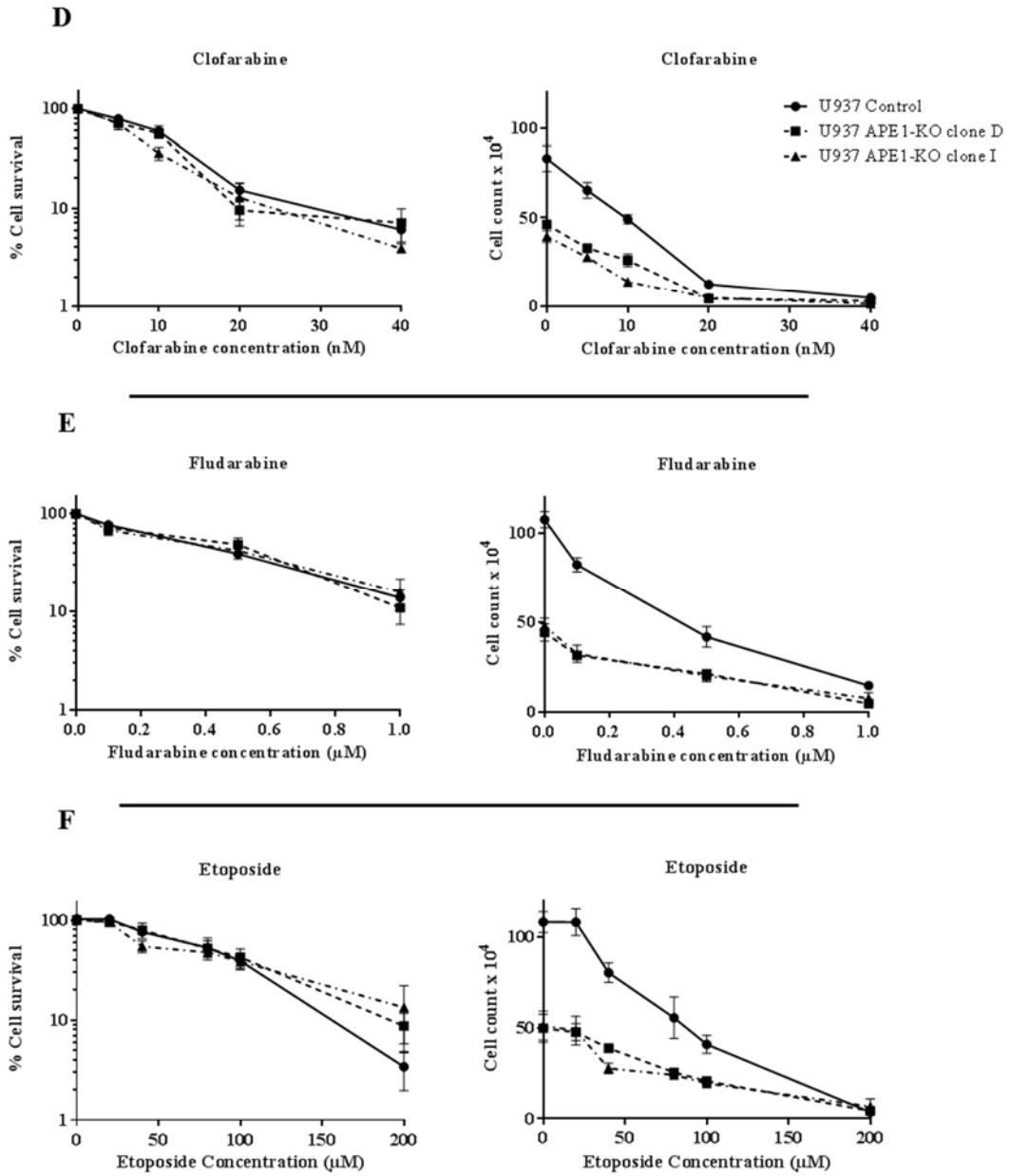


Figure 4.13: Growth inhibition in response to anti-AML treatment in APE1 knockdown U937 cell line (Continued from previous page).
Cytotoxicity was assessed by growth inhibition assay. Two independent APE1 knockdown (APE1-KO) clones with off-target shRNA control cells were treated with clofarabine, fludarabine and etoposide. Data in left panel figures represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. Right panel figures shows the absolute cell count for each cell line/drug dose. **(D)** Cytotoxicity in response to clofarabine **(E)** Cytotoxicity in response to fludarabine and **(F)** Cytotoxicity in response to etoposide. In each case, results represent the mean and standard deviation of three independent experiments.

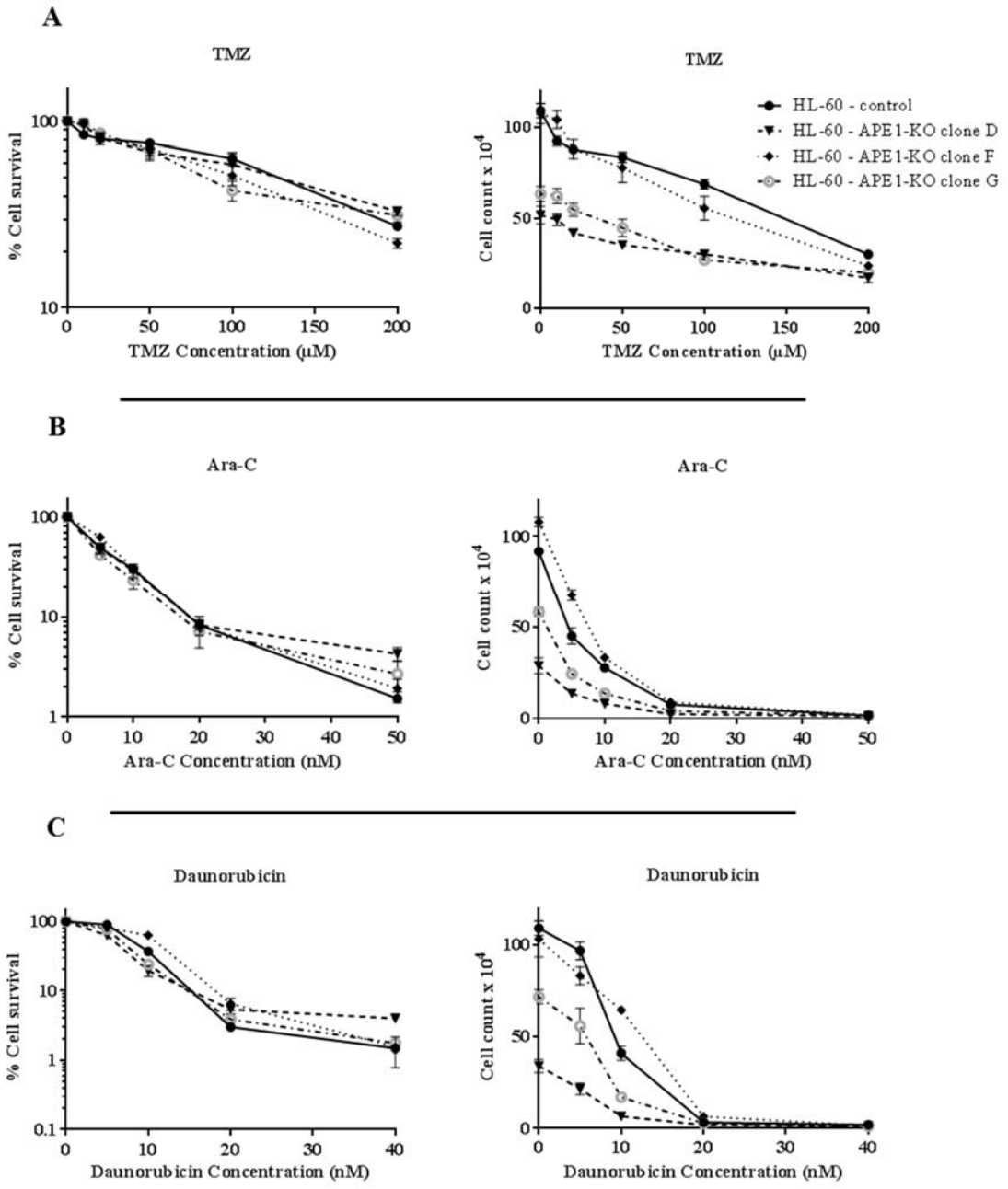


Figure 4.14: Growth inhibition in response to anti-AML treatment in APE1 knockdown HL-60 cell line. (Continued on next page).
Cytotoxicity was assessed by growth inhibition assay. Three independent APE1 knockdown (APE1-KO) clones with off-target shRNA control cells were treated with temozolomide (TMZ), Ara-C and daunorubicin. Data in left panel figures represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. Right panel figures shows the absolute cell count for each cell line/drug dose. **(A)** Cytotoxicity in response to TMZ. **(B)** Cytotoxicity in response to Ara-C **(C)** Cytotoxicity in response to daunorubicin. In each case, results represent the mean and standard deviation of three independent experiments.

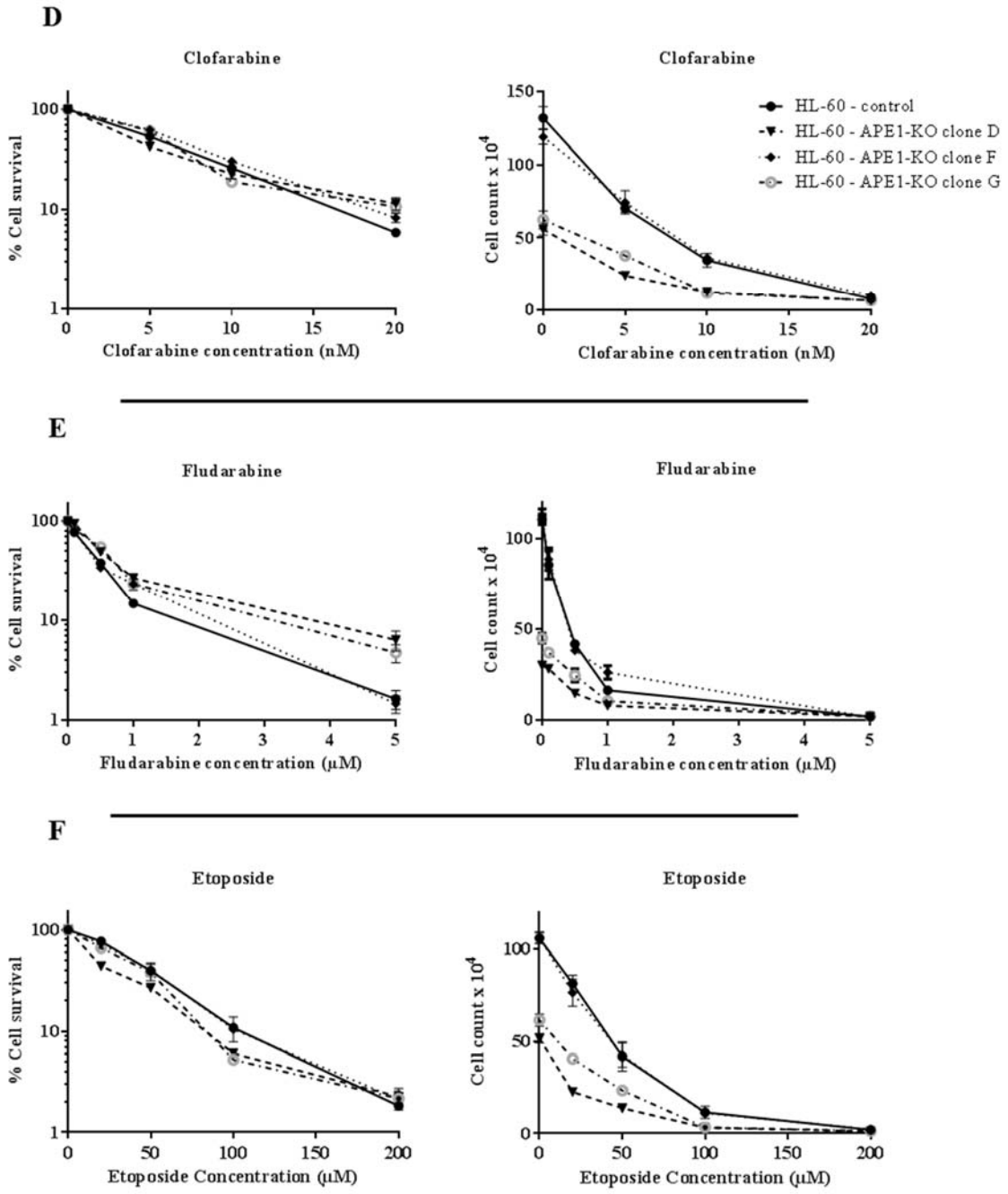


Figure 4.14: Growth inhibition in response to anti-AML treatment in APE1 knockdown HL-60 cells. (Continued from previous page).
Cytotoxicity was assessed by growth inhibition assay. Three independent APE1 knockdown (APE1-KO) clones with off-target shRNA control cells were treated with clofarabine, fludarabine and etoposide. Data in left panel figures represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. Right panel figures shows the absolute cell count for each cell line/drug dose. **(D)** Cytotoxicity in response to clofarabine **(E)** Cytotoxicity in response to fludarabine and **(F)** Cytotoxicity in response to etoposide. In each case, results represent the mean and standard deviation of three independent experiments.

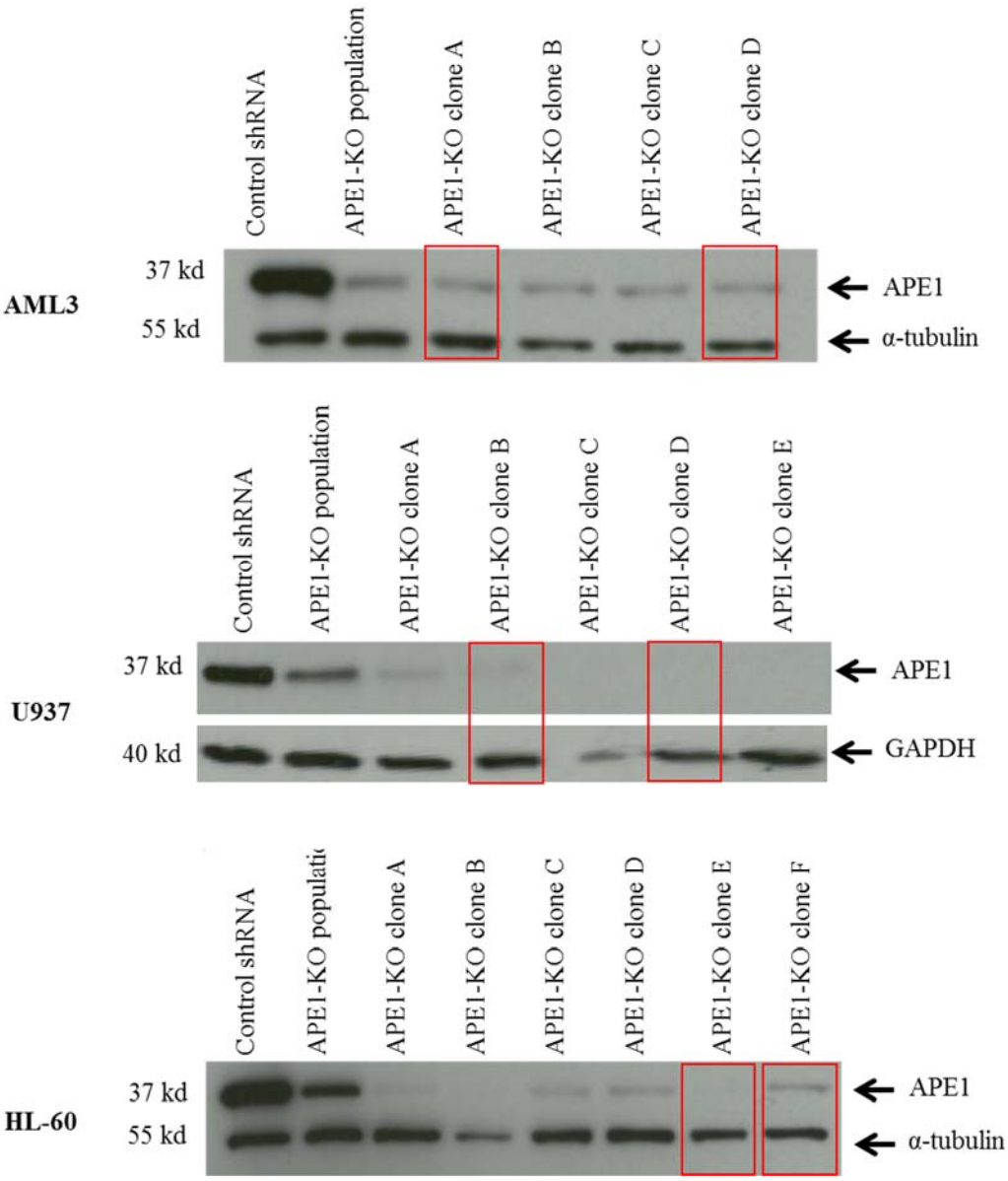


Figure 4.15: APE1 protein expression in AML cells following APE1 knockdown using shRNA.

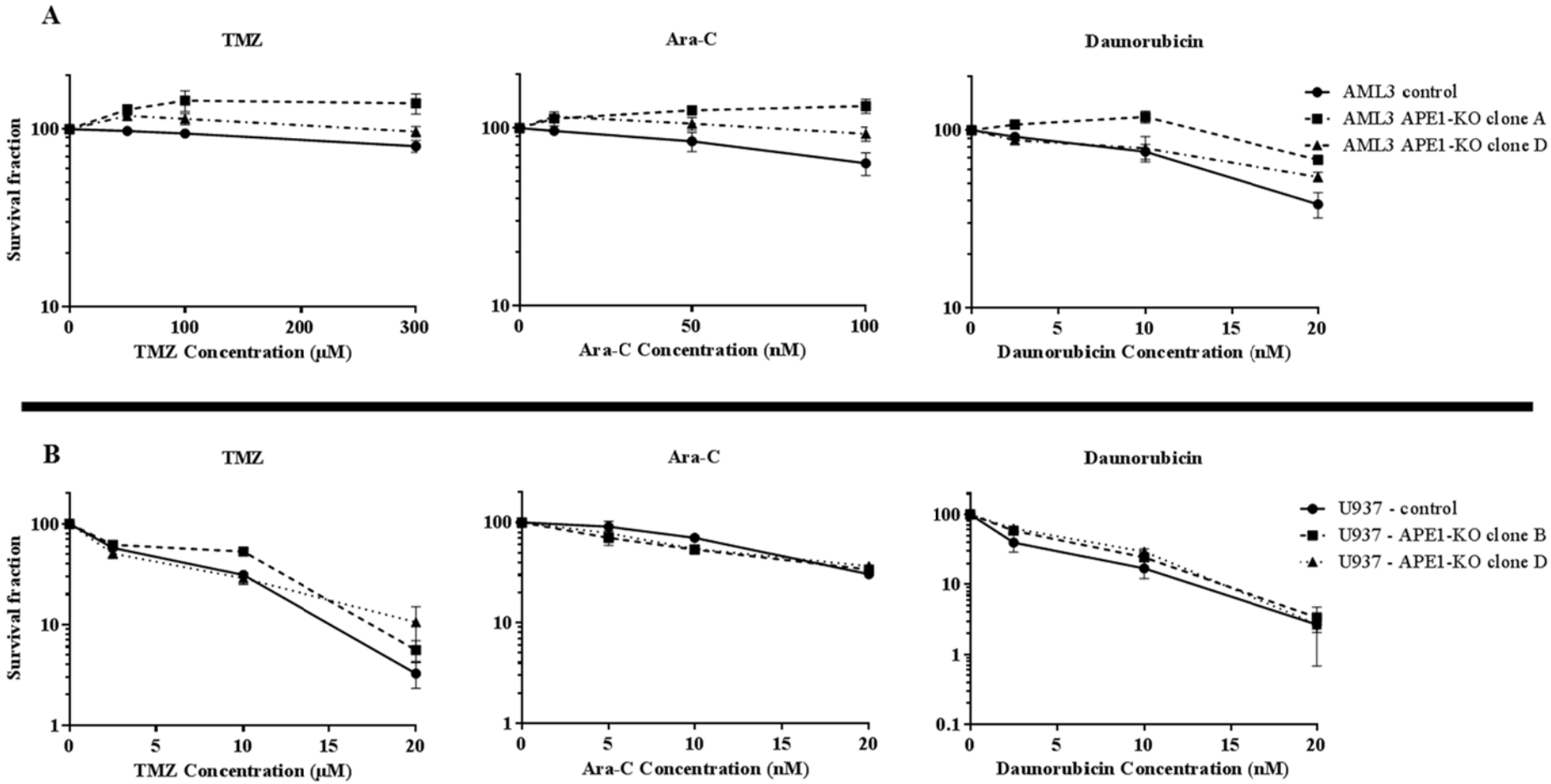


Figure 4.16: Effect of APE1 knockdown on AML cells response to chemotherapy induced cytotoxicity. (continued on next page).

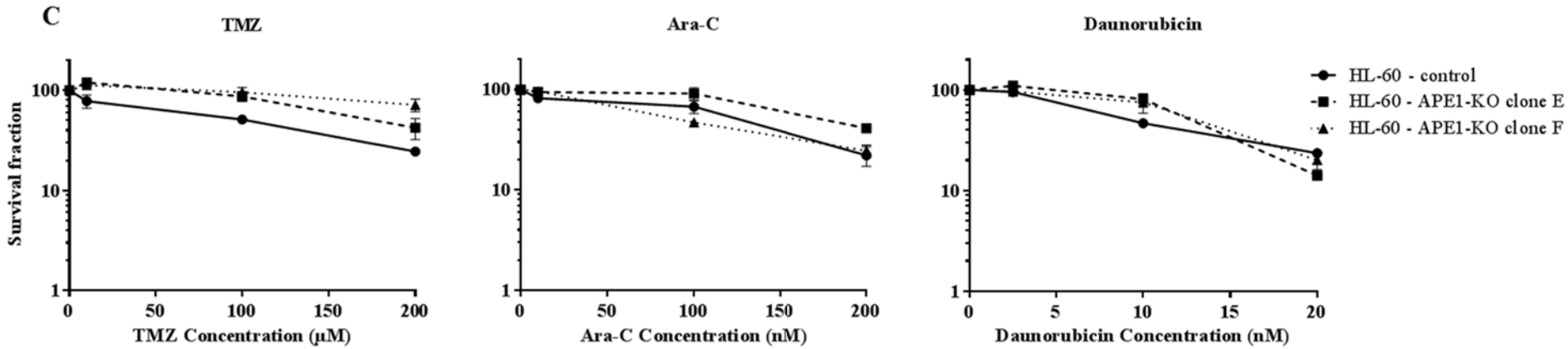


Figure 4.16: Effect of APE1 knockdown on AML cells response to chemotherapy induced cytotoxicity (continued from previous page).

Cytotoxicity via colony formation assay was performed to investigate the influence of APE1 knockdown on the sensitivity of two independent APE1 knocked down clones (APE1-KO) of **(A)** AML3, **(B)** U937 and **(C)** HL-60 cells to temozolomide (TMZ), Ara-C and daunorubicin. Data is presented as the number of viable colonies from treated cells as percentage of the number of colonies of vehicle control cells. APE1 knockdown in AML cells demonstrated antagonistic effect across all investigated cell lines. In each case, results represent the mean of three independent experiments.

4.3.7. Effect of APE1 knockdown on AP sites accumulation

AP site quantification, using the aldehyde reactive probe (ARP) assay, was undertaken to determine the ability of APE1 deficient cell clones to resolve AP sites after induction of DNA damage by hydrogen peroxide (H_2O_2). ARP reacts specifically with the aldehyde group that present on the open ring form of AP site. After treating DNA with ARP, AP sites are tagged with biotin residues, and can then be quantified by colorimetric detection via the biotin/avidin reaction.

HL-60, AML3 and U937 APE1 knockdown cells and their respective controls were treated with 50 μM H_2O_2 for one hour to induce DNA damage, and then prepared for AP site assay as described in section 2.10.

The ARP assay was performed using two independent AP site assay kits supplied by Abcam, UK and Cell Biolabs, Inc, UK. These kits share the same assay principle discussed in section 2.10, and utilise the same reagents and controls. AP sites were determined in 3 technical replicate for each sample with each assay kit. There was no difference in AP site frequency between untreated APE1 proficient and deficient cell clones. AP sites were detectable at low levels in all cell lines prior to H_2O_2 treatment, indicating that these AP sites normally exist in cellular DNA. Induction of AP sites was observed 2 hours following treatment with H_2O_2 in all cell lines, with decreasing numbers of AP sites thereafter (Figure 4.17). Based on a single replicate experiment APE-1 knockdown cells appeared to display higher accumulation of AP sites after two hours, but this would need to be confirmed by further repeat experiments. Intriguingly, the AP site frequency returned to control background levels very rapidly (within 4 hours of H2O2 treatment) in all APE1 deficient cell clones. Again, this observation is from a single replicate and requires additional work for confirmation.

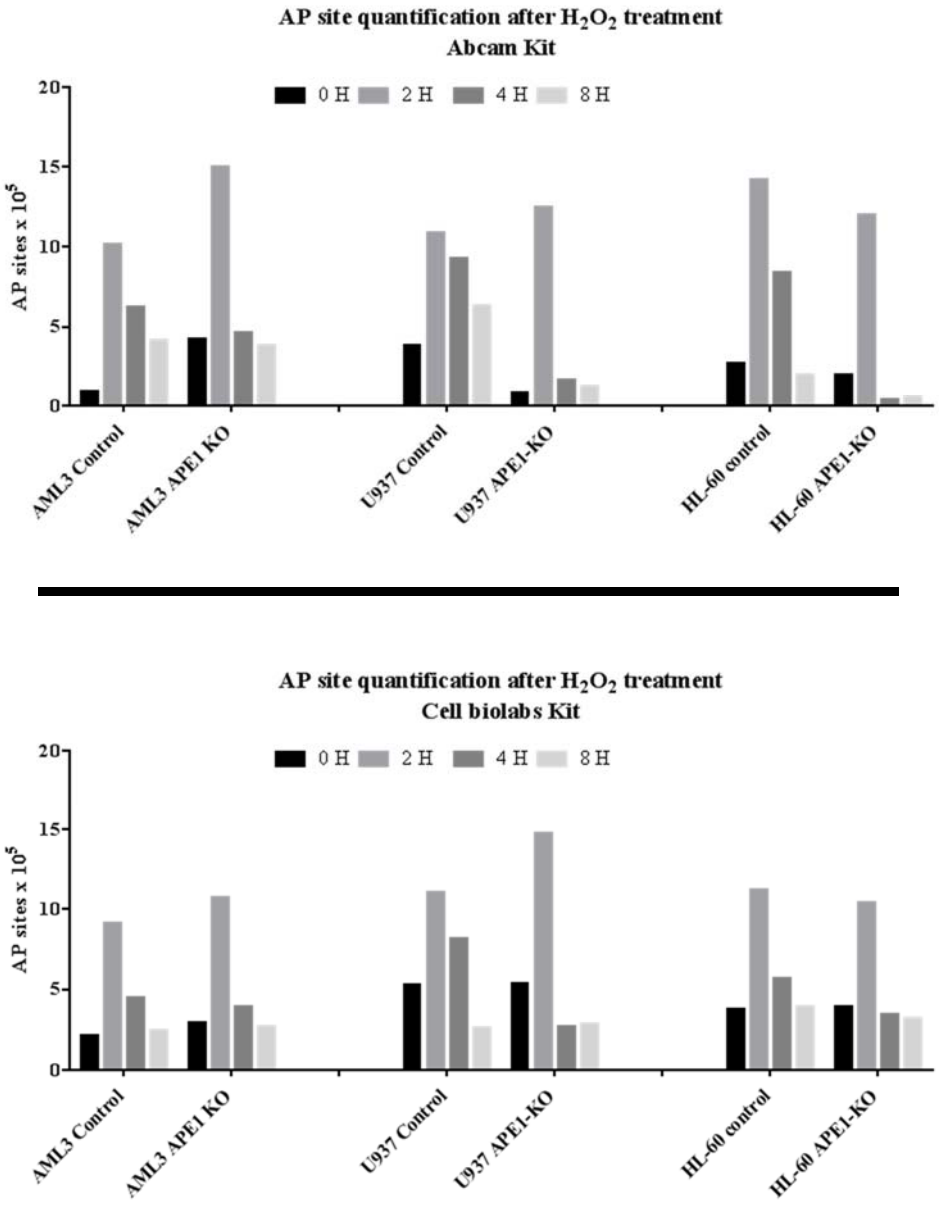


Figure 4.17: AP sites quantification after APE1 silencing in AML cells.

AP sites determination in AML3, U937 and HL-60. Control cells and APE1 knockdown cells (APE1 KO) were pre-treated with 50 µM of hydrogen peroxide (H_2O_2) for 1 hour. AP sites were quantified using aldehyde reactive probe (ARP) based assay prior treatment and after 2, 4 and 8 hours after treatment. AP sites were quantified using two independent AP site kit assay from two independent suppliers. Data represent single experiment.

4.3.8. Determination of TOP2 complexes following exposure to Etoposide using TARDIS assay (Trapped in agarose DNA immunostaining)

The aim of this section was to investigate whether APE1 knockdown contribute to accumulation of TOP2A and TOP2B complexes in AML cells following treatment with etoposide, a topoisomerase poison.

APE1 knockdown in HL-60 and U937 was confirmed by western blot analysis (Figure 4.18). Two independent clones from each cell line and their appropriate controls were used in the TARDIS assay. U937 (clones E and H) and HL-60 (clones D and H) were exposed to 100 μM of etoposide (topoisomerase II poison) for 1 hour. Cells were then harvested to perform TARDIS as described in section 2.11. GraphPad prism software was used to analyse data and to calculate P values. Unpaired parametric t test was used to calculate p values. P values less than 0.05 were considered significant.

HL-60 and U937 APE1 knockdown cell clones had lower TOP2A complex accumulation following etoposide treatment compared to APE1 proficient control cell clones. For example, APE1 deficient HL-60 clones D and H showed significant lower TOP2A complexes (22% and 15%, respectively) following incubation with etoposide (Figure 4.19). Similarly, U937 clones E and H also demonstrated 22% and 19%, respectively, less TOP2A complexes compared to controls (Figure 4.19). When combining all data of HL-60 and U937, APE1 deficient cells showed 18.84±3.514 STD (P value = 0.002) less TOP2A complexes compared to APE1 proficient control cells. Although inconsistent with the observation by colleagues in the Institute for Cell and Molecular Biosciences, these data are consistent with the result described in section 4.3.6 demonstrating reduced sensitivity of APE1 knockdown cells to etoposide induced cytotoxicity.

Stabilisation of TOP2B complexes was observed in APE1 knockdown cells which led to an increased accumulation of these complexes compared to APE1 proficient control cell clones. Generally, APE1 knockdown in HL-60 and U937 increased TOP2B complex accumulation, but this was not true in HL-60 clone D which showed less complex accumulation compared to control cells (Figure 4.20). When combining all data for HL-60 and U937, TOP2B complex accumulation in AML cells was not statistically significant (P value = 0.06) when stratified by APE1 status, even after excluding the HL-60 clone D result as an outlier.

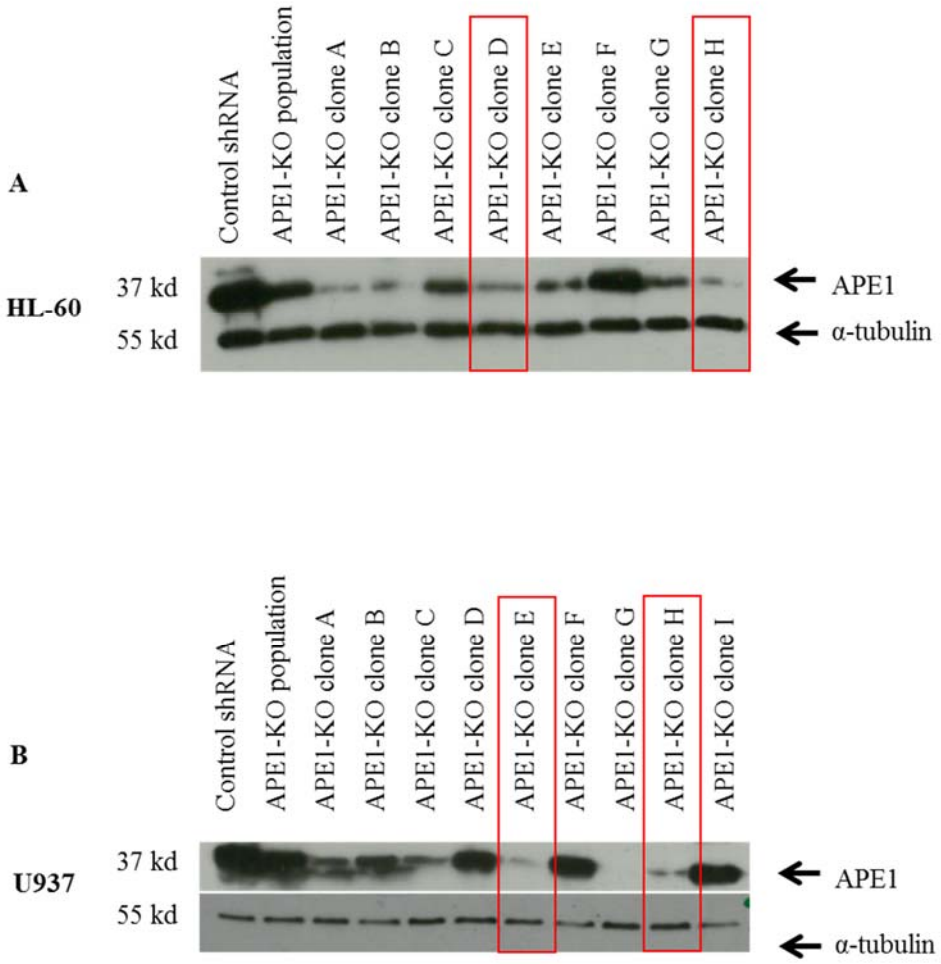


Figure 4.18: APE1 knockdown in AML cells used in TARDIS assay.

Western blots shows the level of APE1 knockdown in **(A)** HL-60 and **(B)** U937 cells used in TARDIS assay. Clones highlighted in red boxes were used in TARDIS experiments.

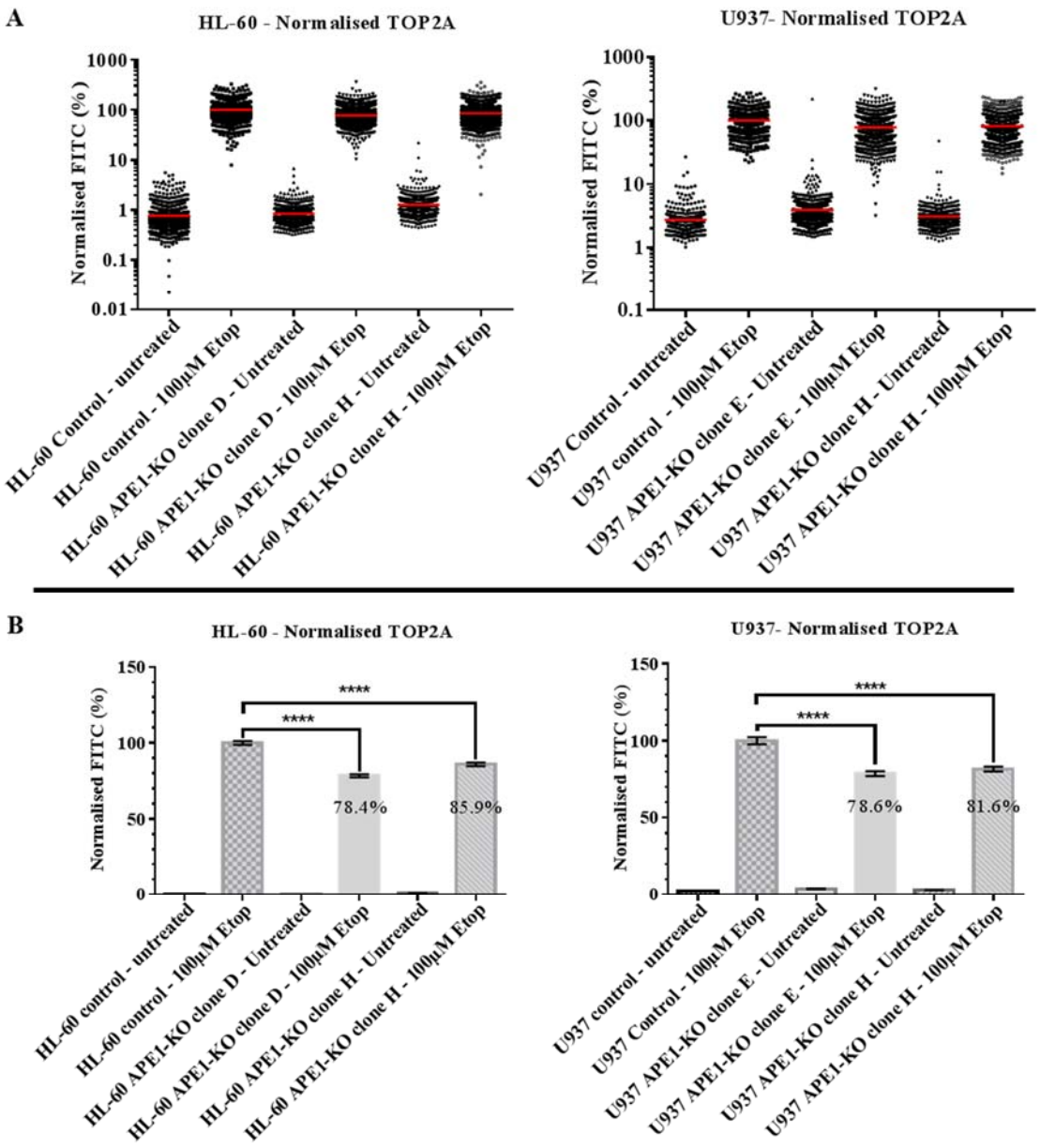


Figure 4.19: Effect of APE1 knockdown on cellular TOP2A levels.

The TARDIS assay was performed to investigate the role of APE1 knockdown on etoposide induced TOP2A-DNA complex formation.

(A) Dot blot represent TARDIS fluorescence intensity measurement on single cell level in HL-60 (left) and U937 (right). Data are normalised to control cells treated with etoposide. Red line represent the median of fluorescence intensity.

(B) Mean of integrated FITC of HL-60 (left) and U937 (right) treated APE1-KO cells and control cells of as percentage of mean of integrated FITC of etoposide treated control cells. This result represent the mean of the median of three independent experiments. Unpaired parametric t test was used to calculate P values. **** indicate P value < 0.00005.

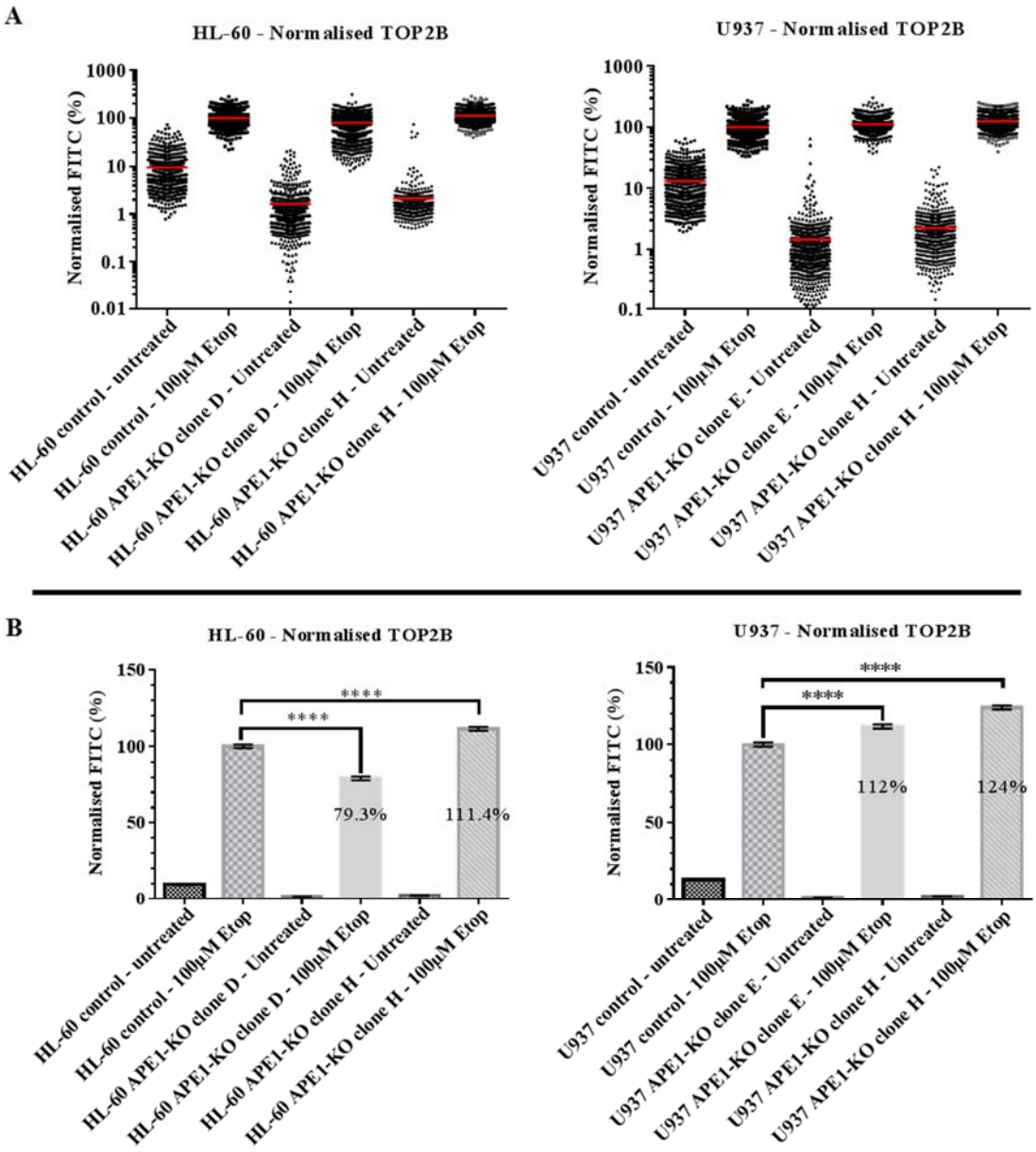


Figure 4.20: Effect of APE1 knockdown on cellular TOP2B levels.

The TARDIS assay was performed to investigate the role of APE1 knockdown on etoposide induced TOP2A-DNA complex formation.

(A) Dot blot represent TARDIS fluorescence intensity measurement on single cell level in HL-60 (left) and U937 (right). Data are normalised to control cells treated with etoposide. Red line represent the median of fluorescence intensity.

(B) Mean of integrated FITC of HL-60 (left) and U937 (right) treated APE1-KO cells and control cells of as percentage of mean of integrated FITC of etoposide treated control cells. This result represent the mean of the median of three independent experiments. Unpaired parametric t test was used to calculate P values. **** indicate P value < 0.00005.

4.3.9. RNA sequencing reveals upregulation of genes involved in cell cycle control and leukaemia pathogenesis

RNA sequencing using Illumina HiSeq 2500 was performed to explore the effect of APE1 knockdown in AML cells, as well as potentially identifying possible mechanism of antagonism in APE1 knockdown cells following exposure to anti-leukaemic chemotherapy agents.

Two AML cell lines were used in this experiment HL-60 and AML3. APE1 knockdown was confirmed in APE1 shRNA transduced cells and expression confirmed in their respective control cells by assessment of protein level using western blotting (Figure 4.21). APE1 deficient cells, including AML3 clone 5 and HL-60 clone 7, were chosen for RNA sequence analysis. RNA quality was assessed using the Agilent RNA 6000 Nano Kit (Agilent Technologies, UK) and the Agilent 2100 Bioanalyzer, as described in section 2.7. The results represent the average of 3 technical replicates for two cell lines. To exclude cell-type-specific effects of APE1 knockdown, upregulated/downregulated genes common to both cell lines are reported and discussed here, and p value < 0.05 was deemed to be statistically significant. P values were calculated using Wald parametric test, and adjusted for multiple testing using the Benjamini-Hochberg algorithm. RNA sequencing data were analysed using DESeq2 software V 3.2. This analysis was kindly performed by Dr Yaobo Xu, (Institute of Genetic Medicine, Newcastle University).

APE1 knockdown significantly upregulated 176 genes and downregulated 191 genes in both cell lines (listed in tables in appendices D and E). APE1 knockdown was confirmed by RNA sequencing in both AML cell lines, HL-60 and AML3, by 12.6 and 8.6 folds respectively, and with a p value < 0.0001 in both cell lines. Interestingly, no significant differences in gene expression were found in those genes involved in the BER pathway, with the exception of *NEIL2* that was significantly downregulated approximately 2 fold in APE1 deficient HL-60 cells compared to control cell clones. Furthermore, there was no significant alteration in the expression of the majority of genes involved in cellular DNA repair, with the exception that *GTF2H1*, *GTF2H2C* and *GTF2H3,* which encode protein products involved in nucleotide excision repair, was significantly downregulated in APE1 deficient cell clones. In addition, *XRCC6,* which plays a role in DNA non-homologous end joining (NHEJ) required for double-strand break repair, was significantly downregulated in APE1 deficient cells.

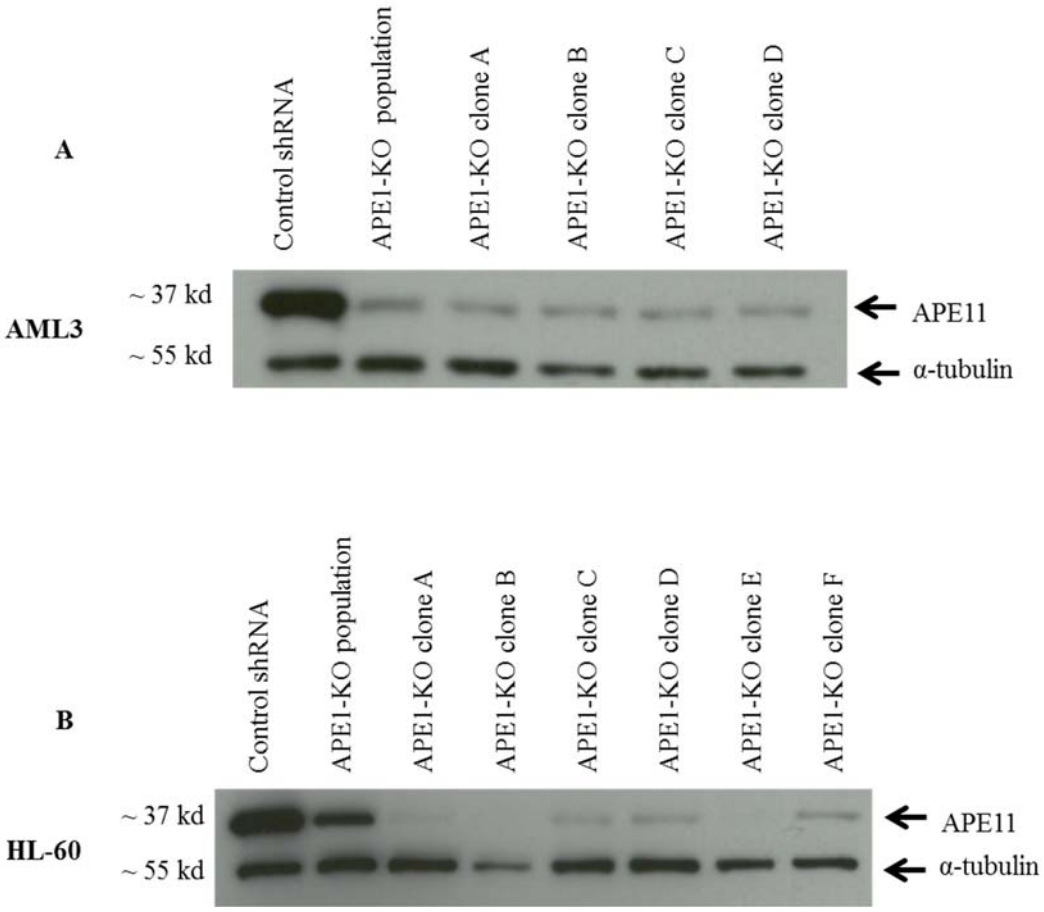


Figure 4.21: APE1 knockdown in AML3 and HL-60 cells used for RNA sequencing.

Cell clones were generated on semi-solid soft agar from AML3 and HL-60 cells populations transduced with shRNA C5. APE1 knockdown was confirmed on protein level in **(A)** AML3 and **(B)** HL-60 cells, which were used for RNA sequencing. AML3 clone C and HL-60 clone E were used for RNA sequencing.

Several genes identified as being indirectly transcriptionally regulated byAPE1 were not changed following APE1 knockdown, including *NF-κB*, *JUN, FOS, TP53*, *YBX1*, *HIF1α*, *SIRT1*, *GAPDH*, *STAT3*, *CBP/p300*, *HDAC1 and NRF2.*

The *EGR1* transcription factor that has previously been reported to interact with APE1 was significantly upregulated 2.3 and 1.8 fold in AML3 and HL-60, respectively (P values <0.0001 for AML3 and p=0.002 for HL-60). Furthermore, *PAX5* transcription factor and *CDKN1A (p21)* cell cycle regulator were differentially overexpressed 2 to 3 fold in both cell lines. Western blotting was performed to confirm PAX5 and CDKN1A upregulation on protein level. PAX5 protein level upregulation was confirmed in APE1-deficient HL-60 cells. However, CDKN1A was undetectable at the protein level in AML3 cells (Figure 4.22A), which is explained by low RNA transcripts reads in this cell line (Figure 4.22B). Additionally, CDKN1A protein was upregulated in AML3 (Figure 4.22A). HL-60 cell showed negligible CDKN1A protein due to low RNA transcripts read (Figure 4.22C).

Notably, two *BCL2* family genes, *BCL2A1* and *BCL2L11*, were differentially upregulated in APE1 knockdown cells. The *BCL2* gene family plays an essential role in cell survival via anti-apoptotic function and impairment of the G0/G1 transition (Zhao *et al.*, 2008). In addition, *ALDH3B1*, which is thought to be involved in the defence against oxidative stress and lipid peroxidation (Marchitti *et al.*, 2007), was overexpressed in APE1 deficient cells. Collectively, BCL2 and ALDH3B1 activation may support AML cells survival advantage after *APE1* knockdown and may explain the tolerance/resistance observed when exposed to cytotoxic therapy.

Some transcription factors and tumour suppressor genes that have been previously linked to AML pathogenesis, such as *FOXO1*, *CUX1*, *NPM1*, and *DNMT3B*, were also altered following APE1 knockdown. The *FOXO1* (Forkhead Box O1) transcription factor, that has been reported to enhance AML transformation and progression (Sykes *et al.*, 2011; Kode *et al.*, 2016), was upregulated 2 fold in HL-60 and AML3 cell lines. APE1 knockdown also led to upregulation of the *CUX1* (Cut-Like Homeobox 1) transcription factor. NPM1 and DNMT3B were significantly downregulated in both cell lines. NPM1 has previously been shown to interact with APE1 and modulate its function (Vascotto *et al.*, 2013; Poletto *et al.*, 2014). However, *NPM1* downregulation following *APE1* knockdown may provide further evidence of direct interaction with NPM1.

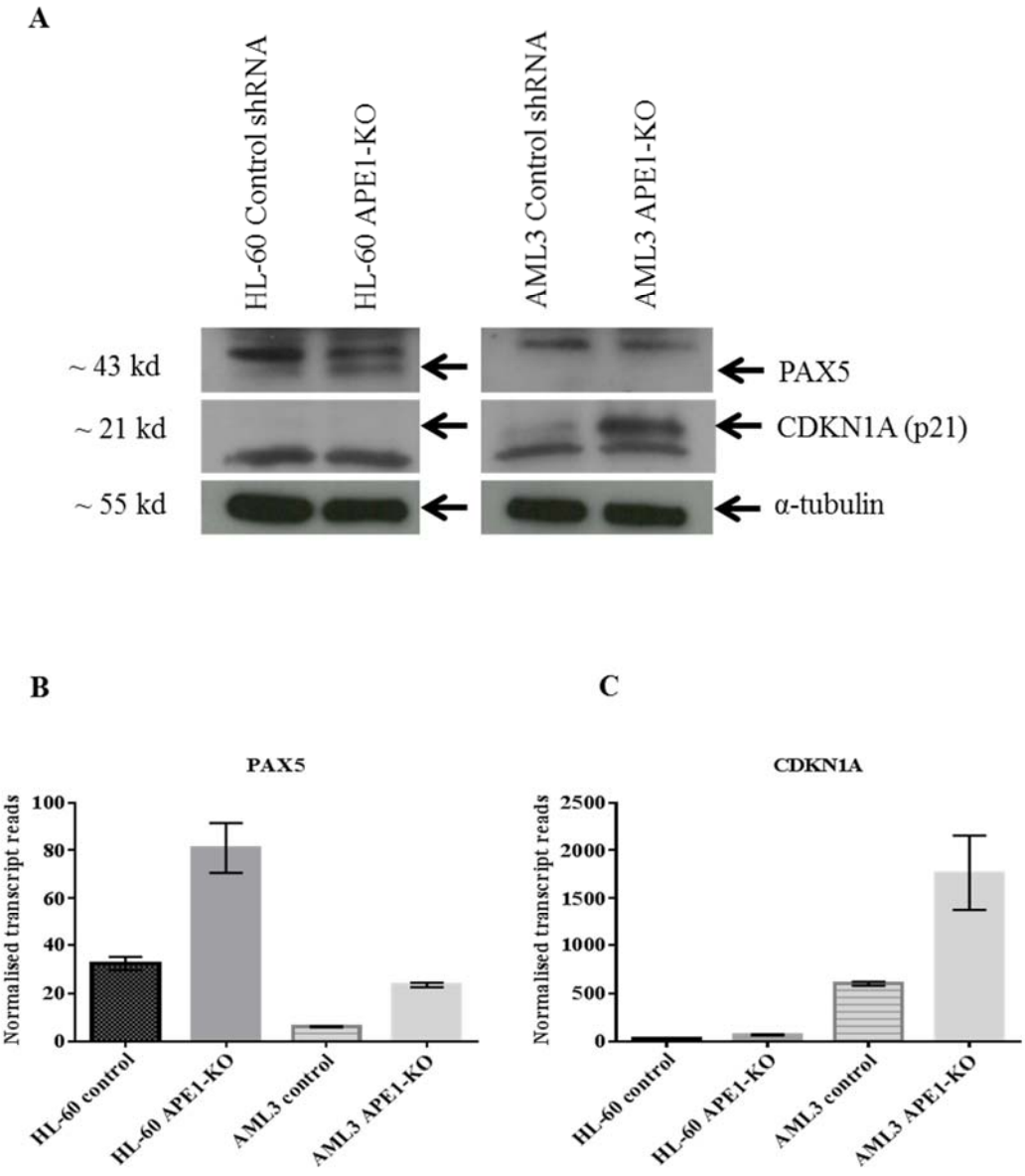


Figure 4.22: PAX5 and CDKN1A protein and transcripts levels in HL-60 and AML3 cells.

RNA sequencing data showed upregulation of *PAX5* and *CDKN1A* expression after APE1 knockdown in HL-60 and AML3 cell lines. **(A)** Determination of PAX5 protein expression by western blotting showed upregulation of protein level in HL-60 only. CDKN1A was upregulated on protein level in AML3 cells only. **(B)** *PAX5* normalised transcripts reads were relatively low in AML3 cells compared to HL-60, therefore were undetectable by western blotting. **(C)** Normalised transcripts reads of *CDKN1A* in HL-60 cells were also relatively low compared to AML3, thus corresponding with undetectable protein levels shown in western blotting result.

Intriguingly, *FLT3* was significantly downregulated 1.7 fold in APE1 deficient AML3 cell clones compared to APE1 proficient control clones (p value <0.00001). In contrast, FLT3 expression was upregulated 2 fold in APE1 deficient HL-60 cell clones, but was not statistically significant due to very low RNA transcript levels (P value = 0.4). Furthermore, APE1 knockdown led to significantly down regulated Bruton's tyrosine kinase (*BTK*) expression. BTK plays an important role in normal B-cell differentiation and hematopoietic signalling and is a potential targets for AML therapy (Rushworth *et al.*, 2014; Pillinger *et al.*, 2015).

Etoposide treatment had no effect on AML cells following APE1 knockdown as shown in section 4.3.6. Furthermore, the TARDIS assay revealed a significant reduction in TOP2A complex formation, but not TOP2A, following etoposide treatment (section 4.3.8). These result suggest downregulation of TOP2A and no alteration in TOP2B expression in APE1 knockdown cells. Consistent with this notion, APE1 knockdown was associated with downregulation of *TOP2A* expression in both cell lines but this was not statistically significant. Moreover, TOP2B was also downregulated 1.1 to 1.3 fold in both cell lines but this was not statistically significant.

Interestingly, APE1 knockdown was associated with reduced bleomycin hydrolase (*BLMH*) expression, which encodes a protein that catalyses inactivation of bleomycin and reduces its cytotoxicity. This result supports the observation that APE1 overexpression confers bleomycin resistance and *APE1* silencing enhances bleomycin induced cytotoxicity (Schild *et al.*, 1999; Robertson *et al.*, 2001; Fung and Demple, 2011).

4.4. Discussion

4.4.1. APE1 knockdown impairs AML cell growth

APE1 knockdown has previously been demonstrated to reduce cell proliferation in several cancer models (Fung and Demple, 2005; Vascotto *et al.*, 2009b; Jiang *et al.*, 2010; Cun *et al.*, 2013). Herein, it was clearly evident that shRNA-mediated APE1 knockdown significantly impaired cell proliferation and cloning efficiency of AML cell lines HL-60, AML3 and U937. Moreover, the reduction in cell proliferation approximately correlated with APE1 protein levels. Furthermore, spontaneous re-expression of APE1 led to restoration of normal proliferation in AML cells, confirming that the reduction in proliferation was due to loss of APE1 expression.

RNA sequencing data demonstrated significant reduction of *MAPK3* expression after APE1 knockdown in AML cells. MAPK3 play vital role in a variety of cellular processes such as proliferation, differentiation and transcription regulation. Consistently, downregulation of MAPK3 has been reported previously following APE1 knockdown (Juliana *et al.*, 2012; Wang *et al.*, 2013b). It has been demonstrated that APE1 overexpression enhances cell proliferation via activation of IL-21 induced MAPK3/1 (Juliana *et al.*, 2012). APE1 knockdown dramatically reduced IL-21 induced MAPK3/1 and impaired cell proliferation and enhanced cell death, but these effects were reversible by APE1 re-expression (Juliana *et al.*, 2012). In addition, it is highly possible that upregulation of CUX1 (Cut-Like Homeobox 1) expression inhibits proliferation following APE1 knockdown. CUX1 is thought to negatively regulate hematopoietic cells proliferation and its knockdown induced over-proliferation of haemocytes in a *Drosophila* model and enhanced engraftment of human haematopoietic cells in immunodeficient mice (McNerney et al., 2013). A putative role for *CUX1* and other genes as regulators of AML cell proliferation is discussed in more detail in section 4.4.5.

APE1 is essential for embryonic development, cell survival and cell viability; *Ape1* loss/mutation in mouse models induces embryogenic lethality at day 5 to 9 (Xanthoudakis *et al.*, 1996; Ludwig *et al.*, 1998; Meira *et al.*, 2001). Intriguingly however, complete loss of APE1 protein by shRNA mediated knockdown was not lethal to some AML clones shown in Figures 4.15 and 4.21. It is feasible that APE1 expression was not completely abolished and was below the detection level of western blotting. RNA sequencing data showed that APE1 transcript was very low but detectable in APE1 knockdown cells. More likely, APE1 expression is essential for cell viability in an embryonic context, but not in transformed fully differentiated AML cells. Consistent with this notion, a recent study demonstrated no effect

on survival or growth in a mouse B cell line (CH12F3) following *APE1* gene deletion (Masani et al., 2013). However, the mechanism by which APE1 loss induces embryonic lethality remains to be fully delineated.

These findings therefore add to a growing body of evidence that APE1 is an important modulator for cell proliferation and provide protection against DNA damage and oxidative stress. Since shRNA is not a specific tool to inhibit a particular protein function, it was not possible to differentiate between the contribution of different APE1 functions (namely DNA repair and redox functions) on cell phenotype. Although both functions are critically important to cellular physiology, the precise mechanisms by which APE1 perform its vital functions are still not fully understood. Nevertheless, APE1 downregulation reduces DNA repair capacity as well as impairing redox regulation function, and may trigger activation of alternative mechanisms to allow cells to repair accumulating damage and circumvent cell death.

4.4.2. APE1 and cell cycle regulation

Despite impaired cell proliferation and dysregulation of several genes involved in cell cycle modulation such as CDKN1A, E2F7 and CDC42 in APE1 knockdown cells, this seems not sufficient to induce cell cycle arrest in AML cells, where there was no clear alteration in cell cycle distribution shown by cell cycle analysis. In addition, there was no evidence of increased cells in subG1 phase, as indication of apoptosis, in cell cycle analysis. Given that APE1 is highly expressed in the early and middle S phase (Fung *et al.*, 2001) and maintains the integrity of DNA by preventing mutagenic events during DNA synthesis. It is plausible that APE1 downregulation triggers cell cycle modulators such as CDKN1A and other cell cycle checkpoints to prevent cell transition into S phase, thus slowing proliferation of cells that harbour pro-mutagenic DNA lesions. However, activation of G1 checkpoints by CDKN1A is predicted to result in G1 arrest but this was not observed in APE1 knockdown cells. Alternatively, reduced DNA repair capacity and higher DNA damage could simultaneously activate G1, S and G2/M checkpoints, result in slower transit through all phases of the cell cycle. As such, cell cycle analysis using propidium iodide would not be sufficiently sensitive to identify such an effect. Rather cell cycle analysis using bromodeoxyuridine (BrdU) could be used to track the transit of cells through the different cell cycle stages. Alternatively, the DNA fibre assay (Nieminuszczy *et al.*, 2016), which provides a direct measure of replication fork speed, could also be used to investigate this phenomenon.

However, APE1 involvement in cell cycle control has been well established in several studies (Fung *et al.*, 2001; Zou *et al.*, 2007; Jiang *et al.*, 2010), but the exact mechanism by which it modulate cell cycle is yet to be defined (Fishel *et al.*, 2008). Available evidence is conflicting and inconclusive regarding the influence of APE1 on cell cycle kinetics. APE1 knockdown in ovarian cell line SKOV-3X altered several components of cell cycle, including S phase progression, S phase exit and transition to G1 (Fishel *et al.*, 2008). In addition, APE1 thought to regulate the G0/G1-S cell cycle transition in embryonic stem cells, where APE1 knockdown compromised transition of cells from G0 to S phase (Zou *et al.*, 2007; Fishel *et al.*, 2008). Other studies reported cell cycle arrest at G2/M in HEK-293T cells (Sengupta *et al.*, 2011) or impaired S to G2M transition in HeLa cells after APE1 knockdown (Vascotto *et al.*, 2009b). In contrast, APE1knockdown did not affect cell cycle kinetics in T98G and U87MG glioblastoma cell lines (Montaldi *et al.*, 2015). These conflicting reports possibly suggest tissue specificity in terms of APE1 function and its role in regulating the cell cycle.

4.4.3. Cytotoxic effect of AML chemotherapy was not enhanced in APE1 knockdown cells

Sensitivity of APE1 knockdown cells to chemotherapy induced cytotoxicity was not evident in this study. This suggests that APE1 and BER do not play a major role in the repair of chemotherapy-induced DNA damage, despite some of these agents inducing base damage known to be repaired by BER, specifically TMZ. Alternatively, there are possible backup mechanisms that could bypass APE1 knockdown.

The finding that APE1 knockdown confers tolerance/resistance to TMZ is in contrast to published evidence that APE1 loss sensitises cancer cells to TMZ (Silber et al., 2002; Luo and Kelley, 2004; Montaldi et al., 2015). TMZ was included here as a positive control drug. The adducts that are generated by TMZ are mainly repaired by two mechanisms, including direct reversal via the activity of O^6-methylguanine-DNA methyltransferase (MGMT) and also BER via the action of the alkyl adenine DNA glycosylase/methyl purine DNA glycosylase. TMZ induces O^6-methylguanine DNA adducts, which are mainly repaired by MGMT. TMZ also induces N3-methyladenine and N7-methylguanine DNA adducts that are mainly repaired by the BER pathway (Zhang et al., 2012). N3-methyladenine is cytotoxic via DNA replication inhibition, whereas N7-methylguanine is relatively inert (Fronza and Gold, 2004; Shrivastav et al., 2010). Indeed, inappropriate initiation of N7-methylguanine repair may convert this inert lesion to a pro-cytotoxic AP site or strand break (Shrivastav et al., 2010; Wirtz et al., 2010). Unrepaired O^6-methylguanine due to MGMT deficiency leads to mismatching of O^6-methylguanine with thymine during DNA replication, which are recognised by MMR (Zhang

et al., 2012). MMR activity leads to repeated attempted cycles of repair which ultimately lead to the accumulation of DNA double strand breaks and apoptosis (Zhang et al., 2012). Therefore, TMZ-induced DNA adducts are largely removed and excised by MGMT and their cytotoxicity is primarily dependant on the MMR status of the cells. Thus, APE1 function could be bypassed by MGMT and MMR after TMZ treatment. Consistent with this conclusion, AML3 cells, which have high MGMT levels, were less sensitive to TMZ-induced growth inhibition. In contrast to AML3, U937 cells are MGMT deficient and showed corresponding hypersensitivity to TMZ. Furthermore and consistent with this result, early clinical trials indicated that TMZ has greater anti-leukaemic efficacy in patients with low MGMT expression (Brandwein et al., 2007; Brandwein et al., 2014).

Daunorubicin is an essential AML treatment and acts on cancer cells through various mechanisms including inhibition of DNA synthesis by DNA intercalation, induction of oxidative DNA damage through free radical formation, topoisomerase II inhibition and direct damage to cellular structures such as the cell membrane (Agrawal, 2007). However, the exact mechanisms by which daunorubicin induces cell killing in AML remain unclear. Based on the ability of APE1 to act through BER to remove oxidised DNA bases and its involvement in regulation of transcription factors under oxidative stress, it was hypothesised that oxidative DNA damage induced by free radicals following daunorubicin treatment will be particularly damaging to APE1 deficient AML cells. Unexpectedly, APE1 knockdown did not sensitise AML cells to daunorubicin, but conferred tolerance/resistance to the cytotoxic effect of this anthracycline. This observation suggests that APE1 does not play a major role is mediating cellular response to the DNA damage and oxidative stress induced by daunorubicin. More likely, the primary mechanism of daunorubicin cytotoxicity is not via induction of oxidative DNA damage and the DNA damage resulted from daunorubicin action is not a substrate for BER. Furthermore, redox regulation of transcription factors in response to oxidative stress induced by daunorubicin is not only modulated by APE1, but can be regulated by multiple and complex signalling transduction network (Trachootham *et al.*, 2008; Mikhed *et al.*, 2015).

HL-60, AML3 and U937 APE1 deficient cells and their relevant controls did not differ in sensitivity to the cytotoxic effects of Ara-C, clofarabine or fludarabine. This could be due to the nature of DNA damage induced by these agents and the ability of BER, particularly APE1, to recognise and remove that damage. Consistent with this notion APE1 inhibition did not sensitise cancer cells to Ara-C (Lam *et al.*, 2006; McNeill and Wilson, 2007; McNeill *et al.*, 2009). A mutated form of APE1 protein, which lacks the endonuclease function but possesses higher AP-DNA binding affinity, was used to inhibit APE1 endonuclease function

in vitro and to identify APE1 biological substrates (McNeill and Wilson, 2007; McNeill *et al.*, 2009). Mutant APE1 expression in Chinese hamster ovary cells enhanced the cytotoxicity of methyl methanesulfonate and dideoxycytidine, but not Ara-C, etoposide or cisplatin (McNeill and Wilson, 2007; McNeill *et al.*, 2009). This may possibly be due to low APE1 efficiency in removing Ara-C nucleotides (Chou *et al.*, 2000; Lam *et al.*, 2006). Furthermore, fludarabine nucleotide has been shown to be substrate for uracil-DNA glycosylase (UDG) (Bulgar *et al.*, 2010), but no evidence was found to suggest APE1 involvement in fludarabine nucleotide removal. The growth inhibitory effects of the topoisomerase II inhibitor etoposide were also diminished in APE1 knockdown HL-60, AML3 and U937 cell clones. Moreover, an inability of etoposide to sensitise APE1 knockdown cells is partially explained by efficient resolution of TOP2A complexes in HL-60 and U937 APE1 deficient cell clones following etoposide treatment. Moreover, nucleoside analogues and topoisomerase poisons operate more efficiently in exponentially proliferating cells, and their efficacy is compromised in cells with a low proliferative index, as seen in APE1 knockdown cell clones.

Taken together, these data strongly suggest that APE1 is not involved in the recognition and removal of DNA damage induced by TMZ, Ara-C, daunorubicin, fludarabine, clofarabine and etoposide. However, further investigation is required to interrogate this hypothesis.

Furthermore, targeting APE1 in cancer cells could be challenging in some circumstances because of potential redundancy of APE1 function where backup mechanisms are present to maintain genome stability and bypass APE1 inhibition. For example, removal of a damaged base by the AP lyase function of bifunctional glycosylases such as OGG1 and NEIL-1 creates a 3'PUA that can be processed via PNKP and repair via long-patch BER (Figure 1.4), therefore bypassing APE1 function (Wiederhold et al., 2004). Moreover, loss of APE1 function in AML cells following APE1 knockdown might be compensated by APE2, which exhibits strong 3'-5' exonuclease and 3'phosphodiesterase activities and a weak AP endonuclease activity (Tsuchimoto *et al.*, 2001; Burkovics *et al.*, 2006; Burkovics *et al.*, 2009). Tsuchimoto and colleagues (2001) demonstrated that APE2 is mainly localised in nuclei and mitochondria and interacts with PCNA *in vitro* and possibly participates in long patch BER. In addition, impaired BER could also be compensated for by an alternative pathway. For example, mismatched 5-fluorouracil:G and U:G, which are predominantly removed by BER could also be efficiently removed by MMR (Fischer *et al.*, 2007; Schanz *et al.*, 2009). Collectively, these observations indicate redundancy in BER to maintain genome stability and could explain the lack of sensitivity of AML cells to chemotherapeutic agents following APE1 knockdown.

Given that APE1 has a role in redox regulation, it is difficult to delineate the exact contribution this function makes to determining cellular response to any given agent. Nevertheless, alterations in gene expression revealed by RNA sequencing may prove useful in this regard.

4.4.4. Effects of APE1 on AP site accumulation

AP sites are intermediate repair products generated after excision and removal of damaged bases by DNA glycosylases. It is estimated that around 10,000 AP sites/cell/day are spontaneously generated under normal physiological conditions (Abbotts and Madhusudan, 2010). AP sites are mutagenic and cytotoxic if unrepaired, and are primarily cleaved and removed by APE1. However, targeting APE1 using RNAi or small molecule inhibitors has been reported to enhance chemotherapy induced cytotoxicity by promoting AP site accumulation and apoptosis activation (Fung and Demple, 2005; Sultana *et al.*, 2012; Abbotts *et al.*, 2014).

Enhanced AP site accumulation was consistently observed in APE1 knockdown cells relative to controls following 2 hours of H_2O_2 treatment. Furthermore, the evidence from these limited experiments suggests that AP sites are rapidly resolved in APE1 knockdown to basal level within 4 hours of treatment, whereas this process was delayed in control cells, taking up to 8 hours post-treatment. This unexpected observation suggests two possible mechanisms by which AML cells may respond to APE1 depletion. Although unlikely, AP sites may be efficiently removed by alternative mechanisms. Alternatively, AP sites may be rapidly converted to single strand or double-strand breaks in APE1 deficient AML cells and repaired through NHEJ or homology directed repair.

It should be noted, however, that the AP site assays undertaken in this project were limited in scope and scale. As such, additional experiments are required to further confirm whether these mechanisms may be operating. Specifically, the comet assay (alkaline and neutral) could be performed to detect the nature of strand breaks induced after APE1 knockdown/inhibition and after treatment with cytotoxic agents. Detection of phosphorylated gamma-H2AX by western blotting could also be a useful biomarker for DNA damage and double strand breaks after APE1 knockdown/inhibition and after treatment with cytotoxic agents.

Widespread upregulation of alternative DNA repair mechanisms to compensate for APE1 deficiency was not supported by RNA sequencing data. Specifically, there was no significant

alteration in expression of the majority of genes involved in DNA repair pathways. Rather, a small number of genes constituting the core element of TFIIF transcription factor involved in nucleotide excision repair were significantly downregulated. These genes include *GTF2H1*, *GTF2H2C* and *GTF2H3*. In addition, *XRCC6,* that plays a crucial role in non-homologous end joining (NHEJ) required for double-strand break repair, was significantly downregulated.

4.4.5. Gene expression analysis after APE1 knockdown

APE1 is an essential modulator and coordinator of the BER pathway, as well as playing a key role as a redox regulator for several transcription factors involved in cell proliferation, survival and cell cycle. APE1 downregulation reduces DNA repair capacity and is predicted to trigger gene expression changes to circumvent accumulating DNA damage and promote cell survival. RNA sequencing analysis of APE1 knockdown cells revealed alterations in the expression of specific genes involved in cell proliferation, cell cycle, tumour suppression, cell survival, RNA metabolism, DNA methylation and tumour invasion, including *BCL2*, *ALDH3B1*, *PAX5*, *CDKN1A*, E2F7, *EGR1*, *FOXO1*, *CUX1, NPM1, DNMT3B, and VEGFB*. As APE1 knockdown was associated with differential expression of numerous genes in AML cells, only those genes putatively involved in AML pathogenesis/prognosis, DNA repair, cell proliferation, survival, differentiation and apoptosis will be discussed. Also, it should be noted that the data and discussion presented in this section require further validation, because the RNA sequencing analysis undertaken in this thesis was relatively limited in scope.

Upregulation of anti-apoptotic genes *BCL2A1* and *BCL2L11* in APE1 knockdown cells may explain the lack of sensitivity to anti-leukaemic agents. Furthermore, *ALDH3B1* upregulation, which is involved in protection against oxidative stress and lipid peroxidation (Jin *et al.*, 2015), may contribute to the resistance of APE1 knockdown cells to daunorubicin induced cytotoxicity. Daunorubicin induced cytotoxicity occurs via induction of oxidative stress, lipid peroxidation and other mechanisms discussed in section 1.1.6.3.

CDKN1A

CDKN1A is an essential protein that plays a key role in regulating cell cycle, DNA replication, apoptosis and transcription (Cazzalini et al., 2010). Transcriptional activity of CDKN1A is primarily regulated by p53, but is also regulated in a p53 independent manner (Abbas and Dutta, 2009; Cazzalini et al., 2010). APE1 has dual regulatory roles in CDKN1A-

mediated transcriptional activation in a p53 dependent manner (Gaiddon *et al.*, 1999; Sengupta *et al.*, 2013). It has also been reported that APE1 down regulation leads to upregulation of CDKN1A transcript and protein levels in several cancer cell models (Vascotto *et al.*, 2009a; Jiang *et al.*, 2010). Consistent with these reports, APE1 knockdown in AML cells upregulates CDKN1A expression at both the transcript and protein level. CDKN1A upregulation the AML cell lines used in this study may be p53 independent given that the HL-60 cell line is deleted for the *TP53* gene and that AML3 is dysfunctional for p53 function (mediated via over-expression of MDM2) despite being wild-type for *TP53* (Sutcliffe *et al.*, 1998; Drexler *et al.*, 2000). It is possible that activation of CDKN1A transcriptional activity following APE1 knockdown is mediated by DHRS2 (HEP27), which was significantly upregulated after APE1 knockdown. Limited studies have demonstrated that DHRS2 expression stabilises p53 by attenuation of MDM2-mediated ubiquitination and degradation, therefore leading to an accumulation of MDM2 and CDKN1A (Deisenroth *et al.*, 2010).

EGR1

Early growth response protein 1 (EGR1) transcription factor was upregulated following APE1 knockdown in HL-60 and AML3 APE1 knockdown cell clones. APE1-EGR1 interaction has been demonstrated *in vitro* in limited studies and thought to stimulate APE1 expression under oxidative stress to induce *PTEN* tumour suppressor gene activation (Pines *et al.*, 2005; Fantini *et al.*, 2008). Interestingly, EGR1 acts as tumour suppressor gene in leukaemia and haploinsufficiency in murine mice model enhances AML transformation (Joslin *et al.*, 2007; Stoddart *et al.*, 2014). Therefore, upregulation of *EGR1* after APE1 knockdown may be a protective mechanism for AML cells in response to elevated oxidative stress and DNA damage.

FOXO1

Forkhead box protein O1 (FOXO1) transcription factor was upregulated in APE1 knockdown AML cell clones and possibly contributed, at least partly, to attenuated cell proliferation and resistance/tolerance to chemotherapeutic agents. FOXO1 has been implicated in cancer as both an oncogene and tumour suppressor gene (Fu and Tindall, 2008; Sykes *et al.*, 2011; Lin *et al.*, 2014; Zhu, 2014; Kode *et al.*, 2016). It is normally required for maintenance of haematopoietic stem cells (HPSC) and protects cells against oxidative stress (Tothova *et al.*,

2007). Elevated FOXO1 expression is thought to be a pro-leukaemic event and was shown to enhance leukaemic transformation through its interaction with components of the β-catenin signalling pathway (Kode *et al.*, 2016). In addition, FOXO1 is upregulated in AML1-ETO primary AML samples and is thought to contribute to AML progression (Lin *et al.*, 2014). Furthermore, FOXO1 inhibition enhances myeloid maturation, differentiation and consequently induces apoptosis in human AML cells (Sykes *et al.*, 2011). Collectively, these data suggest that FOXO1 upregulation after APE1 knockdown enhances AML cells survival and resistance to cytotoxic treatment. However, APE1 and FOXO1 interaction is not yet fully characterised and any role in AML pathogenesis would require extensive further investigation.

CUX1

Cut-Like Homeobox 1 (*CUX1*) transcription factor has tumour suppressor properties as well as oncogenic properties (Ripka *et al.*, 2010; Boultwood, 2013; McNerney *et al.*, 2013; Ramdzan and Nepveu, 2014; Wong *et al.*, 2014). It is highly expressed in multipotent hematopoietic stem cells, but reduced expression is commonly observed in myeloid progenitors (McNerney *et al.*, 2013; Ramdzan and Nepveu, 2014). CUX1 is thought to negatively regulate hematopoietic stem cell proliferation and its knockdown induced haemocyte over-proliferation in *Drosophila* model and enhanced engraftment of human haematopoietic cells in immunodeficient mice (McNerney et al., 2013). In contrast, CUX1 overexpression is associated with shorter relapse-free survival and plays an important role in tumour progression in breast and pancreatic cancer (Michl et al., 2005; Ripka et al., 2010). However, a limited study demonstrated enhancement of OGG1 DNA glycosylase activity *in vitro* by CUX1 to accelerate DNA repair following oxidative DNA damage (Ramdzan *et al.*, 2014), although no evidence was found indicating an effect of CUX1 expression on APE1 function. Taken together, CUX1 overexpression in AML cells following APE1 depletion seems to protect cells from oxidative DNA damage by reducing cell proliferation via modulation of cell cycle proteins.

LAIR1

LAIR1 (Leukocyte-Associated Immunoglobulin-Like Receptor 1) expression, which was recently ascribed to have a role in AML development, was differentially elevated in APE1

deficient cell clones. LAIR1 plays an essential role in development of AML and ALL using both *in vitro* and *in vivo* approaches (Poggi *et al.*, 2000; Zocchi *et al.*, 2001; Chen *et al.*, 2015; Kang *et al.*, 2015). It is expressed in $CD34^+$ hematopoietic stem cell progenitors and seems to be expendable for normal haematopoiesis (Chen *et al.*, 2015; Kang *et al.*, 2015). LAIR1 expression enhanced engraftment of AML cells in a mouse model as well as induced leukemic transformation (Kang *et al.*, 2015). Furthermore, LAIR1 knockdown/knockout blocked xenograft of AML and B-ALL cells in a mouse model and induced apoptosis of AML cells (Chen *et al.*, 2015; Kang *et al.*, 2015). In addition, deletion of *LAIR1* in a leukaemia mouse model was associated with rapid remission and longer survival time (Chen *et al.*, 2015). These data clearly demonstrated the pro-leukaemic role of *LAIR1* and suggest a potential role for LAIR1 in mediating resistance of APE1 knockdown cells to chemotherapeutic agents. It is also possible that APE1 acts as repressor for LAIR1 expression, thus investigation of the mechanistic interaction between APE1 and LAIR1 may help delineate the contribution of both proteins to AML pathogenesis and resistance to chemotherapy.

In addition to the upregulation of several tumour suppressor genes/oncogenes that conceivably play a role in AML tolerance/resistance to chemotherapeutic agents, intriguingly, APE1 knockdown was also associated with downregulation of several genes that contribute to AML pathogenesis, prognosis and tumours invasion such as *NPM1*, *DNMT3B* and *VEGFB*.

NPM1 modulates and stabilises BER proteins, specifically APE1, and is thought to orchestrate BER through regulation of protein translocation to the nucleoplasm under conditions of oxidative stress (Vascotto *et al.*, 2009b; Vascotto *et al.*, 2013; Poletto *et al.*, 2014). APE1/NPM1 protein-protein interaction regulate APE1 DNA endonuclease activity as well as RNA riboendonuclease activity (Vascotto *et al.*, 2009b; Poletto *et al.*, 2014). A reduction of *NPM1* expression in APE1 depleted AML cell clones supports the fact that NPM1 interact with APE1 and modulates BER. It appears that APE1 directly interacts with NPM1 to recruit other BER proteins components to sites of DNA damage. APE1 depletion is predicted to reduce APE1-NPM1 protein interactions potentially leading to de-stabilisation of NPM1 protein. However, further validation of NPM1 protein levels in APE1 knockdown cells is essential to confirm this notion.

Interestingly, APE1 knockdown in AML cells resulted in significant downregulation of DNMT3B gene expression, which implies that APE1 could play a role in regulating DNA

methylation. DNMT3B functions in *de novo* methylation and is responsible for introducing cytosine methylation at CpG sites. However, the role of APE1 in DNA methylation/demethylation is still unclear, and reports in this regard are contrasting. For example, it was reported that APE1 depletion in conjunction with TDG (thymine DNA glycosylase) loss enhances global DNA demethylation in HEK293T cells (Jin *et al.*, 2015). In contrast, it is thought that APE1 and TDG are required to activate RNF4 (RING finger protein 4)-induced DNA demethylation. However, it must be noted that apparent DNMT3B upregulation in APE1 knockdown clones as determined from the RNA sequencing data obtained here requires further validation at the protein level. It can be expected that APE1 is required for demethylation, rather than enhancing methylation, and is required to stimulate TDG activation which specifically excises and removes G:T mispairs resulting from deamination of 5-methylcytosine residues (Fitzgerald and Drohat, 2008; Schuermann *et al.*, 2016; Weber *et al.*, 2016). Nevertheless, the role of APE1 in methylation/demethylation is beyond the scope of this thesis and further investigation is required to delineate the role of APE1 in this process.

4.5. Summary of this chapter

In conclusion, despite the cellular and molecular heterogeneity of the AML cell lines models used in this work, the outcome of experimental investigations are consistent across all cell lines. This implies that targeting APE1 in AML would not be expected to have significant improvement in AML treatment, in terms of combination therapy. However, there may be some clinical value in using APE1 inhibitors as monotherapy, with evidence that this approach may potentially be used to control the proliferation of leukaemic cells.

In summary, this chapter has demonstrated the following:

- APE1 knockdown reduces AML cells proliferation and cloning efficiency.
- Despite reduced cell proliferation, there no evidence of significant alteration in cell cycle kinetics.
- No potentiation of cytotoxic induced chemotherapy was evident following APE1 knockdown in AML cells. Rather, loss of APE1 antagonised the cytotoxicity of several anti-leukaemic agents.
- Genomic AP sites were rapidly removed in APE1 deficient cells compared to control APE1 proficient cells. RNA sequencing revealed no obvious alternative mechanism by which APE1 deficient AML cells remove AP sites.

- Upregulation of several genes involved in cycle progression, cell proliferation, and tumour suppressor genes may provide protection for AML cells from accumulating oxidative DNA damage, and likely contribute to the chemotherapy tolerant phenotype observed in APE1 deficient AML cells.

Chapter 5: Targeting APE1 in AML using small molecule inhibitors

5.1. Introduction

The ability of cancer cells to recognise and repair chemotherapy-induced DNA damage could contribute to treatment resistance and relapse. Therefore, inhibition of particular DNA repair proteins using small specific inhibitors can efficiently enhance chemotherapy -induced cytotoxicity. Chapter 4 demonstrated the utility of targeting APE1 in AML cell line models using RNA interference. However, although targeting APE1 using RNAi did not sensitise AML cells to conventional chemotherapy, using pharmaceutical inhibitors may additionally function by trapping APE1 *in situ*, which could prove cytotoxic independent of loss of function via down-regulation of transcript levels. Furthermore, small molecule inhibitors often inhibit their target rapidly and effectively and do not require selection or further cloning to observe a phenotype, which is often required when using RNAi. Finally, delivery of RNAi to some cells can be inefficient and problematic. In contrast, many small molecule inhibitors are readily taken up by cells.

Since RNAi techniques do not differentially inhibit specific APE1 functions, it is important to target specific APE1 functions independently in order to address their individual contributions. To this end, several function-specific inhibitors have been explored and investigated in preclinical settings and categorised into two major subclasses; DNA repair function inhibitors and redox functions inhibitors. More details about specific APE1 inhibitors can be found in section 1.2.1.4.

5.1.1. DNA repair function inhibitors

Targeting DNA repair in leukaemia is a promising approach but has not been extensively investigated, in particular base excision repair. Preclinical studies have investigated the potential utility of PARP-1 inhibition in leukaemia and generated encouraging results (Gaymes *et al.*, 2009; Gaymes *et al.*, 2013; Esposito *et al.*, 2015; Wang *et al.*, 2015). However, targeting APE1 as a therapeutic strategy in AML has yet to be investigated. This might be due to the lack of potent and specific inhibitors for APE1 functions. Furthermore, the exact molecular participation of APE1 in AML pathogenesis and its regulator mechanisms remain elusive.

Several studies have identified a number of potential APE1 inhibitors (Madhusudan *et al.*, 2005; Simeonov *et al.*, 2009; Bapat *et al.*, 2010; Rai *et al.*, 2010; Mohammed *et al.*, 2011; Dorjsuren *et al.*, 2012; Rai *et al.*, 2012; Srinivasan *et al.*, 2012; Raia *et al.*, 2013; Qian *et al.*,

2014). However, none of the endonuclease function inhibitors have been investigated in pre-clinical animal models or clinical trials in humans, presumably due to a lack of specificity, undesirable toxicity or poor drug-like properties and cellular membrane permeability (Wilson and Simeonov, 2010; Al-Safi *et al.*, 2012; Li and Wilson, 2014).

Several studies have investigated the efficacy of targeting APE1 in leukaemia in a limited context using the non-specific APE1 inhibitor methoxyamine (She *et al.*, 2005; Bulgar *et al.*, 2010; Caimi *et al.*, 2014).

Methoxyamine (MX), tradename TRC102, is non-specific inhibitor of APE1 endonuclease activity, which functions by inhibiting APE1 lyase activity on AP sites following removal of damaged DNA bases by glycosylases. MX reacts with the aldehyde group within the AP site that results in the formation of an intermediate adduct refractory to APE1 lyase activity (Rosa *et al.*, 1991; Liu and Gerson, 2004). Pre-clinical studies demonstrated enhancement of manumycin and fludarabine induced cytotoxicity in AML cells and murine xenograft models when used in combination with MX (She *et al.*, 2005; Bulgar *et al.*, 2010). Furthermore, a phase I clinical trial of MX in combination with fludarabine for treatment relapsed/refractory chronic lymphocytic leukaemia (CLL) and lymphoid malignancies revealed good tolerability with no dose limiting toxicities (Caimi *et al.*, 2014). Furthermore, this trial also reported that MX increased the activity of fludarabine with limited additional toxicity (Caimi *et al.*, 2014). These encouraging results led to speculation that MX may be efficacious in combination with standard therapy for AML.

5.1.2. Redox function inhibitors

Unlike DNA repair inhibitors, the development of APE1 redox inhibitors has been more limited, presumably due to the lack of robust screening methods to identify such inhibitors. Nevertheless, several redox inhibitors are now under investigation in pre-clinical settings, including E3330 and its derivative analogues RN8-51, 10-52, and 7-60. Although the ability of E3330 to specifically impair APE1 activity and inhibit downstream target transcription factors has been well demonstrated in several studies (Miyamoto *et al.*, 1992; Goto *et al.*, 1996; Zou and Maitra, 2008; Fishel *et al.*, 2010; Cardoso *et al.*, 2012), the exact mechanism of action is still inconclusive (Manvilla *et al.*, 2011; Zhang *et al.*, 2013). Inhibition of APE1 redox function in AML cells using E3330 in combination with all-trans retinoic acid induced cell differentiation and apoptosis (Fishel *et al.*, 2010). Despite promising reports of the

potential utility of this molecule in cancer treatment, characterisation of its activity in leukaemia is still very limited.

5.2.Aims of this chapter

The overall aim of the work presented in this chapter was to investigate whether APE1 inhibition using small molecule inhibitors affects AML cell line growth and sensitivity to cytotoxic agents used in the treatment of AML. This aim was addressed by the following specific objectives:

- To treat AML cell lines AML3, U937 and HL-60 with APE1 inhibitors methoxyamine, APE1 inhibitor III and E3330 and investigate their single agent cytotoxic effects.
- To investigate effects of APE1 inhibitors as single agents on cell cycle kinetics and AP sites accumulation
- To determine AML cell line sensitivity to APE1 inhibitors in combination with temozolomide, Ara-C, daunorubicin, clofarabine, fludarabine and etoposide.

5.3. Results

5.3.1. Evaluating the anti-leukaemic activity of methoxyamine

5.3.1.1. *Methoxyamine single agent cytotoxicity*

The aim of this section was to evaluate the cytotoxic effect of MX as a single agent on AML cell lines. Assessment of MX single agent cytotoxicity was measured by two methods growth inhibition /trypan blue dye exclusion, and colony formation assay. Prior to performing MX dosing, MX was dissolved in PBS and its pH was adjusted to 7±0.02 using sterile 1 N sodium hydroxide.

BER inhibition by MX appears to have only modest cytotoxic effects at doses less than 1 mM on AML3, U927 and HL-60 cells (Figure 5.1A). GI_{50}, which refers to the concentration of drug that inhibit 50% of cells growth, was determined using GraphPad Prism software 6.0. GI_{50} concentrations for MX in AML3, U937 and HL-60 cells were 1.59 mM, 1.42 mM and 1.47 mM, respectively.

In contrast to the phenotype demonstrated by growth inhibition using trypan blue exclusion, colony formation assay demonstrated low cytotoxicity of MX at high doses and induced a cytostatic phenotype (Figure 5.1B). For example, 20 mM of MX inhibited approximately 47% of AML3 colony formation, whereas this dose reduced cell proliferation to below10% of controls when investigated using the trypan blue exclusion method.

It must be noted that most of the growth inhibition based investigations in this study were performed in 6well plates, with exception of MX based experiments. It was observed that MX may evaporate at 37 °C and reduce cell growth of vehicle control treated cells in adjacent wells (data not shown). Therefore, all MX related experiments were performed in T25 culture flasks instead of 6 well plates.

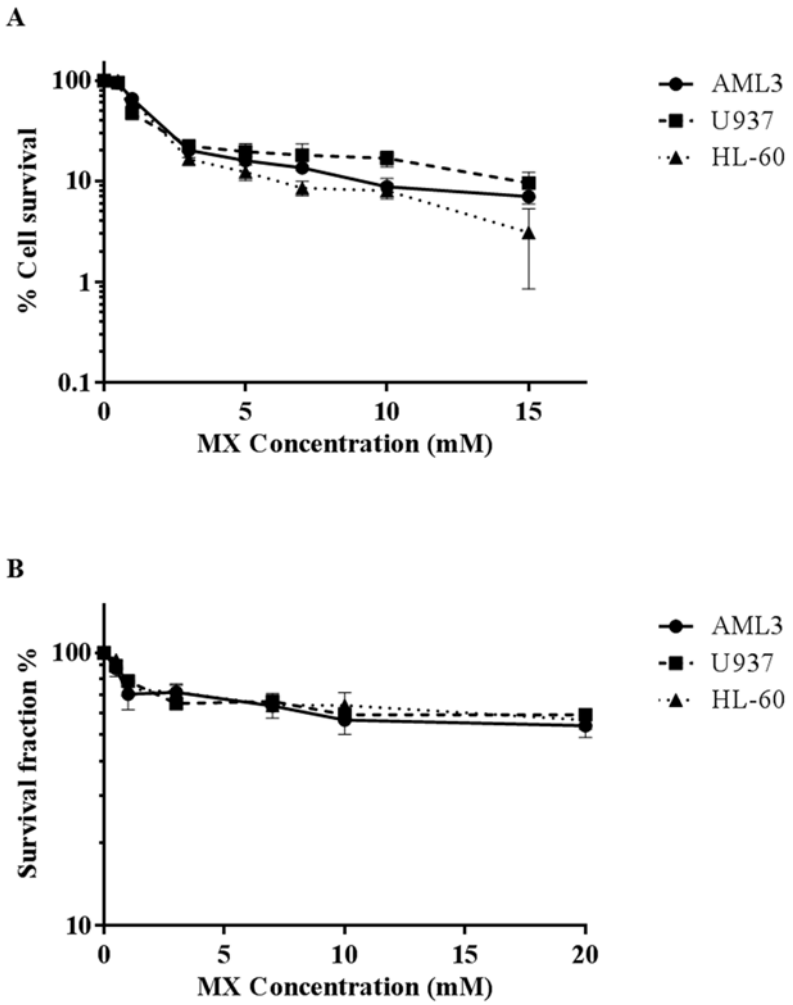


Figure 5.1: Single agent activity of methoxyamine (MX) on AML cells.
(A) Assessment of MX growth inhibition as single agent on AML3, U937 and HL-60 using trypan blue exclusion and cell counting method.
(B) Cytotoxicity of MX as single agent on AML3, U937 and HL-60 using colony formation assay.
In each case, data presented shows the number of viable cells/colonies form each treatment as a percentage of the number of viable cells/colonies from vehicle only treated cells. Results represent the mean and standard deviation of three independent experiments.

5.3.1.2. Optimisation of MX dose for AP site measurement, cell cycle assay and combination with AML treatment.

The aim of this section was to find the optimal biologically effective MX dose that can be used in the assessment of AP site quantification, cell cycle kinetics and in combination with cytotoxic therapies.

It was noted that MX is required in high concentrations (millimolar (mM) levels) to generate a biological effect on cells growth, with a GI_{50} of approximately 1.5 mM. A concentration of 500 μM had no discernible effect on AML cell phenotype and did not alter their growth (Figure 5.1). In addition, low concentration of MX (0.5 mM and 1 mM) did not affect TMZ or fludarabine-induced cytotoxicity (data not shown). Furthermore, several previous reports demonstrated potentiation of MX cytotoxicity at 3 to 6 mM in combination with cytotoxic drugs (She *et al.*, 2005; Yan *et al.*, 2006; Bulgar *et al.*, 2010; Bulgar *et al.*, 2012). Therefore, a dose of 3 mM MX was deemed suitable for studying the effects of this agent on cell cycle, AP site kinetics and enhancement of cytotoxic agent-induced chemotherapy.

5.3.1.3. MX blocks BER by competitive binding to AP sites

MX is not a specific inhibitor for APE1 function, but competitively binds to AP sites generated following the excision of damaged bases by DNA glycosylases. The aim of this section was to investigate the effect of MX treatment on AP sites induction. AML3, U937 and HL-60 cells were pre-treated for 1 hour with 50 μM H_2O_2, then washed with PBS and transferred into RMPI growth media supplemented with 3 mM MX. Control vehicle-treated cells were transferred into RPMI media without MX. Cells were isolated prior to treatment with H_2O_2 (0 hour) and 2, 4 and 8 hours after treatment with H_2O_2. It must be noted that this assay was performed once but with triplicates for each sample, therefore statistical analysis cannot be performed.

Results demonstrated an apparent reduction in AP site frequency in MX/ H_2O_2 treated cells compared to cells treated with H_2O_2 only, implying that MX blocks AP site and prevents aldehyde reactive probe (ARP) reagent from tagging AP sites, thus reducing AP site detection (Figure 5.2). This effects was consistent for all three AML cell lines investigated.

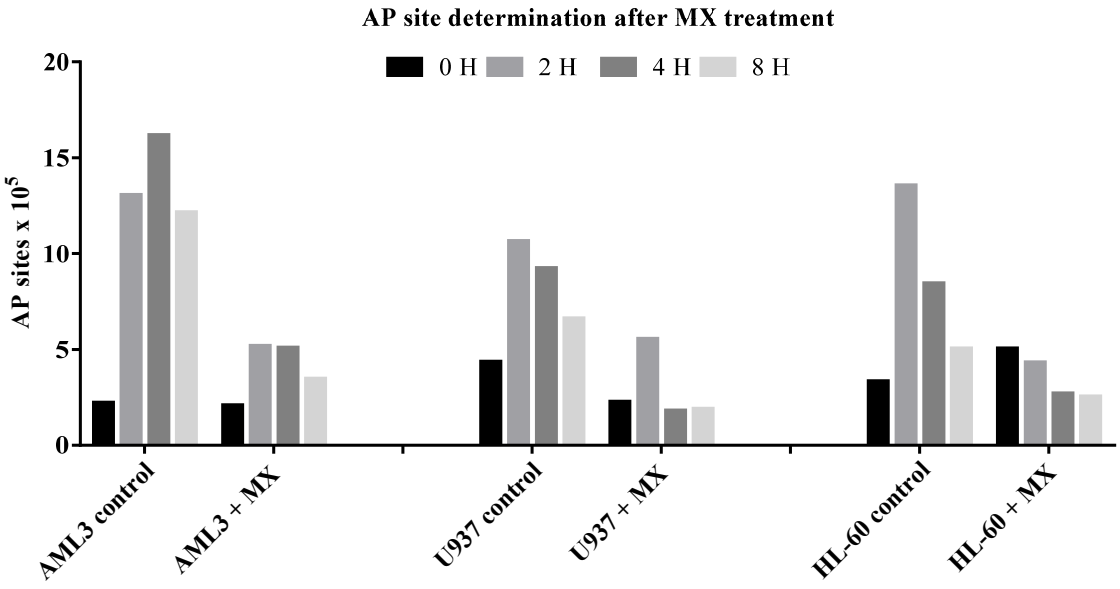


Figure 5.2: AP sites quantification following treatment of AML cells with methoxyamine (MX).

AP sites were quantified following using Aldehyde reactive probe (ARP) assay. AML3, U937 and HL-60 cells were pre-treated with 50 µM of H_2O_2 for 1 hour, to induce DNA damage. Then, cells were washed and treated with 3 mM of MX. Cells were isolated for AP site quantification after prior H_2O_2 treatment (0 hour) and 2, 4, and 8 hours of MX treatment. MX treated cells displayed reduced AP site quantification compared to vehicle control (PBS) treated cells. Data represent single experiment.

5.3.1.4. MX induces cell cycle blockade in S phase

Cell cycle analysis was performed to investigate the effect of MX on AML3, U937 and HL-60AML cells. AML cells were treated with 1 mM and 3 mM MX and isolated at 0, 4, 8, 12, 24, 48 and 72 hours and fixed in 70% ethanol. Cells were stained with propidium iodide (PI) and cell cycle analysis was performed as described in section 2.9.

Three mM MX treatment induced cell cycle blockade in S phase in a concentration and time dependent manner that was apparent between 24 and 72 hours post-treatment (Figures 5.3 and 5.4), but which was not discernible in the first 12 hours post-treatment (data not shown). In contrast, 1 mM MX had no discernible effect on cell cycle kinetics in any of the three cell lines investigated (data not shown).

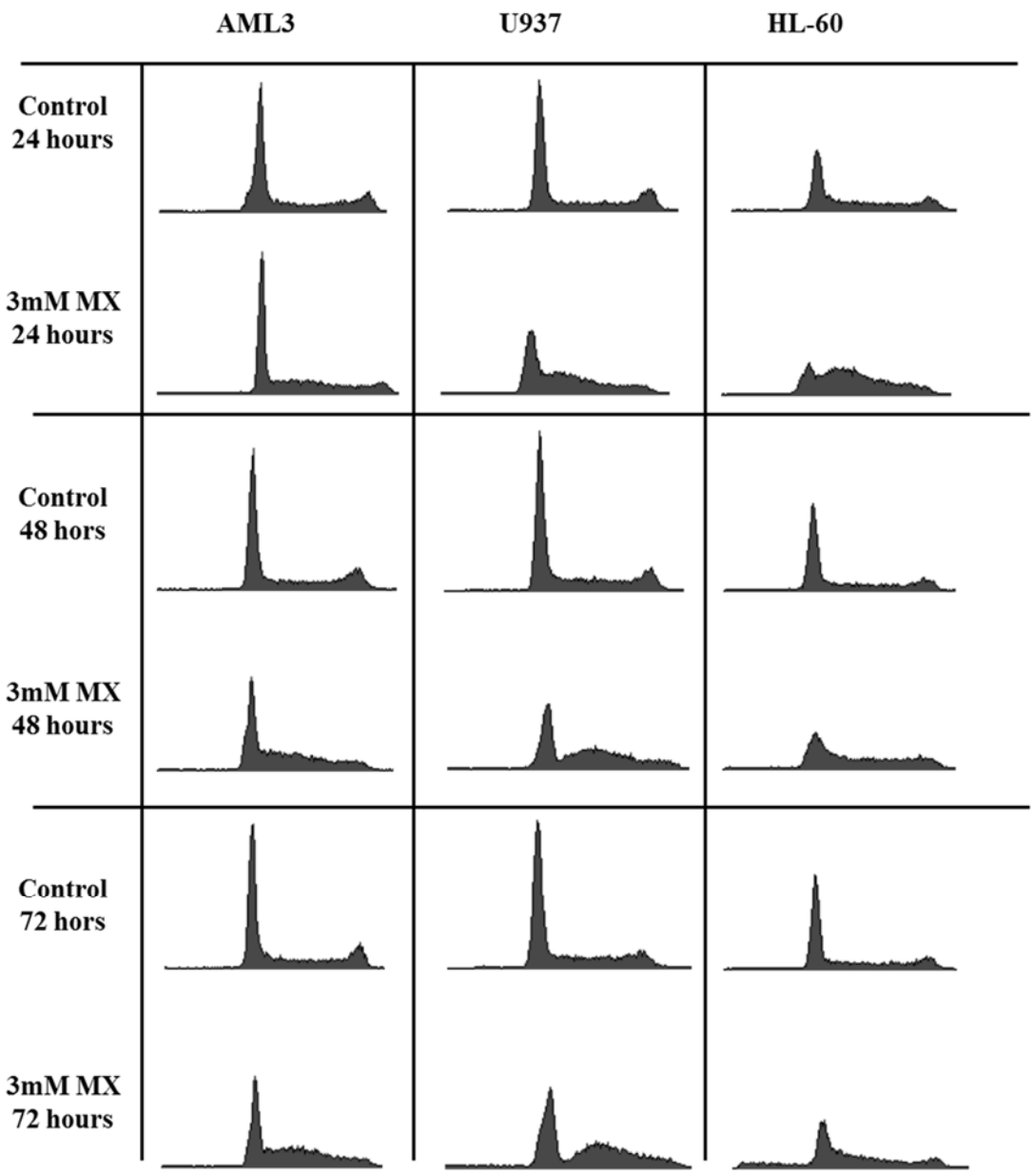


Figure 5.3: Effect of methoxyamine (MX) on cell cycle kinetics of AML cells.

AML3, U937 and HL-60 were treated with 3 mM of MX and cell cycle analysis measured by flow cytometer after 24, 48 and 72 hours of MX treatment. Cell cycle analysis was performed using BD CellQuest Pro software.

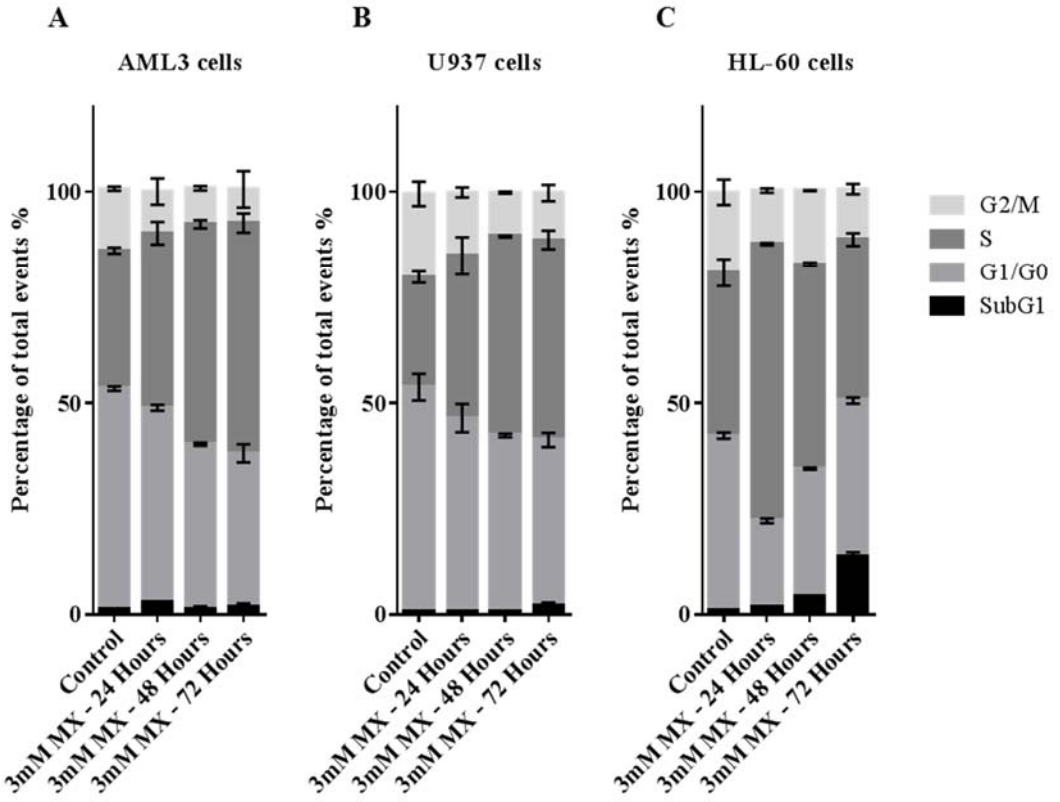


Figure 5.4: Representative quantification of cell cycle distribution following treatment of AML cells with methoxyamine (MX).

Cell cycle analysis was performed on flow cytometer to investigate the effect of methoxyamine (MX) on cell cycle distribution of **(A)** AML3, **(B)** U937 and **(C)** HL-60 cells. Cells were treated with 3 mM of MX and cell cycle was examined after 24, 48 and 72 hours of MX treatment. In each case, results represent the mean and standard deviation of three independent experiments.

5.3.1.5. Cytotoxic effect of MX in combination with cytotoxic chemotherapy

Trypan blue exclusion dye and colony formation were performed to investigate whether inhibition of BER by MX can potentiate the cytotoxicity induced by temozolomide (TMZ), Ara-C, daunorubicin, clofarabine, fludarabine and etoposide. TMZ is not used in AML treatment but is used in this investigation as a control agent because it induces DNA damage that is a substrate for BER (Zhang et al., 2012). In addition, MX is demonstrated to enhance TMZ induced cytotoxicity in several published studies (Fishel et al., 2007; Yan et al., 2007; Reed et al., 2009).

AML3, U937 and HL-60 cells were treated for 4 days with anti-leukaemic agents as single treatment and in combination with 3 mM of MX. Cells were counted using trypan blue exclusion and a TC20 cell counter. For single agent treatments, data are presented as the number of viable cells from each treatment as a percentage of the number of viable cells from vehicle control treated cells. For MX-chemotherapy combinations, data are presented as the number of viable cells form each treatment as a percentage of the number of viable cells from MX only treated cells.

AML3 cells are less sensitive than U937 and HL-60 to TMZ induced cytotoxicity as described in previous chapter. AML3 cells were relatively insensitive to TMZ up to a concentration of 300 μM. A combination of TMZ and 3 mM MX had no effect on TMZ induced cytotoxicity in AML3 cells (Figure 5.5A). Likewise, the addition of 3 mM MX had no effect on the cytotoxic effects of etoposide in AML3 (Figure 5.5F) and MX was antagonistic in combination with daunorubicin in AML3 (Figure 5.5C). In contrast, AML3 cells were sensitised to the cytotoxic effects of nucleoside analogues Ara-C, clofarabine and fludarabine when combined with MX, but that was not statistically significant (Figures 5.5 B, D and E).

MX antagonised the cytotoxic effects of TMZ, Ara-C, clofarabine and particularly daunorubicin in U937 (Figures 5.6 A, B and C). In contrast, MX had no effect on cellular response to fludarabine and etoposide in U937 (Figures 5.6 E and F).

MX had no discernible effect in the cytotoxicity of TMZ, daunorubicin, Ara-C, clofarabine or fludarabine in HL-60, but was significantly antagonistic in combination with etoposide (Figure 5.7).

It has been reported that MX potentiates fludarabine induced cytotoxicity in HL-60 cells (Bulgar et al., 2010). However, attempts to replicate this finding were unsuccessful in HL-60, as well as AML3 and U937.

When investigated using the colony formation assay, MX consistently antagonised the cytotoxicity of TMZ, Ara-C and daunorubicin (Figure 5.8), which was observed in AML3, U937 and HL-60.

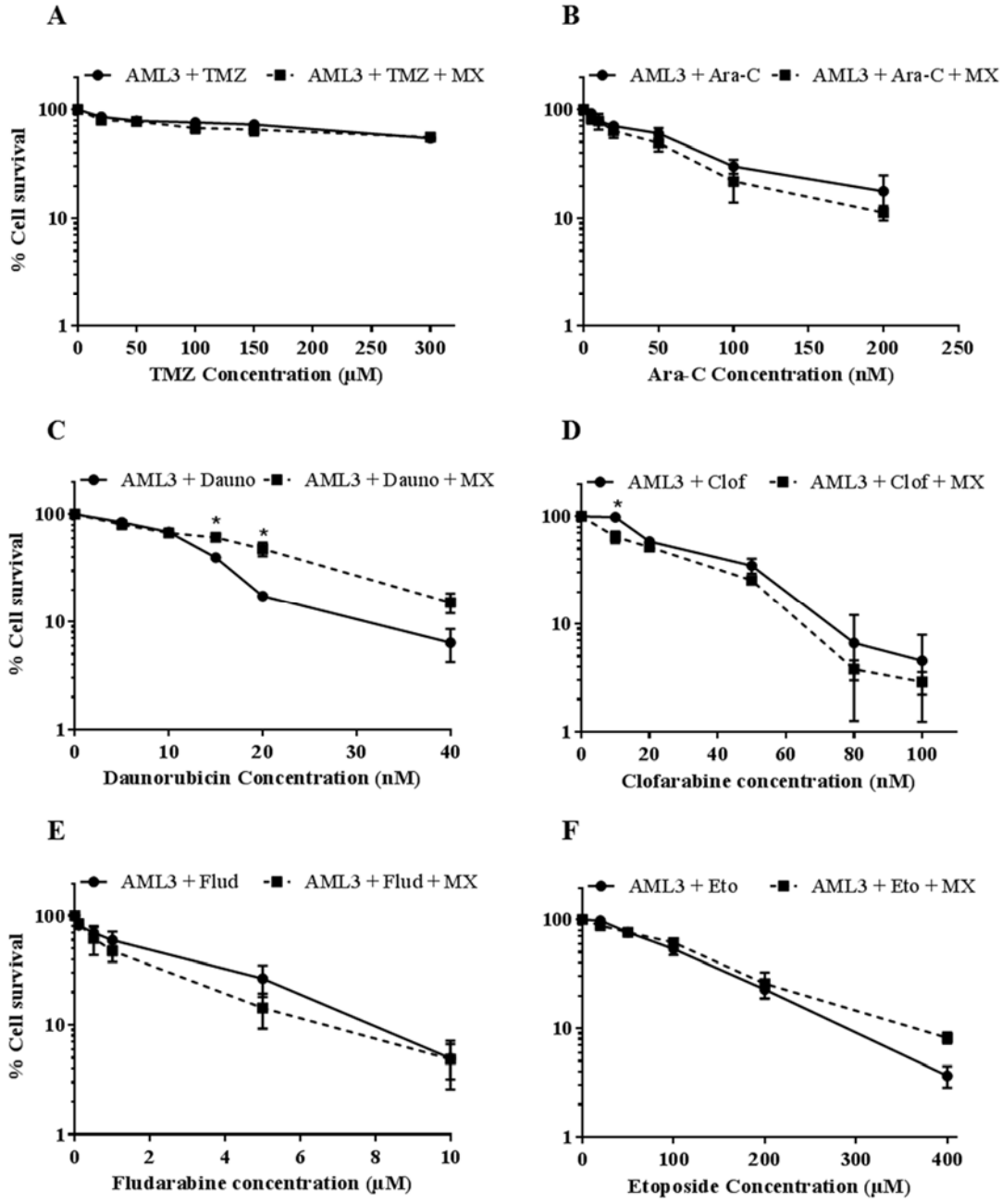


Figure 5.5: Growth inhibition of methoxyamine (MX) in combination with cytotoxic chemotherapy on AML3 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with MX (3 mM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to MX single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

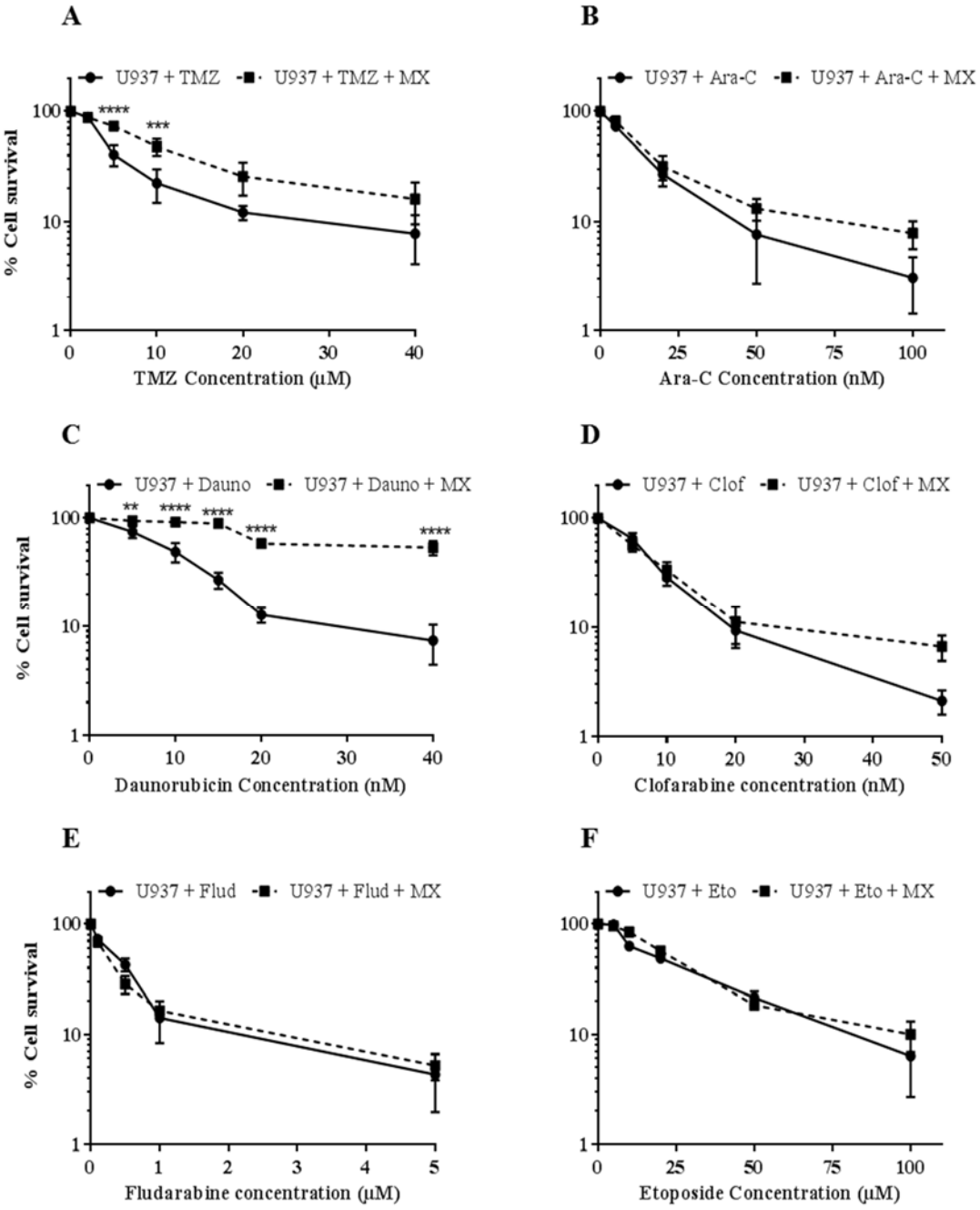


Figure 5.6: Growth inhibition of methoxyamine (MX) in combination with cytotoxic chemotherapy on U937 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with MX (3 mM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to MX single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

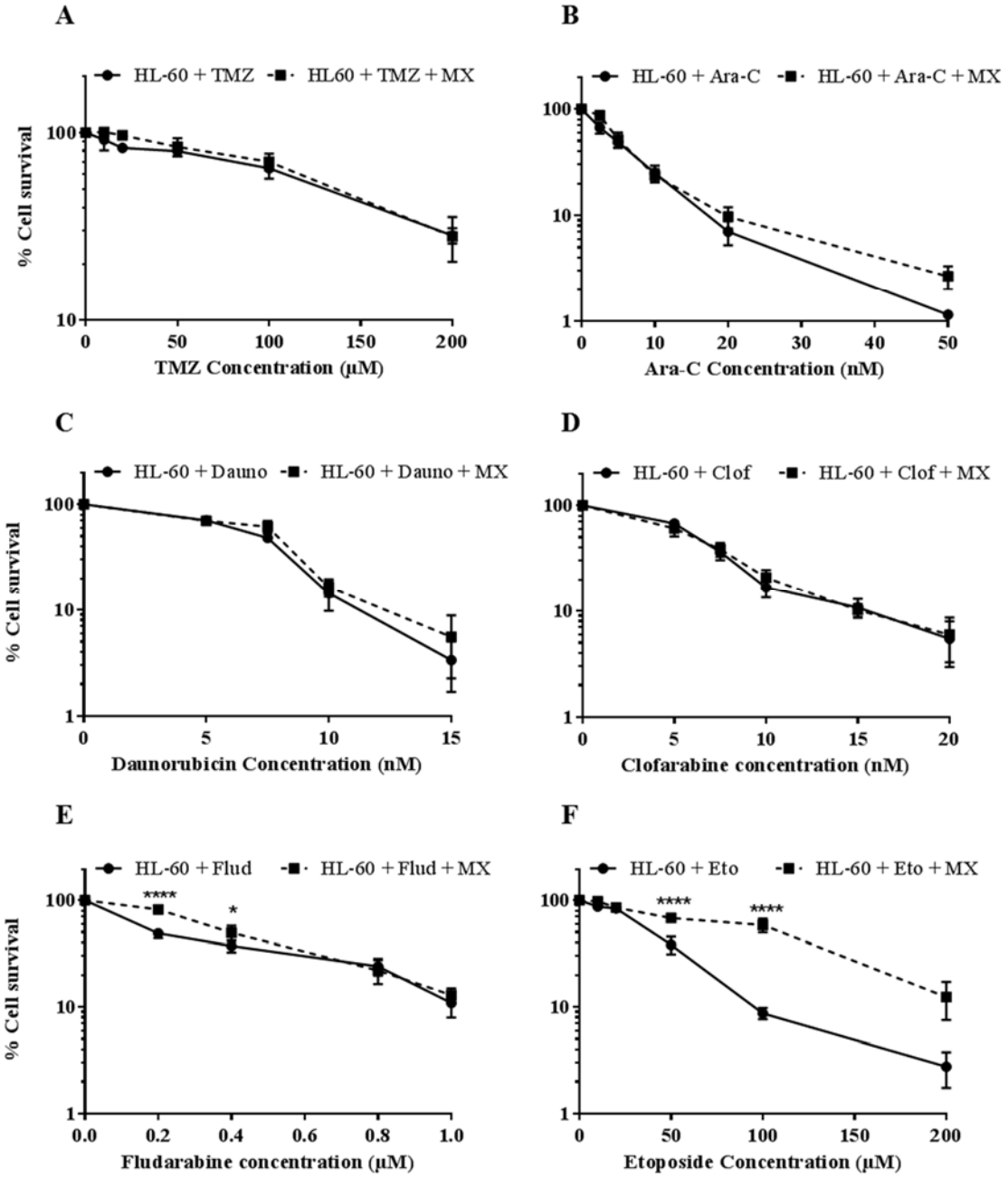


Figure 5.7: Growth inhibition of methoxyamine (MX) in combination with cytotoxic chemotherapy on HL-60 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with MX (3 mM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to MX single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

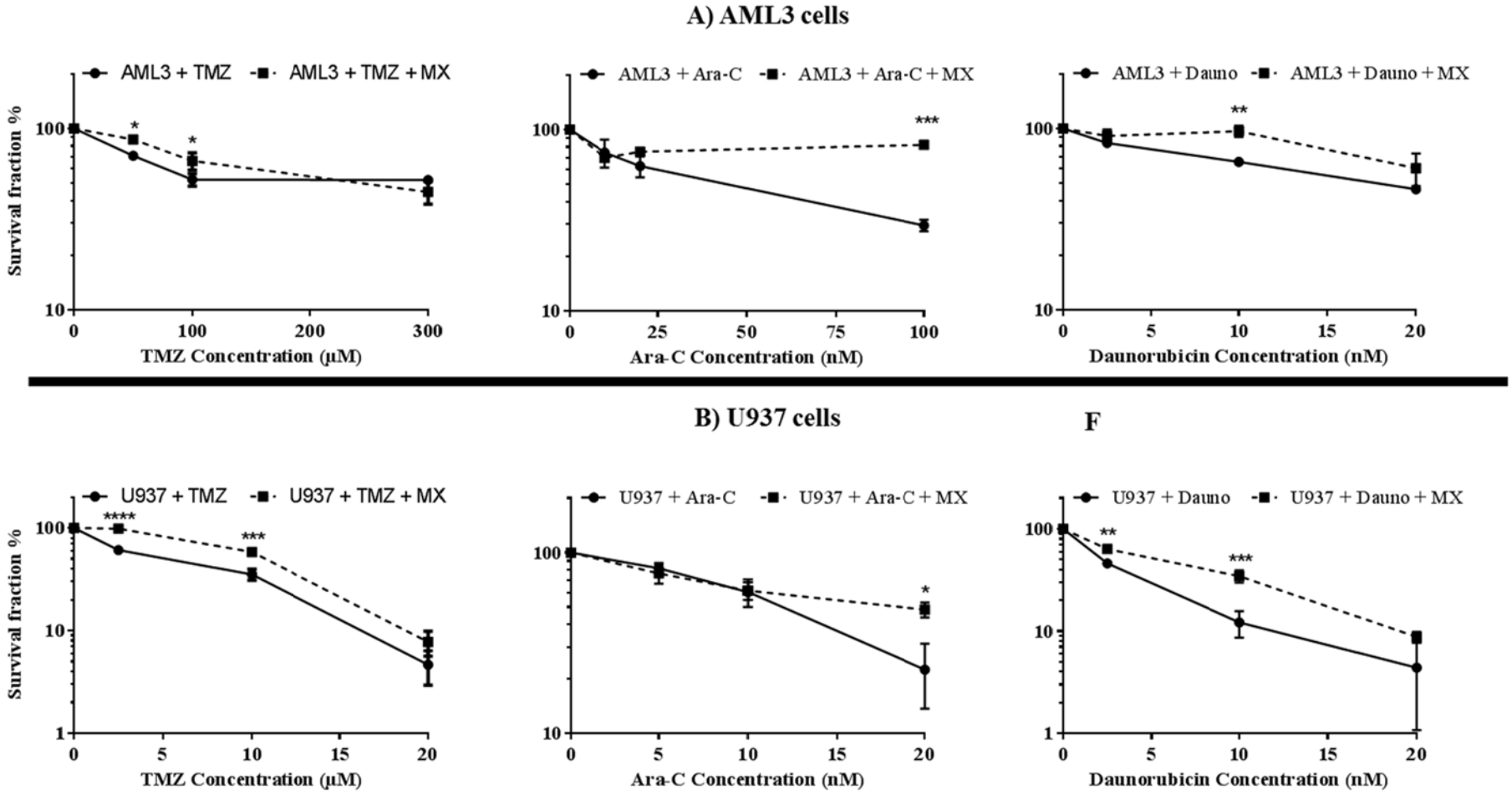


Figure 5.8: Cytotoxicity of methoxyamine (MX) in combination with anti-leukaemia chemotherapy using colony formation assay (continued on next page).

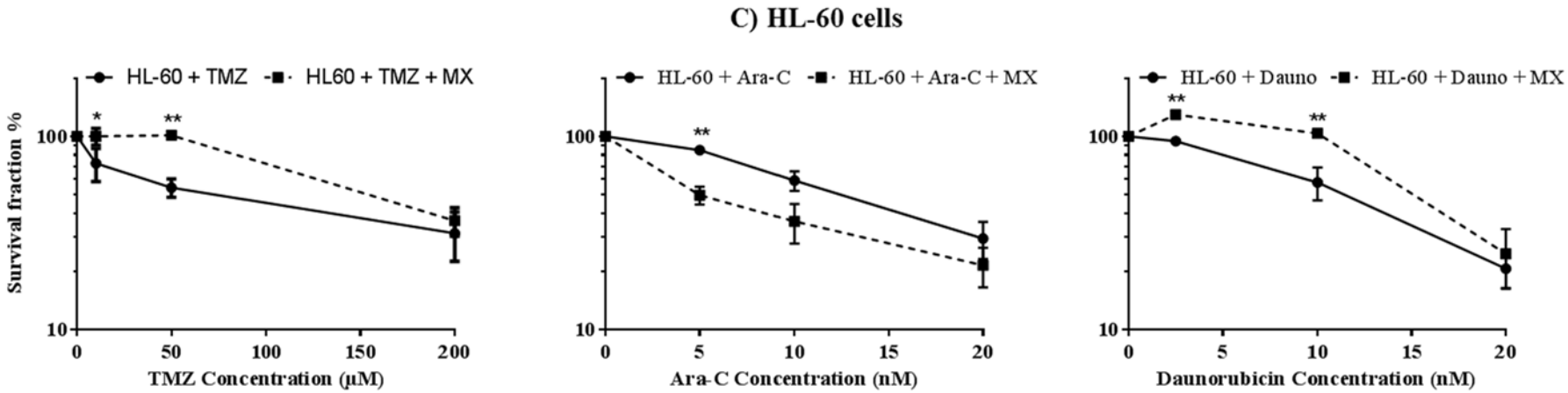


Figure 5.8: Cytotoxicity of methoxyamine (MX) in combination with anti-leukaemia chemotherapy using colony formation assay (continued from previous page).

Colony formation assay was performed to investigate the cytotoxicity of MX (3 mM) in combination with TMZ, Ara-C and daunorubicin on **(A)** AML3 **(B)** U937 and **(C)** HL-60. Data presented for chemotherapy without MX shows the number of viable colonies form each treatment as a percentage of the number of viable colonies from vehicle only treated cells. For combination treatment, data was normalised to single agent cytotoxicity effect of 3 mM MX instead of vehicle treated cells. In each case, results represent the mean and standard deviation of two independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

5.3.2. Inhibition of AP endonuclease function of APE1 using APE1 inhibitor III (APE1-III)

5.3.2.1. Single agent activity of APE1-III in AML cells

Growth inhibition assay using trypan blue exclusion and colony formation assay was performed to measure the cytotoxic effects of the APE1-III inhibitor as a single agent in AML3, U937 and HL-60 cells. Both methods consistently demonstrated the potent cytotoxic effect of APE1-III inhibitor with a GI_{50} of 0.9-1.1 μM in all cell lines (Figure 5.9). APE1-III was used at concentration of 0.7 μM and 0.4 μM to investigate the effects on cell cycle kinetics and AP site formation.

5.3.2.2. Cell cycle effect of APE1-III

Cell cycle analysis was performed to determine effect of APE1-III on cell cycle kinetics. Treatment of AML3, U937 and HL-60 with APE1-III at 0.7 μM had no discernible effect on cell cycle kinetics at 24, 48 and 72 hours post-treatment (Figure 5.10).

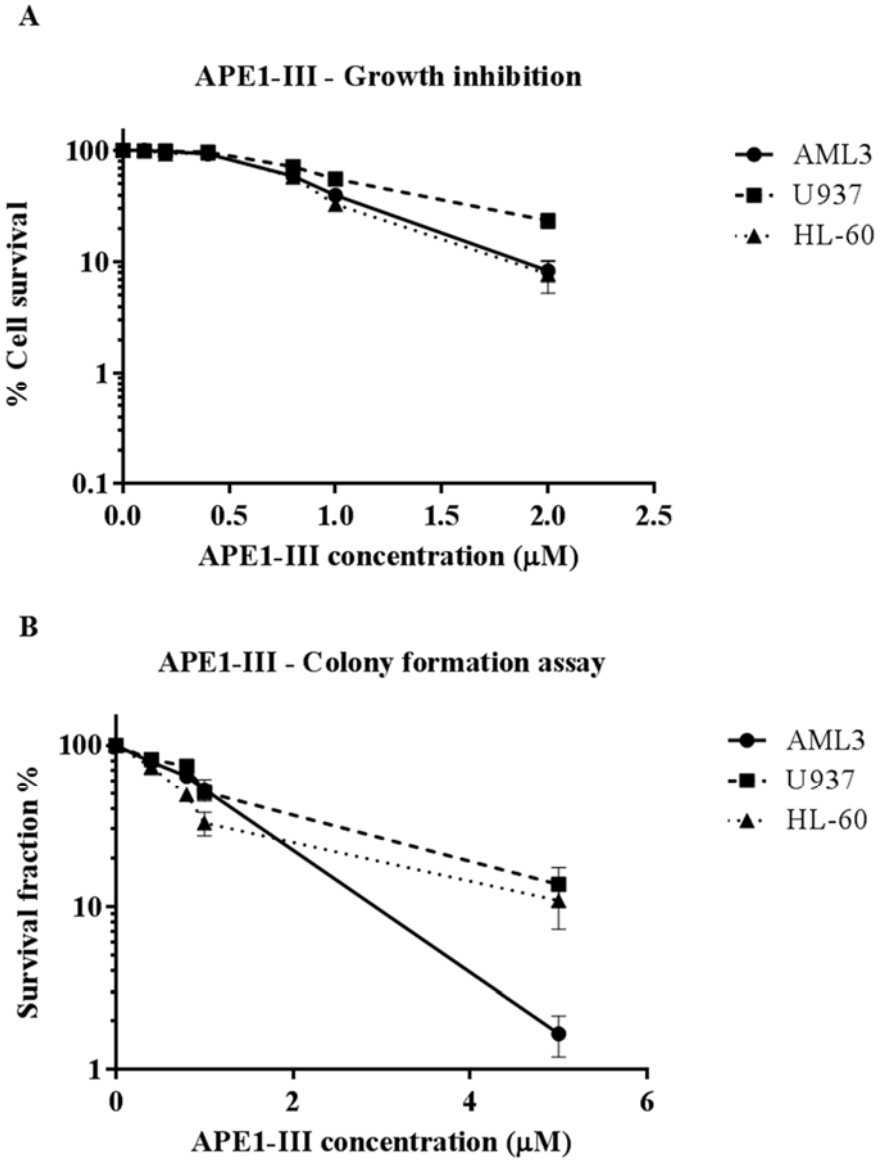


Figure 5.9: Cytotoxicity of APE1 inhibitor III (APE-III) on AML cells.

(A) Assessment of APE1-III growth inhibition as single agent on AML3, U937 and HL-60 using trypan blue exclusion and cell counting method.

(B) Cytotoxicity of APE1-III as single agent on AML3, U937 and HL-60 using colony formation assay.

In each case, data presented shows the number of viable cells/colonies form each treatment as a percentage of the number of viable cells/colonies from vehicle only treated cells. Results represent the mean and standard deviation of three independent experiments.

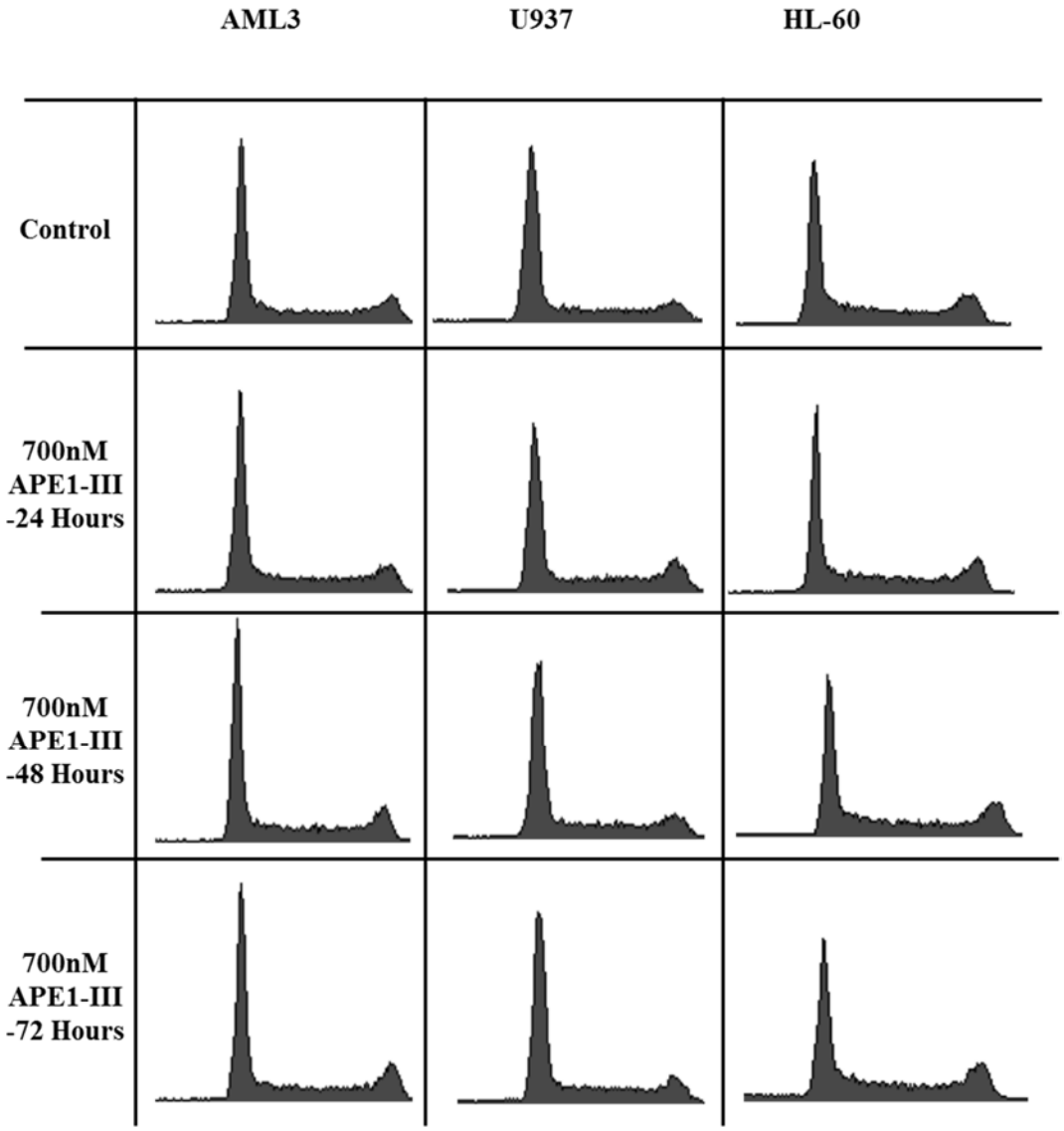


Figure 5.10: Effect of APE1 inhibitor III (APE1-III) on cell cycle kinetics of AML cells.

Cell cycle kinetics for AML3, U937 and HL-60 cells was determined after 24, 48 and 72 hours of treatment with 700 nM of APE1-III. Cell cycle analysis was performed using BD CellQuest Pro software. Cell cycle kinetics for all control cells was performed at 24, 48 and 72 hours (data not shown). APE1-III showed no effect on AML cells cycle distribution.

5.3.2.3. Cytotoxic effect of APE1-III in combination with cytotoxic chemotherapy

The aim of the section was to investigate whether co-incubation of AML cells with APE1-III affects the anti-leukaemic activity of cytotoxic chemotherapy. AML3, U937 and HL-60 cells were treated with TMZ, Ara-C, daunorubicin, clofarabine, fludarabine or etoposide as monotherapy and in combination with 700 nM APE1-III. Monotherapy data are presented as the number of viable cells/colonies form each treatment as a percentage of the number of viable cells/colonies from vehicle only treated cells. For APE1-III-chemotherapy combination treatments, data are presented as the number of viable cells form each treatment as a percentage of the number of viable cells/colonies from APE1-III inhibitor only treated cells/colonies.

Using the growth inhibition assay with trypan blue and cell counting, a low concentration of APE1-III (400 nM) did not affect growth inhibition by any of the chemotherapeutic agents on AML cells (data not shown). However, a combination of 700 nM APE1-III had no added cytotoxicity, but was occasionally antagonistic (Figures 5.11, 5.12 and 5.13).

Consistent with the phenotype observed in the growth inhibition assay above, data derived from the colony formation assay demonstrated antagonism of TMZ, Ara-C and daunorubicin in AML3, U937 and HL-60 in combination with the APE1 inhibition APE1-III (at 700 nM) (Figure 5.14).

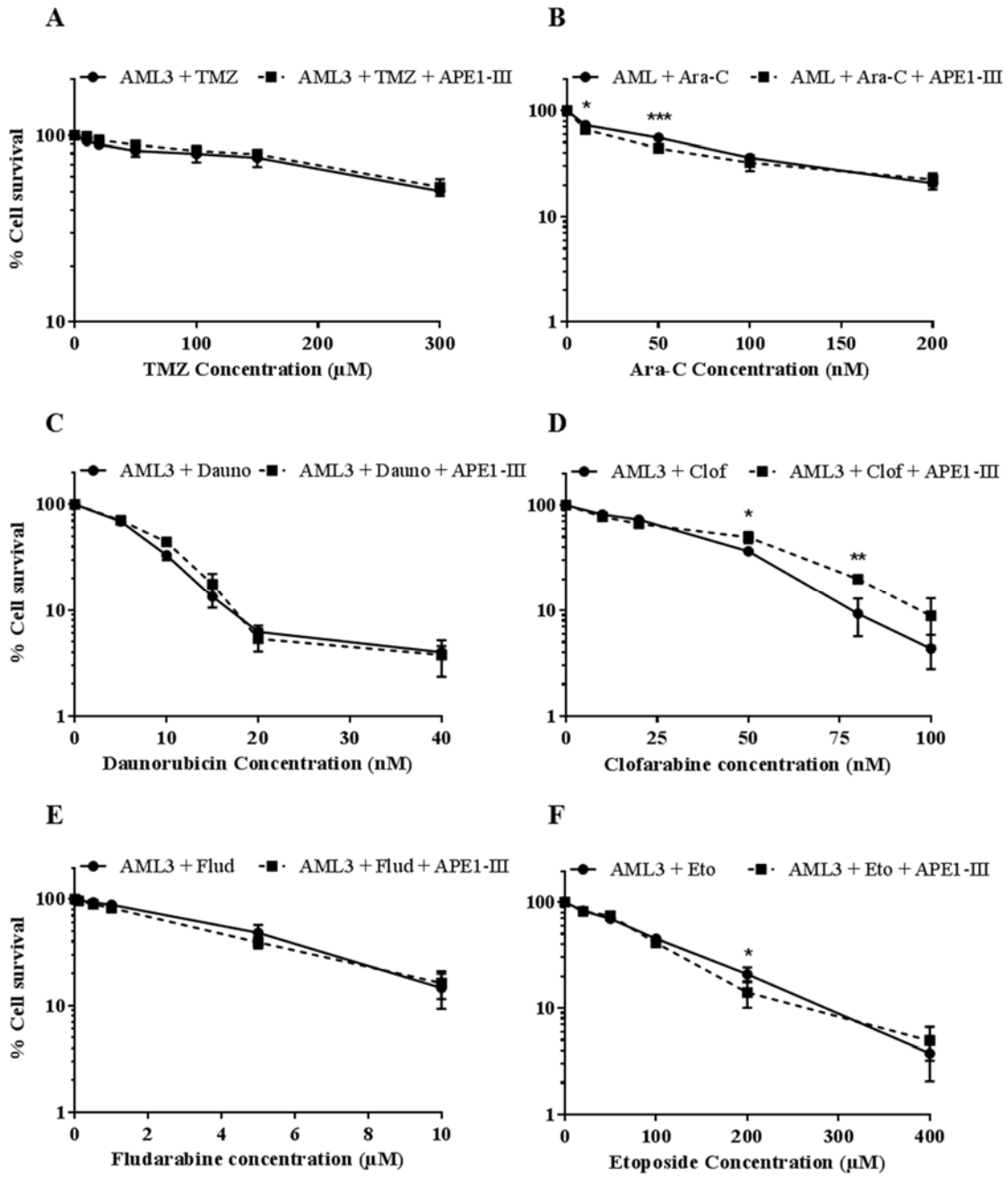


Figure 5.11: Growth inhibition of APE1 inhibitor III (APE1-III) in combination with cytotoxic chemotherapy on AML3 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with APE1-III (700 nM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to APE1-III single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

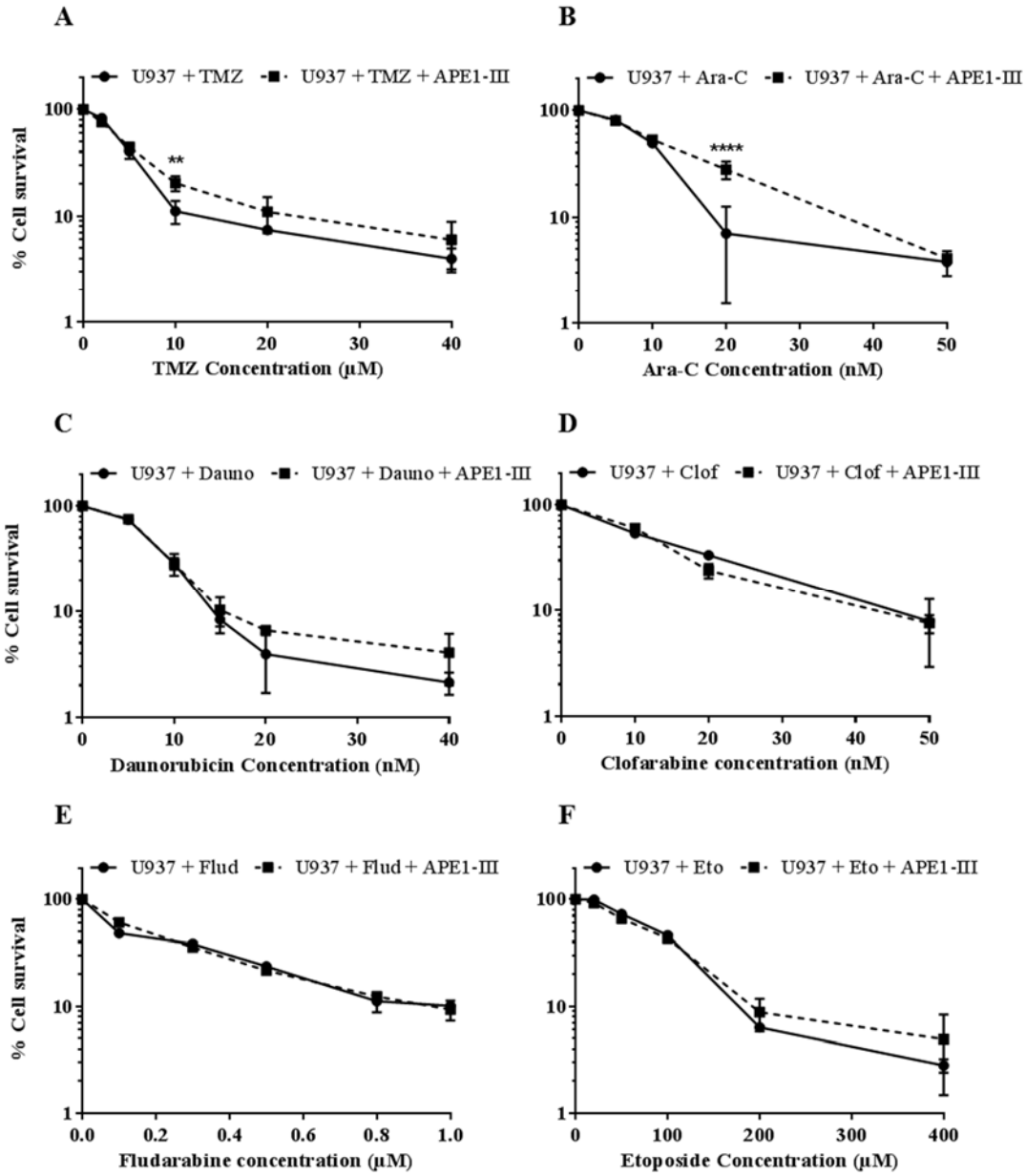


Figure 5.12: Growth inhibition of APE1 inhibitor III (APE1-III) in combination with cytotoxic chemotherapy on U937 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with APE1-III (700 nM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to APE1-III single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

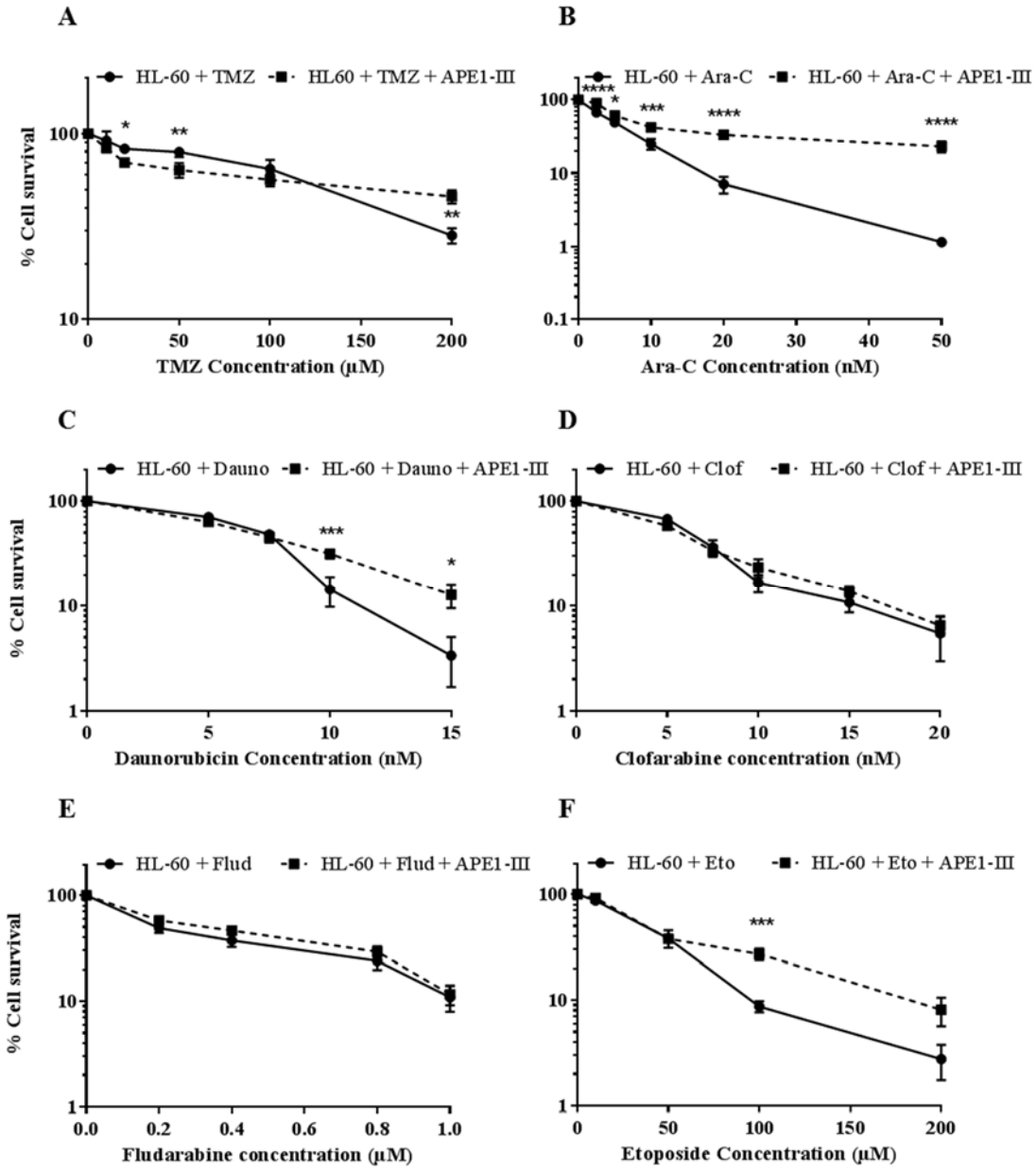


Figure 5.13: Growth inhibition of APE1 inhibitor III (APE1-III) in combination with cytotoxic chemotherapy on HL-60 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with APE1-III (700 nM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to APE1-III single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

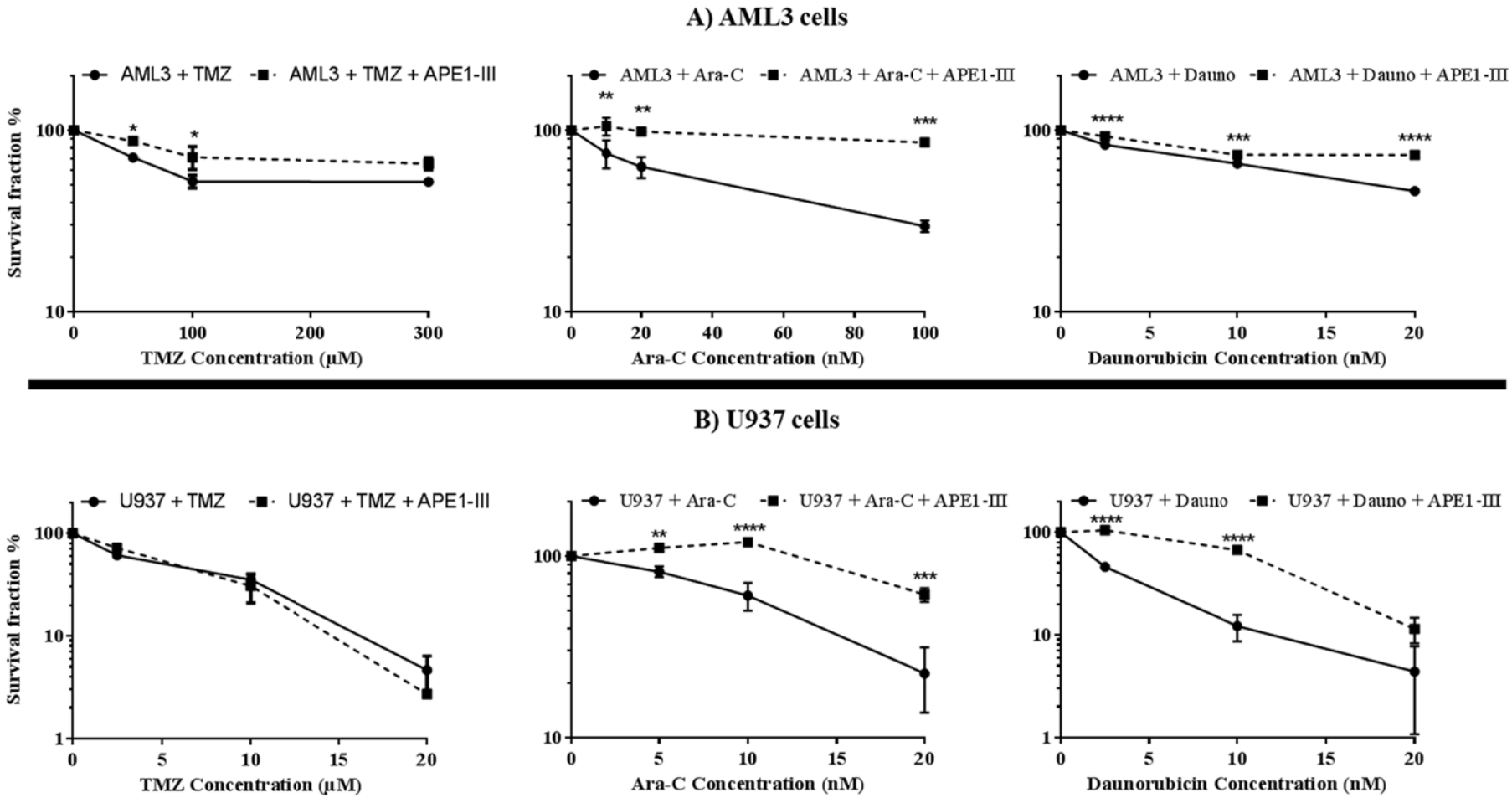


Figure 5.14: Cytotoxicity of APE1 inhibitor III (APE1-III) in combination with anti-leukaemia chemotherapy using colony formation assay (continued on next page).

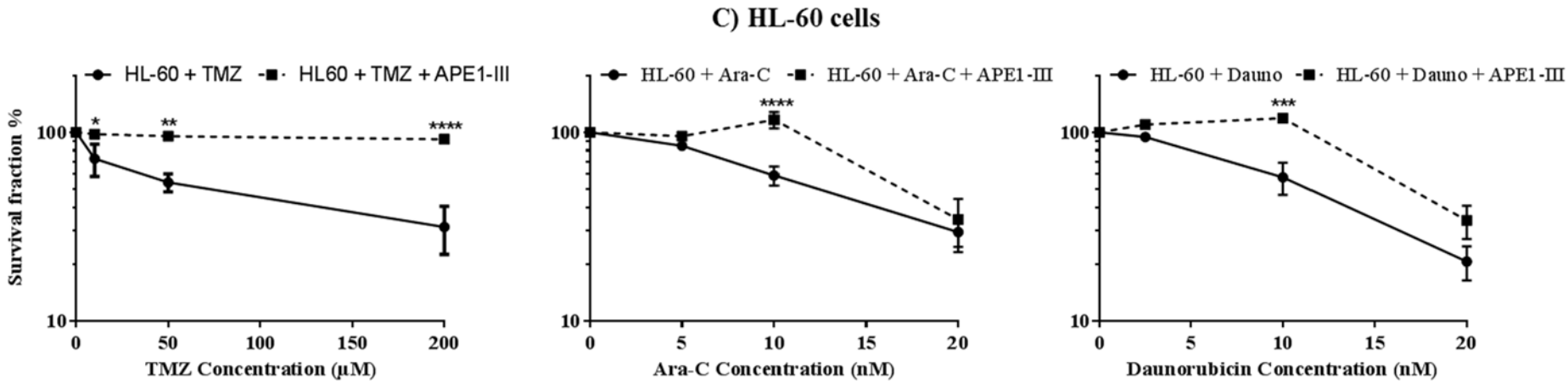


Figure 5.14: Cytotoxicity of APE1 inhibitor III (APE1-III) in combination with anti-leukaemia chemotherapy using colony formation assay (continued from previous page).

Colony formation assay was performed to investigate the cytotoxic effect of APE1-III (700 nM) in combination with TMZ, Ara-C and daunorubicin on **(A)** AML3 **(B)** U937 and **(C)** HL-60. Data presented for chemotherapy without MX shows the number of viable colonies form each treatment as a percentage of the number of viable colonies from vehicle only treated cells. For combination treatment, data was normalised to single agent cytotoxicity effect of 700 nM APE1-III instead of vehicle treated cells. In each case, results represent the mean and standard deviation of two independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

5.3.3. Redox function inhibition using E3330

5.3.3.1. Single agent activity of E3330

Growth inhibition assay and colony formation assay were performed to investigate cytotoxic effect of E3330 alone on AML cells.

It was noted that E3330-induced growth inhibition was attenuated by the addition of FBS in cell culture media (data not shown). Therefore, all experiments described in this section were performed using 5% FBS supplemented cell culture media instead of 10% FBS, which was routinely used in all other experiments.

AML3, U937 and HL-60 cells were treated with E3330 for 4 days and data presented as growth inhibition curves showing the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. E3330 was cytotoxic as a single agent to AML3, U937 and HL-60 cells (Figure 5.15A) with GI_{50} values of 59.4 μM, 65.8 μM and 40.2 μM, respectively.

Similarly, single agent E3330 was cytotoxic effect to AML cells when investigated using the colony formation assay (Figure 5.15B). However, AML3 cells were more sensitive to this APE1 redox function inhibitor compared to U937 and HL-60.

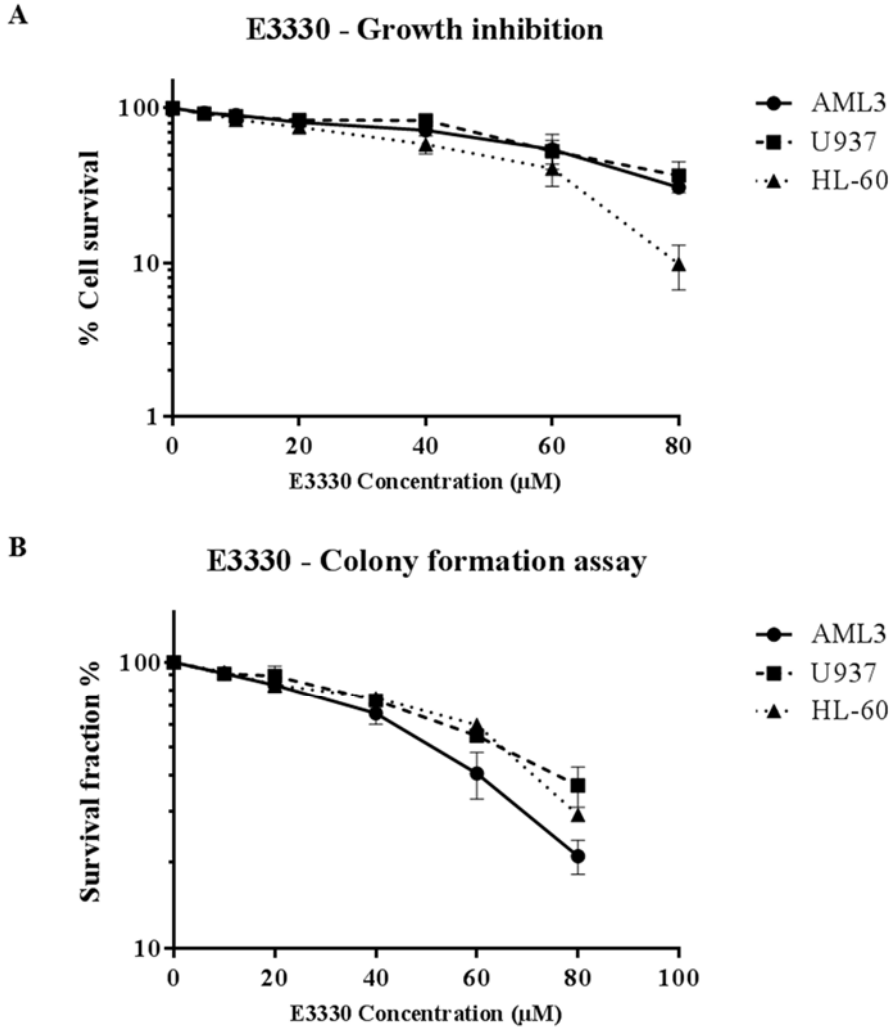


Figure 5.15: Single agent cytotoxicity of E3330 on AML cells.

(A) Assessment of E3330 cytotoxicity as single agent on AML3, U937 and HL-60 using trypan blue exclusion and cell counting method.

(B) Cytotoxicity of E3330 as single agent on AML3, U937 and HL-60 using colony formation assay.

In each case, data presented shows the number of viable cells/colonies form each treatment as a percentage of the number of viable cells/colonies from vehicle only treated cells. Results represent the mean and standard deviation of three independent experiments.

5.3.3.2. E3330 effect on AP sites accumulation

Treatment AML cells with the E3330 inhibitor is not expected to result in AP site accumulation as it only inhibits the redox function of APE1. In order to confirm this, AML cells were pre-treated for 1 hour with 50 μM H_2O_2 to induce DNA damage. Cells were washed and transferred into fresh media supplemented with 2% of FBS and then treated with 40 μM E3330 and AP sites were quantified after 2, 4 and 8 hours of treatment. Again, this was a limited experiment performed only once and the result described here is considered preliminary. Nevertheless, AP sites quantification did not differ in response to E3330 treatment, compared to vehicle control (Figure 5.16). There was a clear increase in AP site levels following treatment with H_2O_2, which then returned to background 8 hours post-treatment.

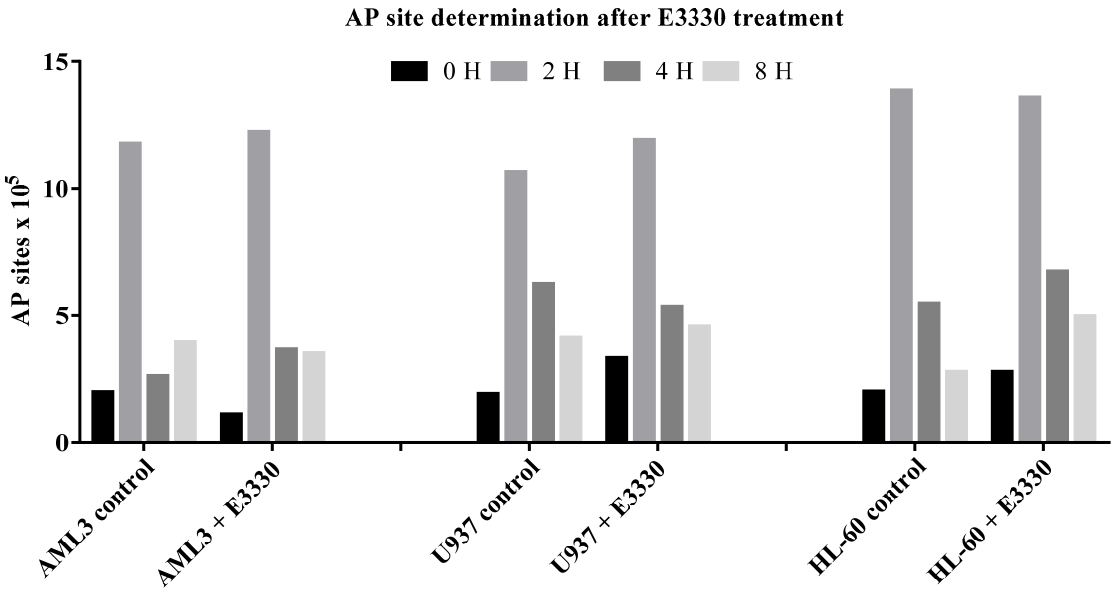


Figure 5.16: AP sites quantification following E3330 treatment.

AP sites were quantified following using Aldehyde reactive probe (ARP) assay. AML3, U937 and HL-60 cells were pre-treated with 50 µM of H_2O_2 for 1 hour, to induce DNA damage. Then, cells were washed and treated with 40 µM of E3330. Cells were isolated for AP site quantification after prior H_2O_2 treatment (0 hour) and 2, 4, and 8 hours of E3330 treatment. E3330 treated cells displayed reduced AP site quantification compared to vehicle control (DMSO) treated cells. Data represent single experiment.

5.3.3.3. Effect of E3330 on cell cycle

The impact of E3330 on cell cycle kinetics is not conclusive with some studies demonstrating no effect on cell cycle profile of pancreatic cells (Zou and Maitra, 2008), and other studies demonstrating impaired transition from G1 to S phase in pancreatic cancer cells after E3330 treatment (Jiang *et al.*, 2010; Fishel *et al.*, 2011). In order to assess the impact of E3330 on AML cells, AML3, U937 and HL-60 were treated with 40 μM E3330, and cell cycle was investigated by flow cytometry 24, 48 and 72 hours after treatment as described in section 2.9. Exposure to E3330 had no discernible effect on cell cycle kinetics of any of the three cell lines investigated (Figure 5.17).

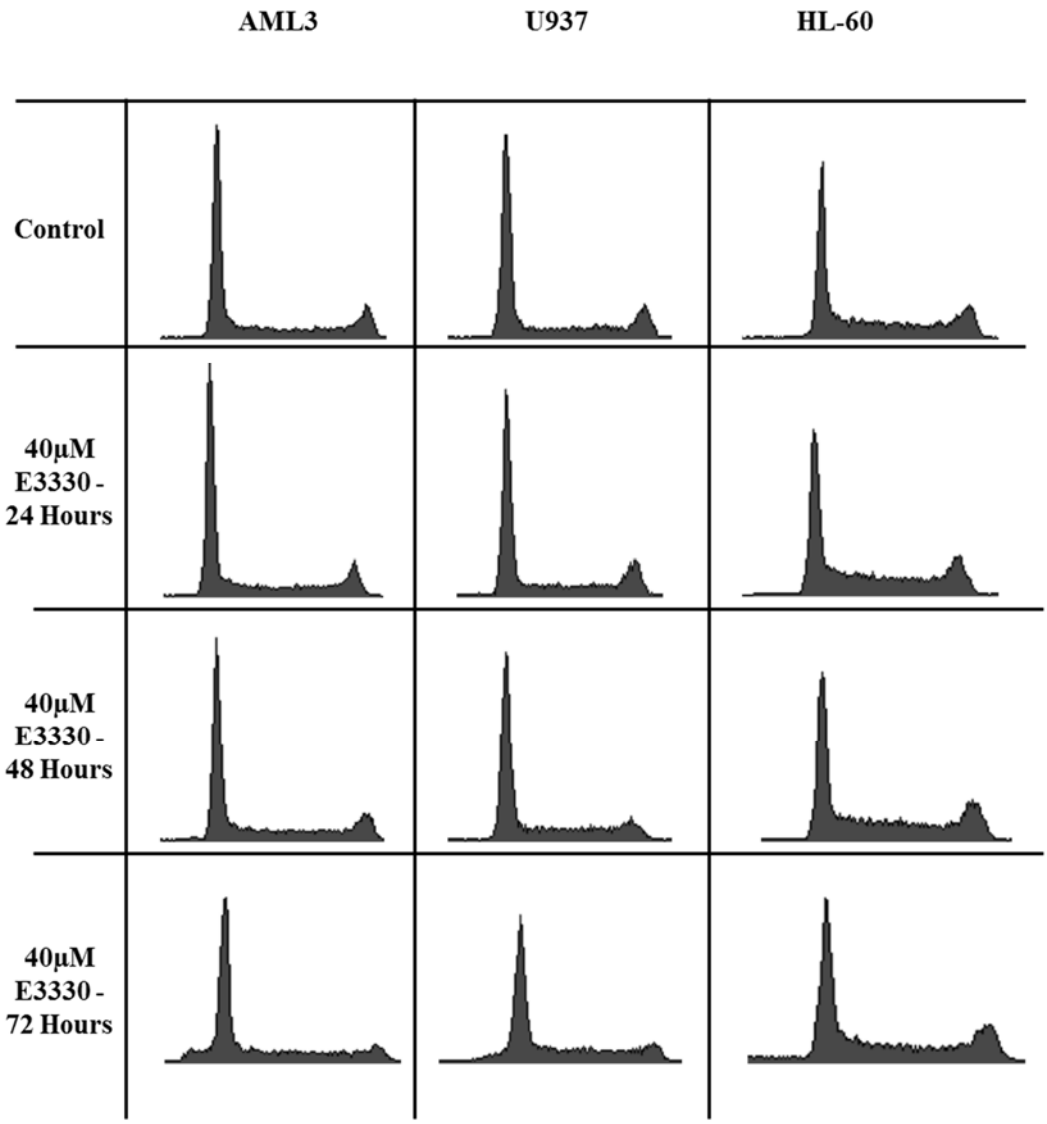


Figure 5.17: effect of E3330 on cell cycle kinetics of AML cells.

Cell cycle kinetics for AML3, U937 and HL-60 cells was determined after 24, 48 and 72 hours of treatment with 40 µM of E3330. Cell cycle analysis was performed using BD CellQuest Pro software. Cell cycle kinetics for all control cells was performed at 24, 48 and 72 hours (data not shown). E3330 showed no effect on AML cells cycle distribution.

5.3.3.4. Cytotoxic effect of E3330 in combination with AML chemotherapy

A limited study demonstrated that inhibition of APE1 redox function using E3330 hyper-sensitised AML cells to retinoic acid induced differentiation and induced apoptosis (Fishel *et al.*, 2010). Growth inhibition and colony formation assays were performed to explore whether E3330 potentiated inhibition of AML cell proliferation in response to cytotoxic chemotherapy.

In order to investigate the effect of E3330 in combination with cytotoxic chemotherapy, cells were treated with chemotherapy alone or in combination with 40 μM E3330. Monotherapy data are expressed as the number of viable cells/colonies form each treatment as a percentage of the number of viable cells/colonies from vehicle only treated cells. For E3330-chemotherapy combination treatments, data are expressed relative to E3330 single agent instead of vehicle-treated control.

Using the growth inhibition assay, treatment with E3330 in combination with several chemotherapeutic agents had no discernible effect on cytotoxicity in most cases (Figures 5.18, 5.19 and 5.20). In some cases, however, E3330 was antagonistic. For example, E3330 displayed no cytotoxicity on AML3 cells when combined with TMZ, clofarabine and fludarabine (Figures 5.18 A, D and E). In contrast, E3330 was antagonistic in combination with daunorubicin and etoposide in AML3 cells (Figures 5.18 C and F).

Data derived from the colony formation assay did not suggest any consistent effect of E3330 in combination with TMZ on AML3 and U937 compared to TMZ alone, but was antagonistic in HL-60 cells (Figure 5.21). Moreover, E3330 in combination with Ara-C or daunorubicin was antagonistic in AML3 and U937 cells (Figures 5.21 A and B).

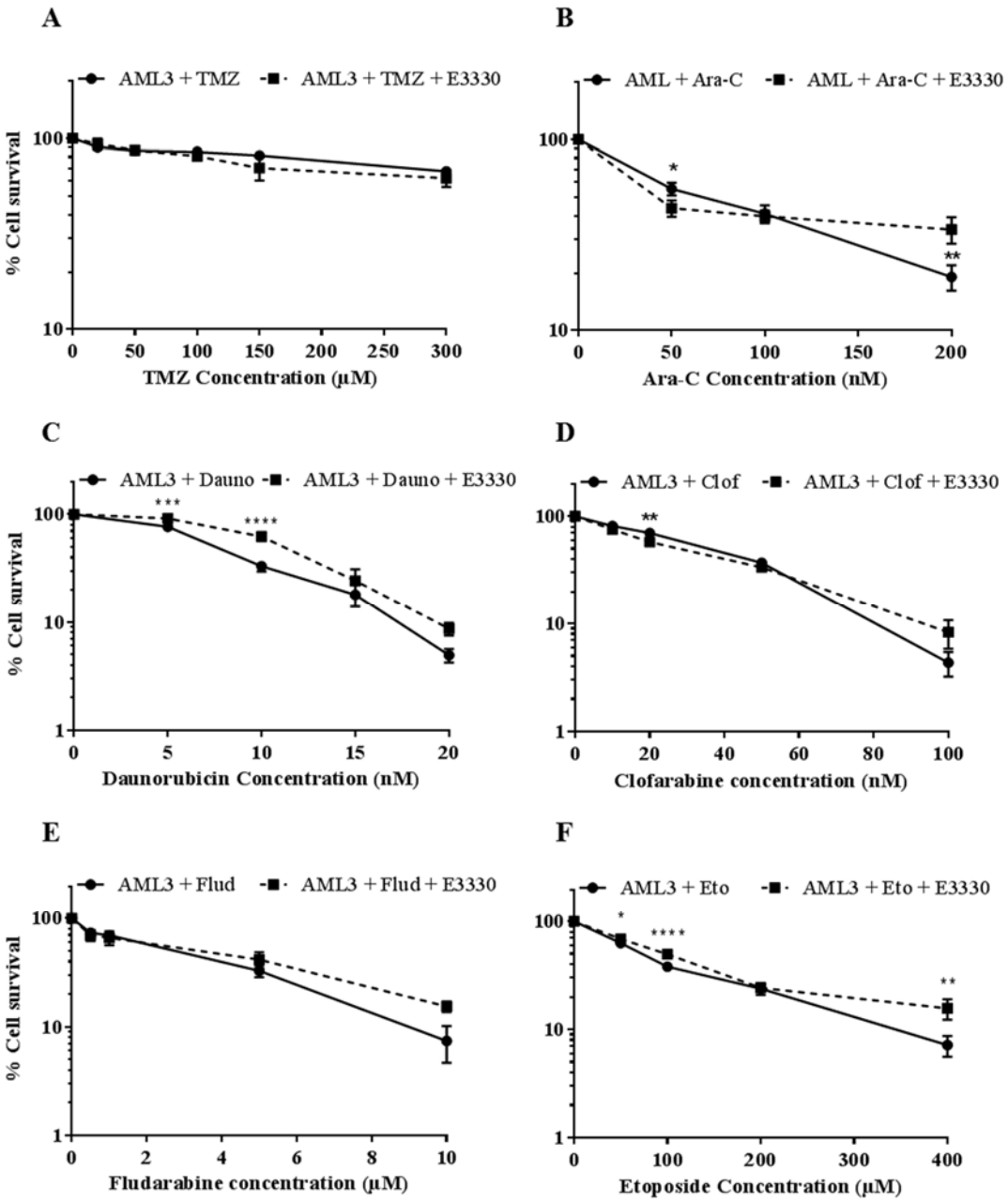


Figure 5.18: Growth inhibition of E3330 in combination with cytotoxic chemotherapy on AML3 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with E3330 (40 µM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to E3330 single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

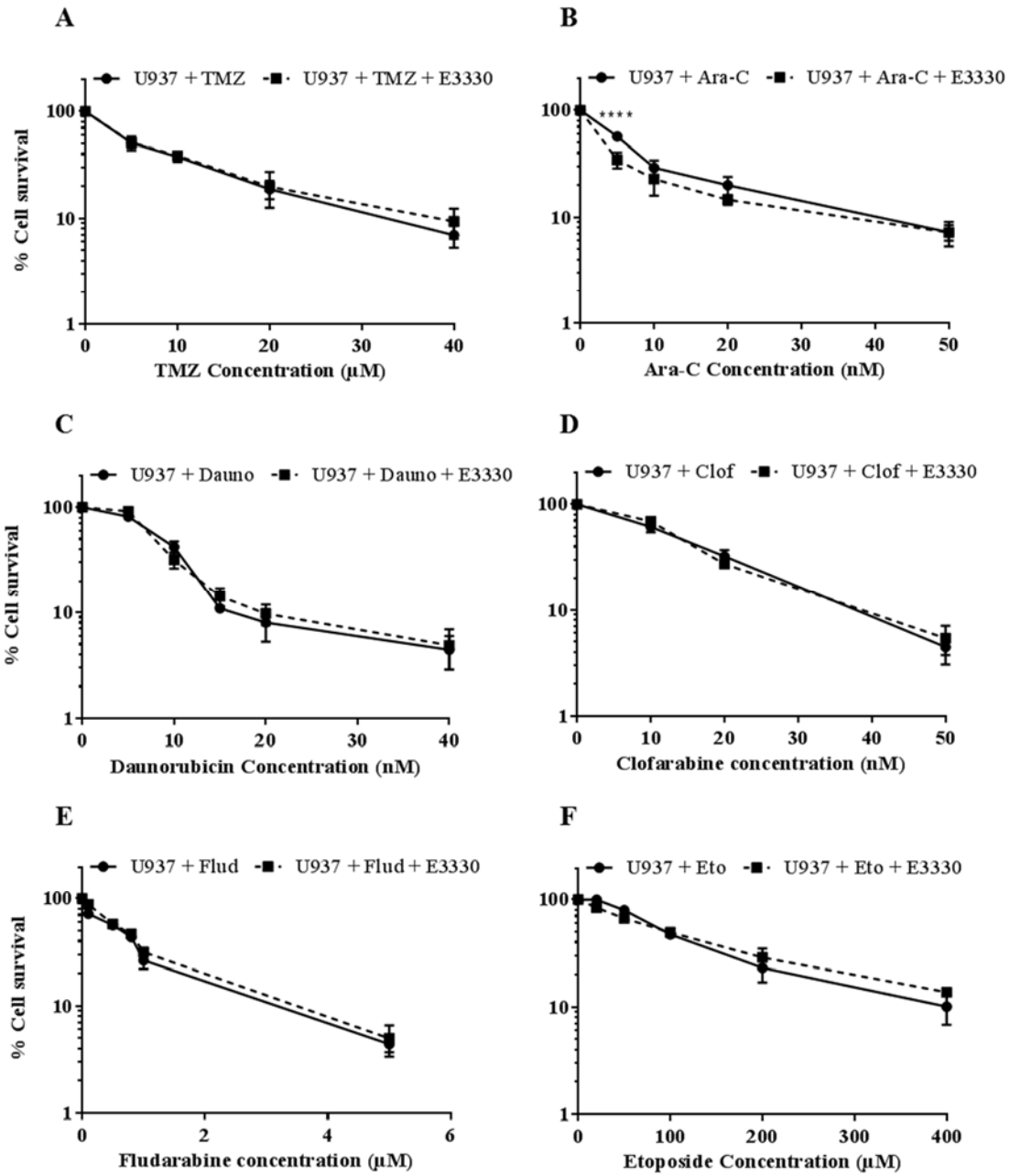


Figure 5.19: Growth inhibition of E3330 in combination with cytotoxic chemotherapy on U937 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with E3330 (40 µM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to E3330 single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

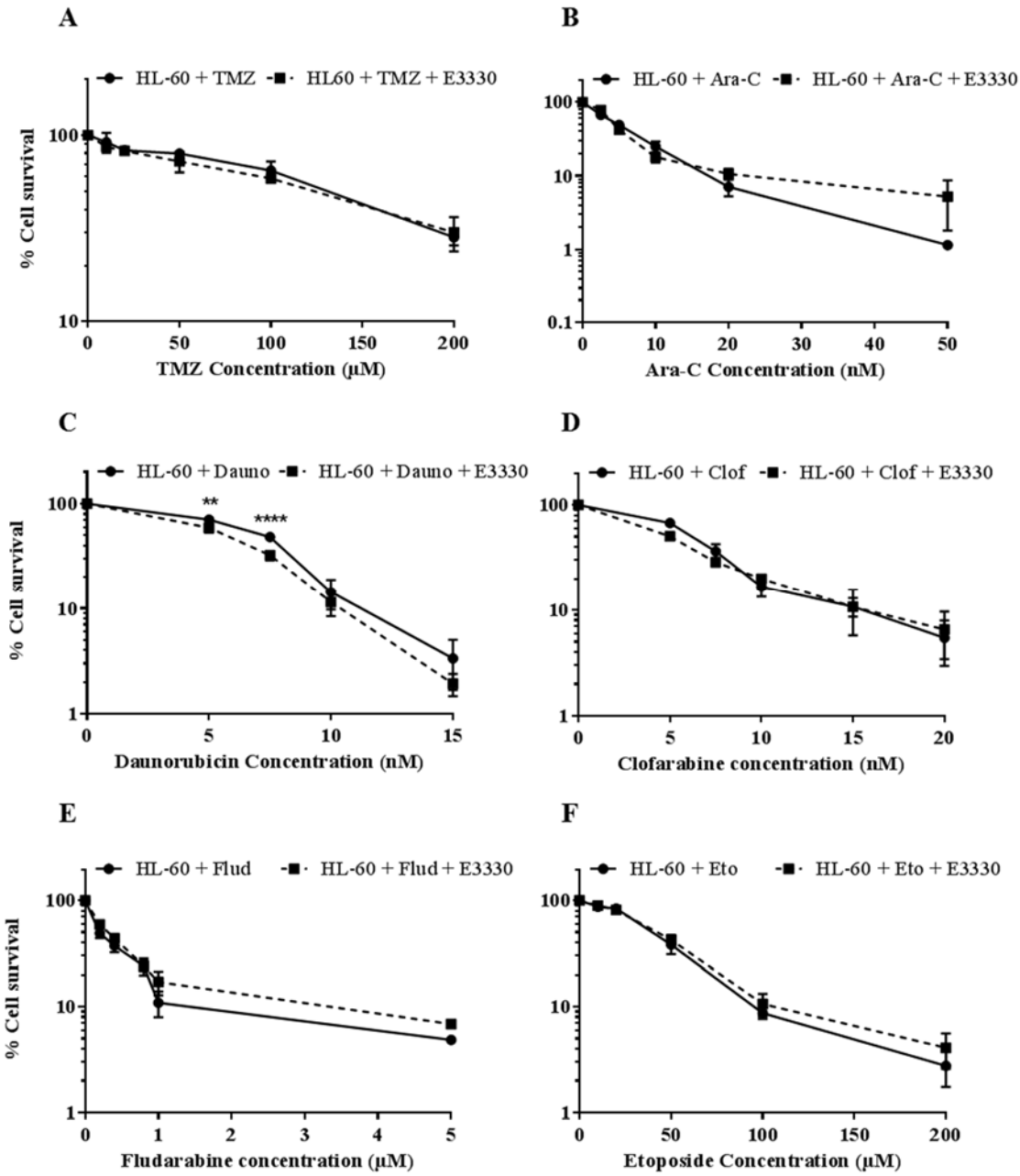


Figure 5.20: Growth inhibition of E3330 in combination with cytotoxic chemotherapy on HL-60 cells.

Growth inhibition assay was performed to measure the cytotoxicity of **(A)** temozolomide (TMZ) **(B)** Ara-C **(C)** Daunorubicin (Dauno) **(D)** Clofarabine (Clof) **(E)** Fludarabine (Flud) and **(F)** Etoposide (Eto) as single agents and in combination with E3330 (40 µM). Drug alone cytotoxicity represent the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. For combination treatment, data was normalised to E3330 single agent cytotoxicity instead of vehicle treated cells. In each case, results represent the mean and standard deviation of three independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

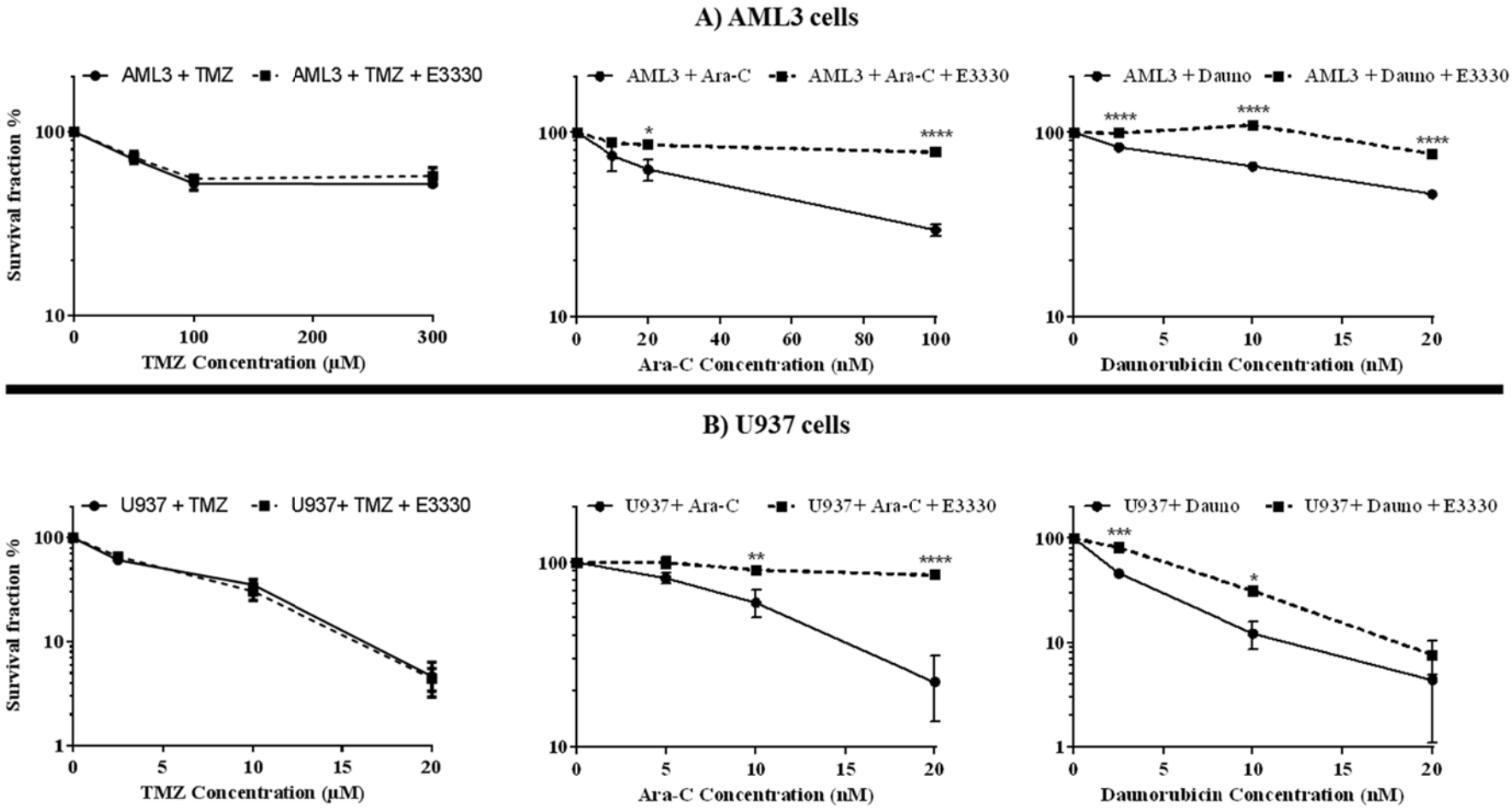


Figure 5.21: Cytotoxicity of APE1 redox inhibitor E3330 in combination with anti-leukaemia chemotherapy using colony formation assay (continued on next page).

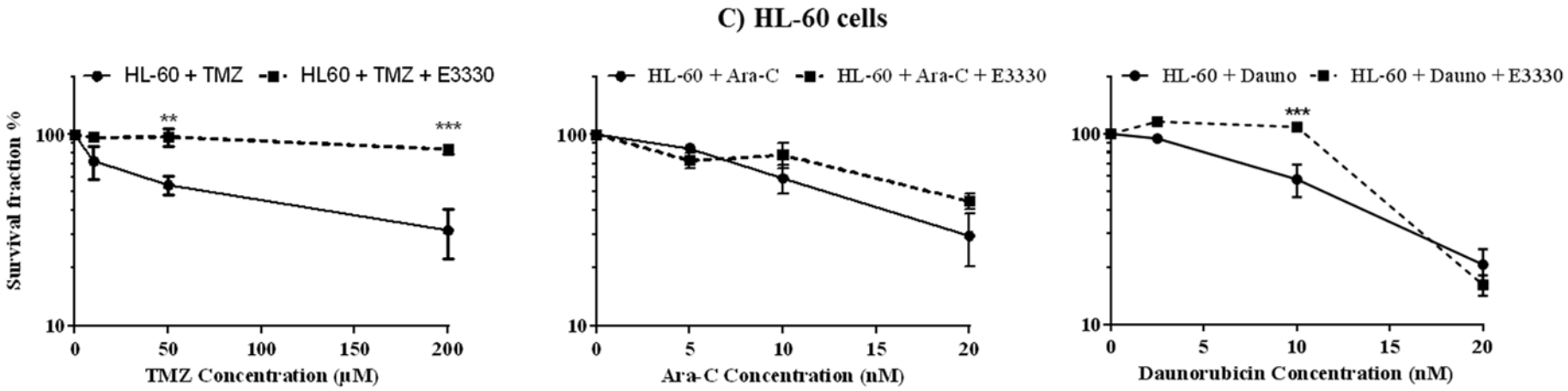


Figure 5.21: Cytotoxicity of APE1 redox inhibitor E3330 in combination with anti-leukaemia chemotherapy using colony formation assay (continued from previous page).

Colony formation assay was performed to investigate the cytotoxic effect of E3330 (600 nM) in combination with TMZ, Ara-C and daunorubicin on **(A)** AML3 **(B)** U937 and **(C)** HL-60. Data presented for chemotherapy without MX shows the number of viable colonies form each treatment as a percentage of the number of viable colonies from vehicle only treated cells. For combination treatment, data was normalised to single agent cytotoxicity effect of 40 µM of E3330 instead of vehicle treated cells. In each case, results represent the mean and standard deviation of two independent experiments. P values were calculated using two-way ANOVA. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

5.4. Discussion

The aim of this chapter was to investigate whether targeting APE1 using inhibitors of its endonuclease activity or its redox capacity would enhance the cytotoxic effects of anti-leukaemic chemotherapy. In order to explore the utility of targeting APE1 functions in AML, three APE1 inhibitors were investigated as single agents or in combination with cytotoxic chemotherapy in different AML cell lines. In addition, cell cycle profile and AP sites accumulation were determined following APE1 inhibition.

Modulation of BER using small molecule inhibitors has generated promising results in several cancer models. Several pre-clinical and clinical studies have shown that targeting APE1 or BER pathway elements as a therapeutic strategy may have clinical utility and may benefit particular subsets of AML patients (She *et al.*, 2005; Gaymes *et al.*, 2009; Horton *et al.*, 2009b; Bulgar *et al.*, 2010; D'Andrea, 2010; Fishel *et al.*, 2010; Vascotto *et al.*, 2013; Caimi *et al.*, 2014; Orta *et al.*, 2014; Esposito *et al.*, 2015; Wang *et al.*, 2015). However, previous studies have focused only on targeting PARP-1 or interruption of BER processing by blocking AP site access using methoxyamine (MX). In contrast, approaches targeting APE1 in AML using RNA silencing techniques and using specific small molecule inhibitors are still relatively undeveloped.

Non-specific inhibition of APE1 by interruption of BER using MX has been demonstrated to enhance fludarabine and manumycin cytotoxic chemotherapy in AML cells (She *et al.*, 2005; Bulgar *et al.*, 2010). In order to investigate MX cytotoxicity, AML3, U937 and HL-60 were treated with MX as a single agent and in combination with anti-leukaemic chemotherapy. MX showed weak single agent activity with a GI_{50} of approximately 1.5 mM. MX in combination with TMZ or fludarabine did not potentiate cytotoxicity, but was occasionally antagonistic, which is contrary to previous studies (Fishel *et al.*, 2007; Bulgar *et al.*, 2010; Caimi *et al.*, 2014). Similarly, MX did not potentiate the cytotoxic effects of Ara-C, daunorubicin, clofarabine or etoposide, but occasionally displayed antagonism. These contrasting data may explained by the molecular and genetic heterogeneity of AML as well as activation of distinct molecular pathways in AML that may change cellular response to APE1 inhibition or BER interruption. In addition, the cell cycle arrest at S phase caused by MX may possibly explain, at least partly, tolerance of AML cells to cytotoxic chemotherapy, which are particularly active against exponentially dividing cells. Furthermore, the different experimental methods used to assess cytotoxicity may contribute to these apparently contradictory results. Attempts to replicate the results presented by Bulgar and colleagues demonstrated no discernible effect of MX on AML cells at 3 mM and also no evidence of fludarabine potentiation.

APE1-III has not been extensively investigated as an anti-cancer agent, despite suggestions that it may be a promising and potent APE1 inhibitor (Vascotto *et al.*, 2013; Poletto *et al.*, 2015). APE1-III was cytotoxic to AML cells as a single agent, and particularly to AML3 cells, which harbour NPM1 mutation. Consistently, APE1-III displayed single agent cytotoxicity against Hela cells and mouse embryonic fibroblasts (MEFs) cells (Rai *et al.*, 2012; Vascotto *et al.*, 2013). Moreover, MEFs with $NPM1^{-/-}$ allele showed hypersensitivity to APE1-III compared to MEFs cells with $NPM1^{+/+}$. However, there was no potentiation of anti-leukaemic chemotherapy in combination with APE1-III in all cell lines investigated in this project. Rather, there was evidence that APE1-III antagonised anti-AML treatment in some cases. Although TMZ is anticipated to induce DNA damage which is recognised by BER, the induced DNA damage is not limited to lesions recognised by BER and which is repaired by other DNA repair pathways (see section 4.4.3 for more details about TMZ cytotoxicity) (Brandwein *et al.*, 2007; Horton *et al.*, 2009b; Zhang *et al.*, 2012). In addition, there are potentially several mechanisms that suggest redundant mechanisms that in principle could compensate for the inhibition of APE1 and explain the resistance/tolerance phenotype demonstrated in cells with APE1 inhibition. These include the AP lyase function of bifunctional glycosylases, which could execute APE1-independent BER. In addition, APE2 could bypasses APE1 function through its exonuclease and phosphodiesterase functions (see section 4.4.3 for more details). Furthermore, APE1 has low affinity to DNA damage induced by Ara-C and etoposide, which may explain the resistant phenotype with APE1 inhibition in AML cells (see section 4.4.3 for more details) (Lam *et al.*, 2006; McNeill and Wilson, 2007; McNeill *et al.*, 2009).

Although the role of APE1 redox function is not yet fully understood in leukaemia, it was hypothesised that specific targeting of this function using E3330 would enhance the cytotoxicity of anti-leukaemic therapy through interruption of survival mechanisms and maintenance of oxidative stress in AML cells. The redox inhibitor E3330 displayed single agent cytotoxicity in AML3, U937 and HL-60 cells, and reduced cells growth. Of note, redox inhibition of APE1 using E3330 mainly had no effect on the cytotoxic effects of anti-leukaemia chemotherapy, but there was some evidence of antagonism rather than potentiation of the chemotherapy as illustrated by colony formation assay in figure 5.21. However, E3330 drug activity was affected by FBS concentration in cell culture media and possibly also affected by oxygen level tension (Rohrabaugh *et al.*, 2011), which may limit its use. A previous study demonstrated that E3330 enhances retinoic acid (RA) induced differentiation and apoptosis in HL-60 AML cells (Fishel *et al.*, 2010). E3330 is thought to potentiate RA

induced differentiation via enhancement of BLR1 expression (a target gene of RA) and inhibition of NF-κB anti-apoptotic pathway activation (Fishel *et al.*, 2010). However, this study did not characterise the impact of E3330 on the cytotoxicity of commonly used anti-leukaemia therapies, specifically Ara-C, daunorubicin, clofarabine and etoposide. Nevertheless, one possible explanation for the resistance phenotype observed in response to E3330 in combination with chemotherapy may be due to, at least in part, to activation of defence mechanisms in order to regulate drug induced oxidative stress and prevent further DNA damage. A recent study showed that inhibition of APE1 redox function induces NRF2 expression at both the protein and transcript level in a dose dependant manner (Fishel *et al.*, 2015). NRF2 is thought to regulate cellular defence in response to oxidative stress (Fishel *et al.*, 2015). Furthermore, siRNA mediated knockdown of APE1 consistently upregulated NRF2 transcriptional activity (Fishel *et al.*, 2015). However, there was no evidence of upregulation of NRF2 following APE1 shRNA silencing in RNA sequencing data discussed in chapter 4. This may imply that upregulation of NRF2 was tissue specific. It is possible that other factors may be involved in the resistance phenotype reported in this study, such as CDKN1A, PAX5, EGR1, FOXO1 or CUX1.

It should be noted that two other APE1 inhibitors were assessed in terms of their cytotoxicity as single agents, but they were excluded from further investigation due to poor solubility in aqueous solution. These inhibitors include CRT0044876 and N-(4-fluorophenyl)-2-(2-phenyl-4-phenylsulfonyl-oxazol-5-yl)sulfanyl-acetamide.

The impact of APE1 inhibition on cell cycle is inconclusive and dependent on the inhibitor used and the targeted tissue. However, previous data discussed in chapter 4 revealed no alteration in cell cycle kinetics of AML cells following APE1 silencing using shRNA. Consistent with this observation, specific inhibition of APE1 endonuclease function by APE1-III or redox function inhibition using E3330 revealed no alteration in cell cycle profile over 72 hours. Previous reports of E3330 on cell cycle profile are inconclusive, with studies of pancreatic and ovarian cancer cells reporting impaired G1/S transition and induced G2 cell cycle arrest in response to E3330 (Jiang *et al.*, 2010; Fishel *et al.*, 2011). In contrast, another study revealed no effect on cell cycle kinetics of pancreatic cancer cell lines PANC1 by E3330 (Zou and Maitra, 2008).

However, monitoring AML cells for 72 hours after MX treatment showed significant cell cycle blockade at S phase. This result may explain the cytostatic effect of single agent MX on AML cells when examined using the colony formation assay, with cells arrested in S phase unable to form colonies (Figure 5.1B). As MX irreversibly blocks AP site and prevents

resolution of BER, cell cycle arrest in S phase may have occurred to prevent replication of possible pro-mutagenic lesions caused by MX incorporation into cellular DNA. However, a previous study has shown that MX treatment alone does not alter cell cycle kinetics of human colorectal cell lines (Yan *et al.*, 2006). MX combination with iododeoxyuridine, slowed cell cycle progression from G1 phase and sensitised cells to radiotherapy (Yan *et al.*, 2006).

BER is initiated following detection of DNA damage and removal of damaged bases by DNA glycosylases. The resulting AP sites are the main substrate for APE1 endonuclease function. Therefore, quantification of AP sites following APE1 inhibition would provide further evidence for inhibition of APE1 endonuclease function. As previously discussed, MX binds to AP sites and interrupts BER, and likely interferes with AP site detection. Preliminary data presented in this study suggests reduced AP site frequency in MX treated cells compared to vehicle control treated cells. This result is in agreement with the fact that MX blocks AP site consistent with reported studies (Liu and Gerson, 2004; Wang *et al.*, 2009b; Bulgar *et al.*, 2012). Determination of AP site frequency following inhibition of APE1 endonuclease function using APE1-III was unsuccessful, which was possibly was due to a technical error during sample preparation and tagging AP sites with the ARP reagent. However, it is expected that APE1 inhibition with APE1-III would lead to increased AP sites accumulation. In contrast, the E3330 redox inhibitor is not expected to impair APE1 endonuclease function, or have an effect on AP site accumulation. Consistent with this hypothesis, there was no alteration in AP site frequency in E3330 exposed cells compared to vehicle control treated cells. It should be noted, however, that the AP sites quantification assays undertaken in this project were limited in scope and scale. As such, additional experiments are warranted to further test whether these mechanisms may be operating.

5.5. Summary of this chapter

APE1 inhibition might not be a suitable generic approach for the treatment of AML, in particularly in combination with anti-leukaemia treatment, given the heterogeneity of genetics and molecular background of this disease. In addition, targeting APE1 may activate signalling pathways that reduce AML cells proliferation in order to prevent further DNA damage. Taking this advantage can be clinically useful by controlling AML blast proliferation and then controlling the disease progression. Furthermore, lack of appropriate and potent APE1 inhibitors may slow the progress of this filed of research.

In summary, this chapter has demonstrated the following:

- APE1 inhibitors are cytotoxic to AML cells as single agents, but in combination with anti-leukaemic cytotoxic chemotherapy either have no effect or are antagonistic.
- MX induced cell cycle blockade at S phase in AML3, U937 and HL-60 cells
- There was no evidence of alteration of cell cycle distribution following APE1-III and E3330 treatment.
- MX prevents resolution of BER via binding to AP sites.
- The E3330 APE1 redox inhibitor has no discernible effect on AP sites induction or resolution following exposure to hydrogen peroxide.

Chapter 6: Targeting 8-oxoguanin DNA glycosylase (OGG1) in AML cells using shRNA interference.

6.1. Introduction

AML is characterised by excessive production of reactive oxygen species (ROS) resulting in high oxidative stress and which can contribute to disease progression and relapse (Zhou *et al.*, 2010; Hole *et al.*, 2011; Udensi and Tchounwou, 2014). Evidence indicates that high oxidative stress induces various forms of DNA damage, which can cause mutations if unrepaired (Sallmyr *et al.*, 2008b; Hole *et al.*, 2011). Therefore, DNA repair systems are important defence mechanisms against genotoxic DNA damage caused by oxidative stress. In particular, base excision repair (BER) is a critical system that mostly repairs oxidised DNA bases generated under high oxidative stress (Figure 1.4). However, one of the most abundant and deleterious oxidative DNA lesions is 8-oxoguanine (8-oxoG), which results from oxidation of guanine, and which is primarily repaired by BER. Specifically, two DNA glycosylases in BER are responsible for excising this base, including 8-oxoguanine DNA glycosylase (OGG1) and MutY DNA Glycosylase (MUTYH) (Cooke *et al.*, 2003; Scott *et al.*, 2014).

OGG1 has been demonstrated previously as a potential prognostic marker in AML (Liddiard *et al.*, 2010). Specifically, *OGG1* expression, determined using Affymetrix expression microarray analysis, correlated with overall survival in AML (Liddiard *et al.*, 2010)(discussed in detail in sections 1.2.2). Patients with high *OGG1* expression were more likely to have adverse cytogenetics risks and significantly reduced overall survival (Liddiard *et al.*, 2010). Conversely, patients with low *OGG1* expression were more likely to have favourable cytogenetics, including t(8;21) (Liddiard *et al.*, 2010). Based on these data it is possible that high OGG1 expression may confer enhanced BER activity and inhibition of OGG1 activity may enhance chemotherapy efficacy in particular subset of AML patients.

Several *OGG1* polymorphic variants have been reported (Hyun *et al.*, 2000; Hill and Evans, 2007; Saitoh *et al.*, 2013), some of which have been linked to reduced OGG1 activity as well as better response to DNA damaging agents (Hyun *et al.*, 2000; Hill and Evans, 2007) (Further details are discussed in section 1.2.2). Therefore, the prevailing evidence implies that OGG1 could be a legitimate therapeutic target in AML. However, OGG1 has yet to be validated as a viable therapeutic target in cancer, possibly due to the current lack of specific inhibitors against OGG1.

6.2. Aims of this chapter

The aim of this chapter was to investigate whether OGG1 knockdown sensitises AML cells to conventional genotoxic anti-leukaemic chemotherapy. In order to investigate this hypothesis, the following specific objectives were defined:

- To silence OGG1 using shRNA and to generate of AML cells with stable OGG1 knockdown.
- To investigate the impact of OGG1 silencing on AML cell proliferation and cloning efficiency.
- To evaluate the impact of OGG1 knockdown on cell cycle kinetics.
- To determine the sensitivity of OGG1 knockdown cells to anti-leukemic DNA damaging agents.

6.3. Results

Part of the results described in the sections below were performed by undergraduate student Ahpa Sae Yeoh, who performed this as part of her undergraduate project under supervision of Professor James Allan. Specifically, Ahpa studied the effect of OGG1 knockdown on proliferation and cloning efficiency of U937 cells. In addition, she investigated the effect of OGG knockdown in U937 cells on cell cycle profile and sensitivity to the anti-proliferative effects of DNA damaging agents.

6.3.1. Generation of AML cell lines with stable OGG1 knockdown using small hairpin RNA (shRNA)

Five shRNA lentiviral constructs (table 2.4) targeting different regions of the OGG1 transcript were used to knockdown OGG1 in AML cell lines including AML3, U937, HL-60 and kasumi-1. Only construct TRCN0000314740 (G5) generated measurable OGG1 knockdown at the protein level, which was observed in U937 and HL-60 cells only (data not shown), and there was no evidence of OGG1 knockdown in AML3 and kasumi-1 cells (data not shown). G5 targets the sequence CGGCTCATCCAGCTTGATGAT of the OGG1 transcript. This region is common between the majority of OGG1 splice transcript variants, specifically isoforms 1a and 2a, which encode nuclear and mitochondrial OGG1 proteins, respectively.

Transduced HL-60 and U937 cell populations are expected to be heterogeneous with respect to the extent of OGG1 knockdown. This is partly due to heterogeneity in terms shRNA integrations per single cell, in addition to differences in shRNA promoter activity due to in the site of integration in the host cell genome. In order to obtain cells with stable OGG1 knockdown, U937 and HL-60 transduced populations were cloned in semi-solid soft agar. Western blotting demonstrated considerable variation in OGG1 protein knockdown in different cell clones with some clones showing minor reductions in OGG1 expression with others showing almost complete loss of OGG1 expression (Figure 6.1).

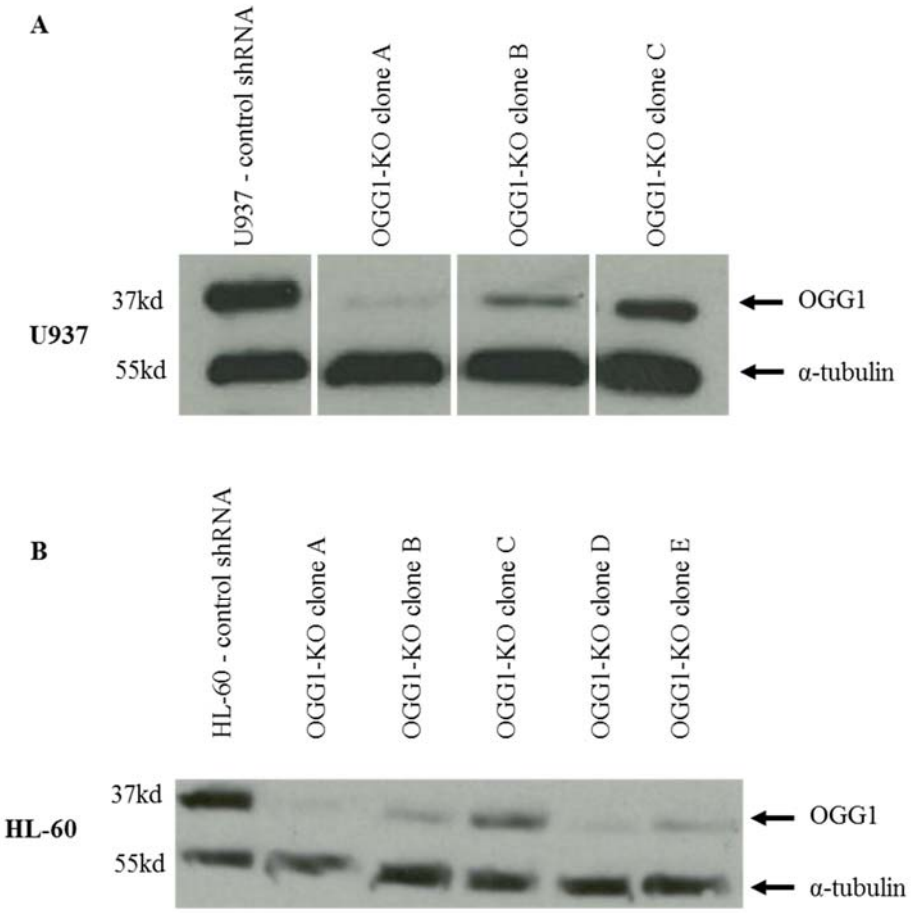


Figure 6.1: OGG1 shRNA induced knockdown in U937 and HL-60 clones.

(A) U937 and **(B)** HL-60 cells were transduced with shRNA to knockdown OGG1. Several independent clones showed variable OGG1 knockdown on protein levels. Alpha tubulin was used as loading control.

6.3.2. Effects of OGG1 knockdown on AML cell proliferation and cloning efficiency

6.3.2.1.Assessment of U937 and HL-60 cell proliferation

In order to assess the impact of OGG1 knockdown on AML cells, OGG1 proficient and deficient U937 and HL-60 cell clones were seeded at a density of $2x10^4$ cells/ml and cell density was measured using trypan blue exclusion and cell counter every 24 hours for 5 days.

U937 and HL-60 cell clones with OGG1 knockdown showed no significant alteration in their proliferation kinetics compared to control shRNA-transduced cells with the exception of HL-60 clones A, C and E (Figure 6.2). Interestingly, HL-60 clone C, which has relatively modest OGG1 knockdown (Figure 6.1), had a slow proliferation rate compared to control and other OGG1 deficient clones. This is possibly due to integration of the shRNA into a critical region in the cells genome which affected proliferation.

6.3.2.2.Assessment of cloning efficiency

Colony formation assay was performed in order to determine the effect of OGG1 knockdown on the ability of U937 and HL-60 cell clones to grow in semi-solid soft agar. U937 clones with OGG1 knockdown displayed a similar cloning efficiency to control cell clones (Figure 6.3 A). In contrast, OGG1 knockdown in HL-60 cells was associated with a modest but significant reduction in cloning efficiency compared to control cells (Figure 6.3B). HL-60 clone C, which had the lowest proliferation rate, also had the lowest cloning efficiency. Likewise, HL60 clone E demonstrated low proliferation rate and low cloning efficiency.

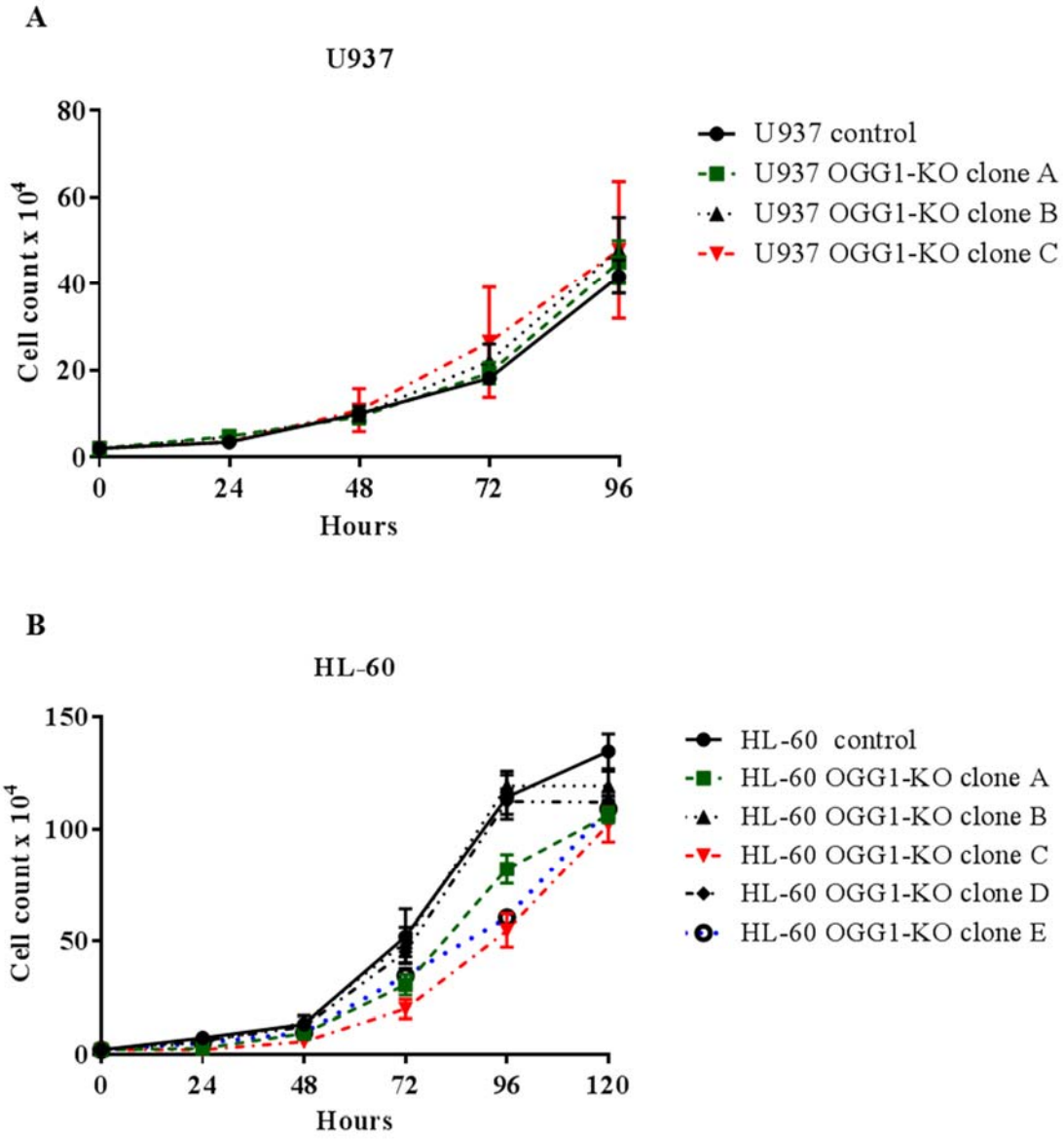


Figure 6.2: Effect of OGG1 knockdown on AML cells proliferation.

Proliferation of **(A)** U937 and **(B)** HL-60 cells after OGG1 knockdown was assessed by monitoring cell density by trypan blue and cell counting every 24 hours for 5 days. Data presented are the mean and standard deviation of 3 experiments.

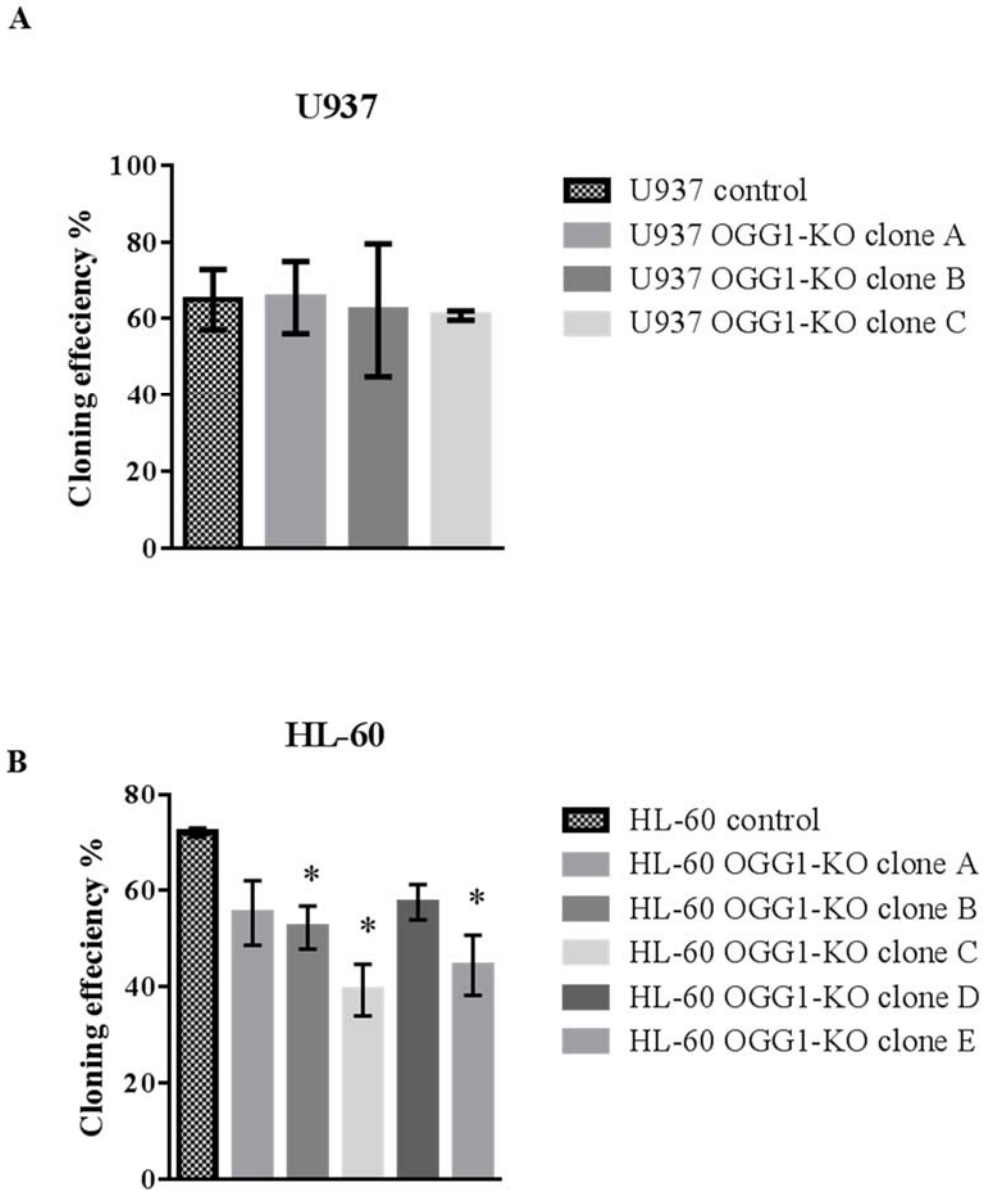


Figure 6.3: Effect of OGG1 knockdown on cloning efficiency of AML cells.

U937 **(A)** and HL-60 **(B)** clones with OGG1 knockdown alongside with their respective controls were grown on semi-solid soft agar to assess the impact of OGG1 knockdown on cloning efficiency. P values were calculated using unpaired t test (*p <0.05)

6.3.3. Effect of OGG1 knockdown on AML cell cycle

Propidium iodide (PI) staining and flow cytometry was performed in order to determine the effect of OGG1 knockdown on cell cycle profile of unsynchronised U937 and HL-60 cells. Flow cytometry demonstrated no alteration in cell cycle distribution in OGG1 knockdown cell clones compared to control cells (Figure 6.4). There was no indication of apoptosis following OGG1 knockdown as the proportion of cells in subG1 was not changed in OGG1 deficient cells compared to their relevant controls.

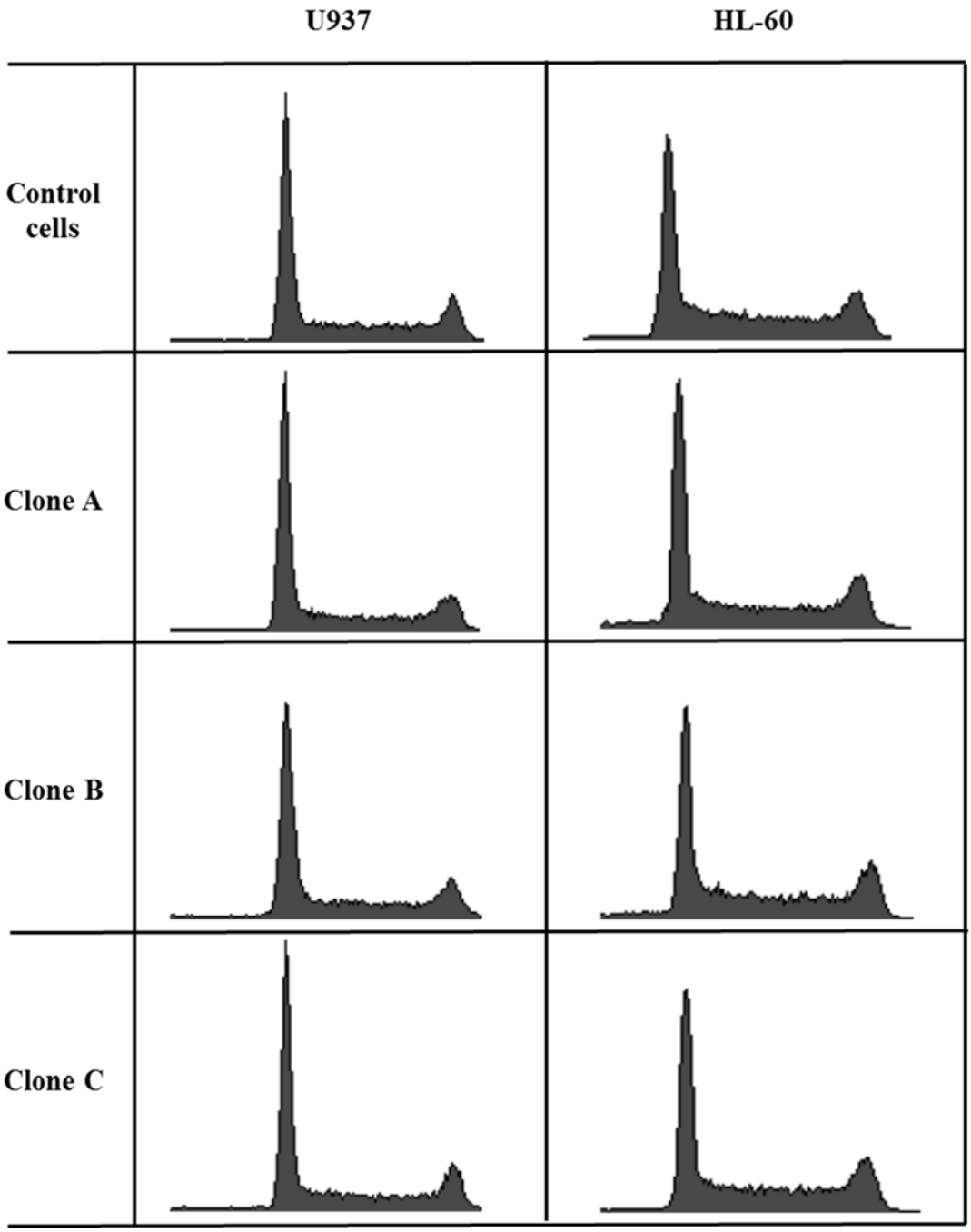


Figure 6.4: Effect of OGG1 knockdown on cell cycle kinetics of AML cells.

Cell cycle analysis measured by flow cytometer for to determine the impact of OGG1 knockdown in U937 and HL-60 cells.

6.3.4. Cytotoxicity of OGG1 deficient cells following treatment with cytotoxic DNA damaging agents

OGG1 knockdown cells and their relevant controls were treated with different DNA damaging agents in order to determine cytotoxicity in response to anti-leukaemic agents. Cells were treated with DNA damaging agents, Ara-C, daunorubicin, clofarabine, fludarabine and etoposide, and cell numbers were determined after 4 days using trypan blue exclusion dye and cell counting. Results are presented as the number of viable cells from each treatment as a percentage of the number of viable cells from vehicle only treated cell populations.

OGG1 knockdown in U937 cell clones did not affect their sensitivity in response to anti-leukaemic agents (Figure 6.5). Similarly, OGG1 deficient and proficient HL-60 showed no significant differential response to anti-leukaemia treatment (Figure 6.6), with the exception that HL-60 clone C, which was moderately tolerant to the anti-proliferative effects of genotoxic anti-leukaemia treatment compared to controls. This may be due to the low proliferation rate of this clone, and concomitant reduction in sensitivity to genotoxic chemotherapy.

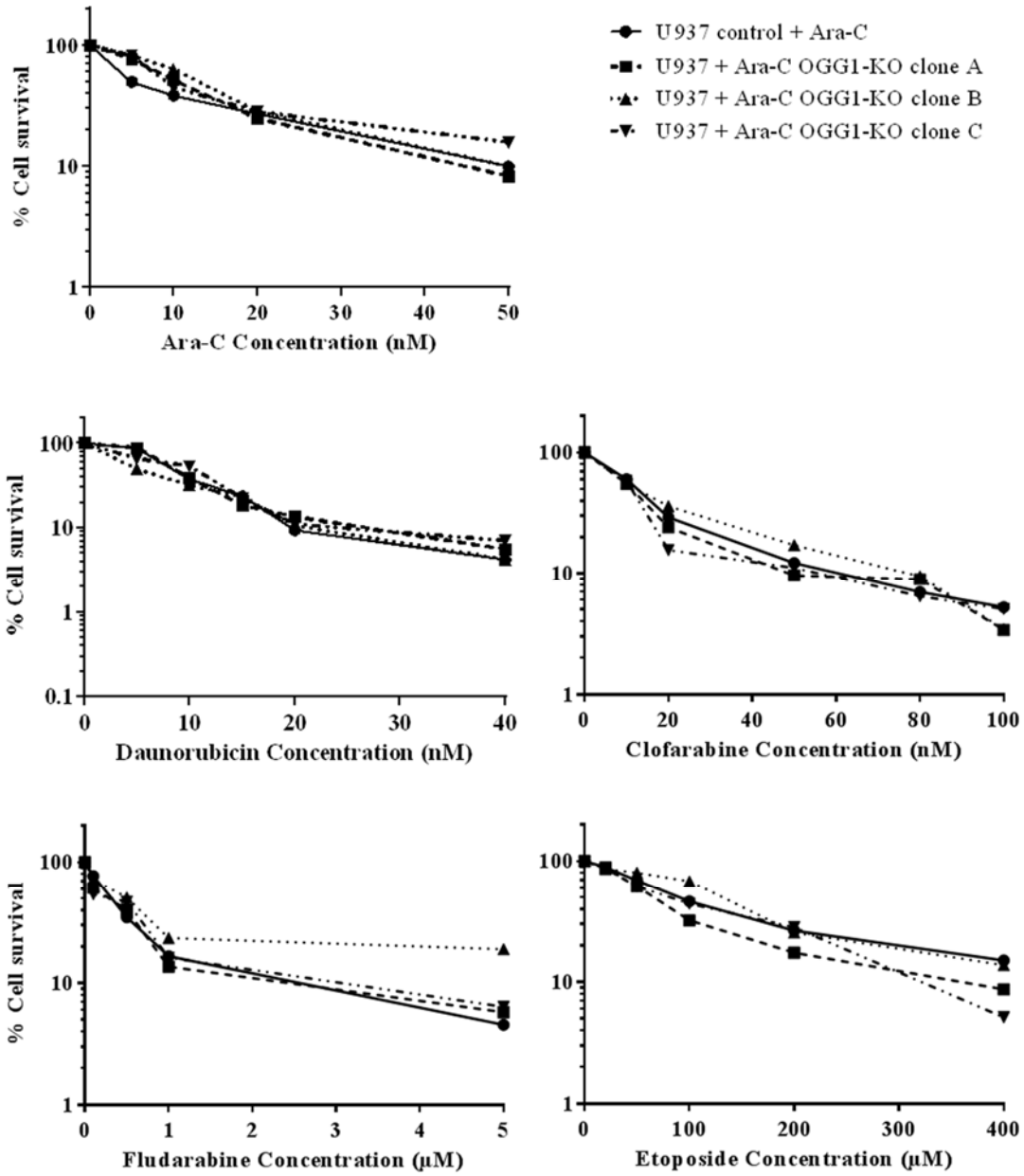


Figure 6.5: Growth inhibition in response to DNA damaging chemotherapy in U937 cells with OGG1 knockdown.

Cytotoxicity assay was performed using growth inhibition assay. Several independent U937 clones with variable OGG1 knockdown and their relevant control were treated for 4 days with Ara-C, daunorubicin, clofarabine, fludarabine and etoposide. Data presented shows the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. In each case, data represent one experiment.

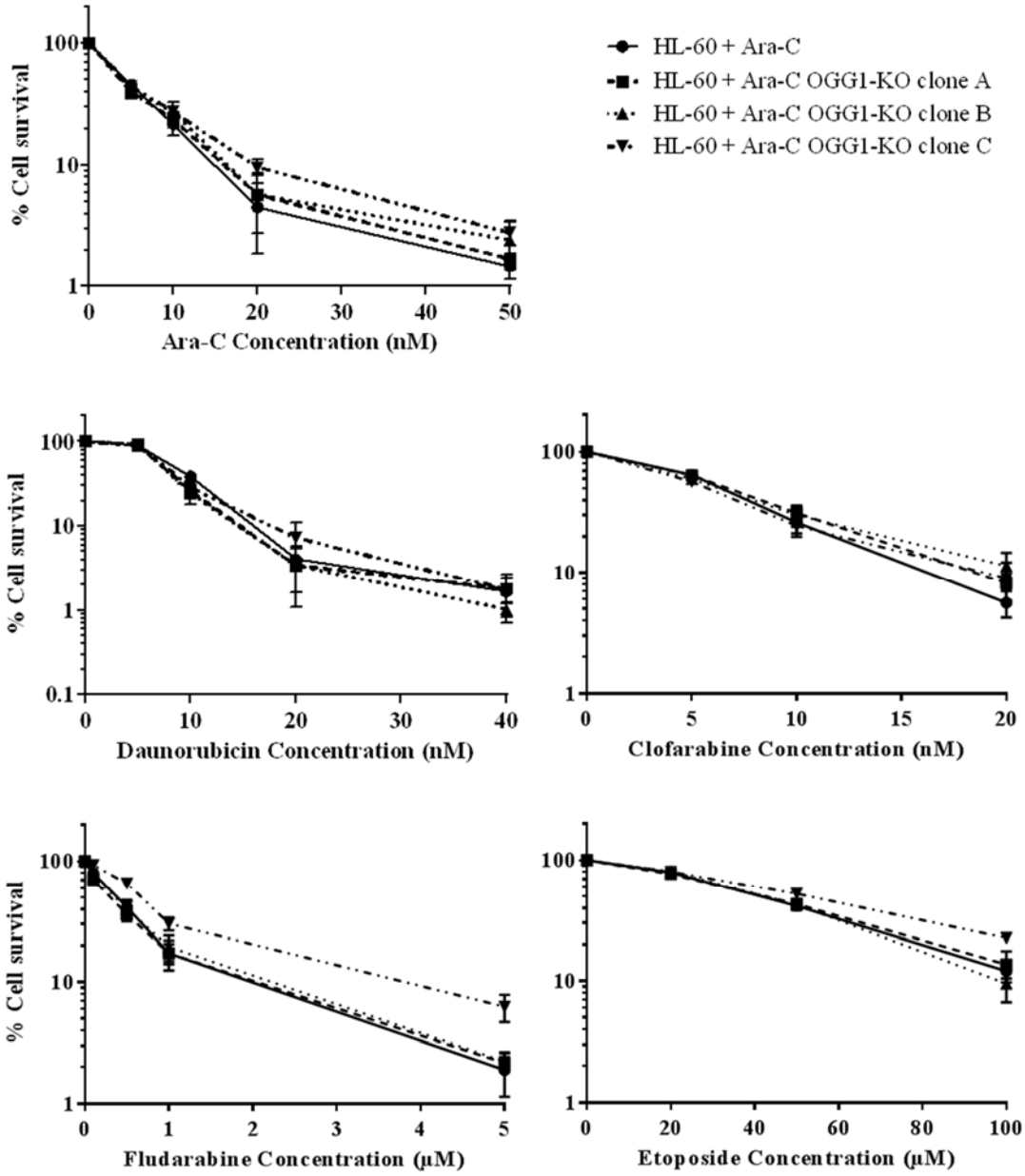


Figure 6.6: Growth inhibition in response to DNA damaging chemotherapy in HL-60 cells with OGG1 knockdown.

Cytotoxicity assay was performed using growth inhibition assay. Several independent HL-60 clones with variable OGG1 knockdown and their relevant control were treated for 4 days with Ara-C, daunorubicin, clofarabine, fludarabine and etoposide. Data presented shows the number of viable cells form each treatment as a percentage of the number of viable cells from vehicle only treated cells. In each case, results represent the mean and standard deviation of three independent experiments.

6.4. Discussion

BER is a highly conserved pathway involved in repairing DNA lesions resulting from oxidation, alkylation and deamination. The OGG1 DNA glycosylase, which is involved in the early steps of BER, removes 8-oxoguanine oxidised DNA bases. High OGG1 expression has been associated with worse prognosis and low overall survival in AML (Liddiard et al., 2010). Thus, OGG1 might be an attractive target in AML and inhibition of its activity could potentially enhance the efficacy of AML chemotherapy.

Targeting OGG1 in AML using shRNA was relatively difficult. AML3, U937, HL-60, Kasumi1 cell lines were transduced with 5 different OGG1 lentiviral constructs, but only one construct generated measurable OGG1 knockdown in U937 and HL-60, as shown in Figure 6.1. One possible explanation for this observation is that AML3 and Kasumi1 cells have relatively lower mRNA *OGG1* transcripts as illustrated in figure 3.4 in chapter 3. OGG1 has several alternative splice variant transcripts which may contribute to difficulty in its silencing. Two predominant mRNA splice variants are exist in human cells including *OGG1* 1a and 2a (Nishioka *et al.*, 1999). These variants encode OGG1 1a protein, which localised to the nucleus and OGG1 2a protein which localised to the mitochondria (Nishioka *et al.*, 1999). However, the main shRNA construct (G5) used in this project targets a common sequence on *OGG1* mRNA transcript variants including 1a and 2a.

OGG1 is crucial for removing the pro-mutagenic oxidised DNA lesion 8-oxoguanine and silencing OGG1 could possibly activate cell signalling pathways to prevent the fixation of damage to mutation and to initiate repair with accumulating 8-oxoguanine lesions. However, shRNA induced silencing of OGG1 had no effect on AML cell growth kinetics and cloning efficiency compared to control cells. Furthermore, OGG1 deficient and proficient cells exhibited similar cell cycle kinetics regardless of OGG1 level. These results imply that OGG1 is not involved in the regulation of cell cycle progression or proliferation.

It was hypothesised that knocking down OGG1 in AML cells would increase 8-oxoguanine DNA lesion levels and sensitise to conventional anti-leukaemic chemotherapy. However, knocking down OGG1 in U937 and HL-60 cells appears to have no differential effect on response to cytotoxic DNA damaging agents. Although 8-oxoG lesions were not measured following OGG1 knockdown, this result suggest presence of alternative mechanism by which cells were able to minimise the level of 8-oxoG deleterious lesion, such as MUTYH (Cooke *et al.*, 2003; Russo *et al.*, 2004; Russo *et al.*, 2007; Scott *et al.*, 2014). *Ogg1* Knockout mice showed considerable increase in 8-oxoG lesions (Klungland *et al.*, 1999; Minowa *et al.*,

2000). *Ogg1* knockout mice also showed tendency to develop lung adenoma/carcinoma spontaneously, but however, *Ogg1* and *Muthy* double knockout mice showed no development of tumours, regardless of 8-oxoG accumulation (Sakumi *et al.*, 2003). Therefore, it might be useful to disable/inhibit the alternative 8-oxoG removal mechanisms in order to efficiently enhance accumulation of 8-oxoG and to determine therapeutic utility and biological effect of OGG1 depletion/inhibition on cancer cells. Consistent with this suggestion, mouse embryonic fibroblasts with *Ogg1*$^{-/-}$ or *Mutyh*$^{-/-}$ alone were not sensitive to hydrogen peroxide and tert-Butyl hydroperoxide induced cytotoxicity (Xie *et al.*, 2008). However, double defective *Ogg1* and *Mutyh* mouse fibroblast cells were hypersensitive to the cytotoxic effects of hydrogen peroxide and tert-Butyl hydroperoxide (Xie *et al.*, 2008).

Tolerance/resistance of OGG1 deficient AML cells to cytotoxicity of anti-leukaemia therapy can also be explained by involvement of OGG1 in biological functions that might be required to facilitate the cytotoxicity of DNA damaging agents. In agreement with this notion, a recent study demonstrated that OGG1 is essential in oxidative stress induced DNA demethylation (Zhou *et al.*, 2016). Knocking down *Ogg1* in mouse embryonic fibroblasts cells conferred resistance to oxidative stress-induced DNA demethylation, but however, restoring *Ogg1* expression enhanced DNA demethylation induced by H_2O_2 (Zhou *et al.*, 2016).

6.5. Summary of this chapter

Although OGG1 is a prognostic marker in AML, the evidence presented in this thesis suggests that loss of OGG1 function does not sensitise cells to anti-leukaemic chemotherapy. OGG1 knockdown in AML cells did not impair cell proliferation, cell cloning efficiency or affect cell cycle kinetics. More importantly, OGG1 knockdown in U937 and HL-60 cells did not enhance the cytotoxic effects of several DNA damaging agents.

Chapter 7: Concluding discussion

7.1. General discussion

AML is a challenging disease to effectively treat with high mortality rates, particularly in elderly patients. The treatment of AML has not changed significantly for several decades and remains primarily dependent on Ara-C and anthracycline containing intensive chemotherapy, which is not generally well tolerated and potentially life threatening for elderly patients. Moreover, the majority of AML patients suffer from disease relapse and develop chemotherapy resistant disease. Nevertheless, combination chemotherapy treatment regimens allow specific targeting of dysregulated molecular pathways in AML which can facilitate the eradication of AML cells. Combination chemotherapy incorporating Ara-C, daunorubicin and other established anti-leukaemia treatment, including FLT3 inhibitors, kinase inhibitors and histone deacetylase inhibitors for example, has led to improvements in disease outcome for some patient groups, but have limited efficacy in other AML subtypes (Burnett *et al.*, 2011a; Swords *et al.*, 2012; Montalban-Bravo and Garcia-Manero, 2014). Therefore it is crucial to explore alternative well tolerated drugs with low cytotoxicity to normal cells as well as minimal side effects.

AML is characterised by elevated levels of reactive oxygen species and oxidative stress, which tightly regulate and support survival of AML cells through a complex network of signalling pathways (Rassool *et al.*, 2007; Dohner and Dohner, 2008; Sallmyr *et al.*, 2008b; Hole *et al.*, 2011; Zhou *et al.*, 2013). AML is a challenging disease with heterogeneous somatic genetic aberrations, which can include the formation of oncogenic fusion genes that contribute to the upregulation/activation of pro-survival signalling pathways. These factors collectively participate in disease progression, relapse and treatment resistance. Of note, some of these oncogenic alterations reportedly induce high oxidative stress and impaired DNA repair mechanisms, which can result in accumulation of DNA damage (Alcalay *et al.*, 2003; Takacova *et al.*, 2012; Yeung *et al.*, 2012; Esposito and So, 2014). Conversely, upregulation of DNA damage response pathways can confer AML cells with protective mechanism to circumvent the cytotoxic effects of DNA damaging agents, and ultimately contribute to treatment resistance (Bagrintseva *et al.*, 2005; Seedhouse *et al.*, 2006; Cavelier *et al.*, 2009). Although DNA repair is compromised in a large subset of AML patients due to acquired genetic alterations and resultant fusion proteins, the opportunities for taking advantage of compromised DNA repair as a therapeutic strategy is being extensively investigating, primarily via synthetic lethal approaches. For example, PARP inhibition is synthetically lethal to AML cells expressing the PML-RARα oncogenic fusion protein, which is thought to impair the DNA damage response (Esposito *et al.*, 2015). Furthermore, targeting DNA

mismatch repair deficient AML cells with PARP inhibitor and temozolomide was successfully identified as a synthetic lethal approach (Gaymes *et al.*, 2009; Horton *et al.*, 2009b; Gaymes *et al.*, 2013). Therefore, targeting DNA repair mechanisms and associated regulatory pathways are, in fact, a promising treatment approach in AML.

Targeting BER pathway proteins, specifically APE1, in cancer has been successfully demonstrated in several cancer models and appears to be a promising target in other cancer settings (Fishel and Kelley, 2007; Abbotts and Madhusudan, 2010; Dorjsuren *et al.*, 2012; Abbotts *et al.*, 2014). Since APE1 has distinct roles in DNA repair, the regulation of several transcription factors (redox function), RNA metabolism and other biological functions, targeting these functions could prove efficacious and could enhance the cytotoxicity of established anti-leukaemia therapy.

OGG1 is another component of the BER pathway that may represent a viable therapeutic target in AML. High OGG1 expression is associated with an adverse prognosis and its downregulation may increase AML cell sensitivity to DNA damaging agents (Liddiard *et al.*, 2010).

7.1.1. Targeting APE1 as therapeutic strategy in AML

Compelling evidence from clinical data strongly links alterations in DNA repair in cancer with prognosis, which can help to determine treatment plans and predict outcome. APE1 over-expression has been associated with adverse prognosis in several cancers including ovarian cancer, breast cancer, pancreatic cancer, lung cancer and multiple myeloma (Yang *et al.*, 2007; Wang *et al.*, 2009a; Al-Attar *et al.*, 2010; Sheng *et al.*, 2012; Woo *et al.*, 2014; Abdel-Fatah *et al.*, 2015). However, APE1 overexpression and its role as a prognostic marker in AML has not yet been established. Evidence presented in this study indicates that APE1 is ubiquitously expressed in AML cell lines and its expression (at the transcript level) does not significantly correlate with overall survival in AML. It is possible that the molecular genetic heterogeneity in AML and activation of multiple oncogenic pathways as well as chromosomal translocations may mask any effect of APE1 as a prognostic marker in AML. Furthermore, the data presented in this study is statistically underpowered to detect anything other than a strong association between APE1 expression and prognosis in AML. It remains possible that APE1 expression may be prognostic in some AML sub-groups but not others, and the accrual of large datasets will be required to test this hypothesis. Furthermore, should APE1

expression have prognostic value, it will be essential to use this in conjunction with other established AML prognostic markers in the clinical setting.

It is becoming clearer that chromosomal translocations, genetic aberrations and epigenetic events can disrupt genome stability and activate DNA damage response pathways, and yet these same alterations also affect treatment efficacy, confer treatment resistance, and promote disease progression. Although mutations in genes involved in DNA damage response are relatively uncommon in AML, with the exception of *TP53*, evidence suggests that DNA damage response dysregulation in AML is caused by two possible mechanisms; disruption of transcriptional regulation and polymorphic variation (Alcalay *et al.*, 2003; Allan *et al.*, 2004; Seedhouse *et al.*, 2004; Rollinson *et al.*, 2007; Voso *et al.*, 2007). The importance of maintaining a balanced DNA damage response is underscored by the observed dysregulation of DNA repair systems in several human cancers. Failure of cells to recognise and proficiently repair DNA damage is a potential cause of cancer initiation and progression. Accumulating evidence demonstrates that loss of DNA repair function in AML increases susceptibility to the acquisition of potentially mutagenic DNA base lesions, genome instability, and may contribute to disease initiation, evolution and relapse (Jankowska *et al.*, 2008; Sallmyr *et al.*, 2008a; Sallmyr *et al.*, 2008b; Schnerch *et al.*, 2012; Stanczyk *et al.*, 2012; Olipitz *et al.*, 2014). Likewise, dysregulated DNA repair in leukaemia could lead to over-activity and low repair fidelity, inducing genome instability and treatment resistance (Bagrintseva *et al.*, 2005; Seedhouse *et al.*, 2006; Fan *et al.*, 2010). Therefore, it is important to consider the wider effects of targeting specific DDR components and impact on the whole pathway, and that targeting the DDR might not be suitable treatment approach in some cases, and particularly where DNA repair is already impaired or not highly upregulated compared to non-leukaemic cells.

One of the major conclusions arising from the study reported here is that APE1 is critical for AML cell proliferation and growth. APE1 knockdown or inhibition significantly impaired cell proliferation and reduced cloning efficiency. Despite impaired proliferation, cell cycle analysis showed no alterations in cell cycle profile of AML cells. However, the exact role of APE1 in cell cycle control and progression is not completely understood. A previous study has demonstrated that APE1 expression is cell cycle dependent, where it is highly expressed in early to mid S phase, possibly to ensure that replicating DNA is free of pro-mutagenic DNA damage (Fung *et al.*, 2001). However, proliferation of AML cells after APE1 knockdown was reduced by affecting all stages of the cell cycle equally, with gene expression data suggesting that this may be primarily due to upregulation of CDKN1A, but upregulation

of other genes could also be a contributing factor. CDKN1A upregulation is associated with cell cycle arrest primarily in the G0/G1 phase, but can induce cell cycle arrest in other phases. CDKN1A is also involved in cell senescence, but there was no indication, from cell cycle analysis and gene expression data, of cell senescence after APE1 knockdown/inhibition. Cells undergoing senescence arrest in the G0 phase, but there was no evidence of G0 cell cycle arrest after APE1 inhibition/knockdown. Furthermore, the gene expression did not show general features characteristic of senescent cells, such as upregulation of CDKN2A (p16), retinoblastoma, PCNA and other factors (Campisi and d'Adda di Fagagna, 2007). Furthermore, CUX1, which thought to prevent cells senescence was significantly upregulated in APE1 knockdown cells (Ramdzan *et al.*, 2015). However, determination of senescence biomarker beta-galactosidase activity is required to further confirm AML cells were not senescent after APE1 inhibition/knockdown.

Although AML cells displayed impaired growth after APE1 knockdown or inhibition, these cells were not differentially sensitive to the cytotoxic and anti-proliferative effects of several anti-leukaemic chemotherapy agents. Rather, there was some evidence of antagonism. This phenotype could be explained by the fact that the drugs used in this study are mainly cytotoxic to exponentially proliferating cells and some drugs utilised here do not generate DNA damage that is recognised and repaired by BER. Furthermore, RNA sequencing analysis of APE1 knockdown AML cells demonstrated upregulation of several genes involved in regulating cell proliferation and cell cycle, in addition to genes previously linked to leukaemogenesis, cell survival and regulation of oxidative stress, such as *CDKN1A, PAX5, BCL-2, FOXO1, CUX1, LAIR1* and *EGR-1*. Of note, RNA sequencing data analysis is preliminary and requires further validation at the protein level. However, impaired cellular proliferation following APE1 knockdown was not due to alteration in cell cycle kinetics but likely due to upregulation of CDKN1A expression and other factors such as PAX5, EGR-1 and CUX1. These factors are collectively involved in sophisticated networks of signal transduction that maintain several cellular functions including cell proliferation and cell survival (see section 4.4.5 for more details).

Impairment of critical DNA repair components reduces cell proliferation and may restrict cell cycle progression to allow DNA damage repair (Viale *et al.*, 2009). As such, this may explain the low levels of DNA damage (AP sites) and topoisomerase II alpha and beta complexes in APE1 knockdown cell clones as determined by the AP site quantification and trapped in agarose DNA immunostaining (TARDIS) assays. However, determination of AP sites using ARP colorimetric assay has been limited in this study and the assay technique may not be

reliable. This is due to potential loss of DNA during tagging with ARP and during washing processes. Such limitations could be avoided by using cell based AP site quantification methods. In addition, further investigation of the nature of DNA damage induced after APE1 knockdown/inhibition is required, which could includedetermining single and double DNA strand breaks using comet assay.

These findings therefore add to a growing body of evidence that APE1 is an important modulator for cell proliferation and provides protection against DNA damage and oxidative stress. Since shRNA is not a tool that can be used to specifically target a specific protein function, it was not possible to differentiate between the contribution of different APE1 functions (namely DNA repair and redox regulation) on cell phenotype. Although both functions are critically important to cellular physiology, the precise mechanisms by which APE1 performs these functions are still not fully understood. Nevertheless, APE1 downregulation reduces DNA repair capacity as well as impairing redox regulation function, and may trigger activation of alternative mechanisms to allow cells to repair accumulating damage and circumvent cell death.

In terms of clinical application, although not explored in this thesis, targeting APE1 in AML may have limited curative potential, based on the observation that cell death is not directly elicited and also that there is no evidence of potentiation of genotoxic anti-leukemic chemotherapy. Rather, APE1 downregulation and inhibition appear to be cytostatic. As such, APE1 inhibition in a clinical setting may slow disease progression via reduction of blast cell proliferation and therefore control disease symptoms, which is a clinically feasible and legitimate treatment approach.

Targeting APE1 in AML may also have adverse effects on normal haematopoietic stem cells and therefore impair haematopoiesis. Although the exact role of APE1 in normal haematopoiesis is still elusive, an early report demonstrated that APE1 is involved in regulation of differentiation of haematopoietic stem cells (Zou *et al.*, 2007). Silencing APE1 expression in embryonic stem cells reduced haemangioblast precursors and resulted in diminished haematopoiesis (Zou *et al.*, 2007). Furthermore, blocking the redox function of APE by E3330, impaired haematopoiesis by inhibition of CD34+ stem cells differentiation down the erythroid and myeloid lineages (Zou *et al.*, 2007). AML patients suffer from anaemia, pancytopenia and thrombocytopenia as a result of infiltration of bone marrow with myeloid progenitors with impaired differentiation, leading eventually to haematopoiesis failure. Therefore, targeting APE1 in AML patients could be challenging and may contribute to the prevailing anaemia by blocking myeloid differentiation. Clinically, one possible

solution to overcome this issue is to co-administer enhancers for haematopoiesis in conjunction with APE1 inhibitors. Therefore, these data suggest that not only cancer cells are dependent on APE1 function, but normal cells (CD34+ cells) are also dependent on APE1 functions. Therefore, targeting APE1 can be cytostatic, or potentially cytotoxic to non-cancer non-target normal cells and it remains to be determined whether there is a potential therapeutic window. However, it should be noted that the presence of a therapeutic window was not directly explored in this work, as such, further investigation using normal CD34+ cells is required to discern this.

In summary, APE1 remains a potential therapeutic target in AML as monotherapy, but not in combination with the established anti-leukaemia therapy. In addition, limited specificity and potency of APE1 inhibitors is currently a major challenge in this particular area of research.

7.1.2. Targeting OGG1 as therapeutic strategy in AML

The BER pathway initiates DNA repair by recognition, excising and removing damaged bases via the action of multiple DNA glycosylases, which have unique DNA lesion specificities. However, one of the key DNA glycosylases involved in this process is the 8-oxoguanine DNA glycosylase (OGG1), which recognises oxidised guanine bases 8-oxoguanine. High OGG1 expression in AML has recently been associated with poor prognosis and identified as a prognostic marker in AML (Liddiard *et al.*, 2010). In addition, AML with the t(8;21) chromosomal translocation is characterised by the AML1-ETO fusion protein, which represses OGG1 expression (Krejci *et al.*, 2008; Forster *et al.*, 2016). Patients with the AML1-ETO fusion protein have a better response to DNA damaging agents and a relatively favourable prognosis, possibly due to low OGG1 activity (Cho *et al.*, 2003; Krejci *et al.*, 2008; Liddiard *et al.*, 2010). Moreover, some polymorphic variants of OGG1, such as S326C and R229Q, are characterised by low OGG1 enzymatic activity and may confer cellular sensitivity to DNA damaging agents (Hyun *et al.*, 2000; Hill and Evans, 2007). This compelling evidence, added to high oxidative burden in AML, strongly supports the hypothesis that targeting OGG1 activity in AML cells may lead to an accumulation of unrepaired 8-oxoG lesions in AML blasts and enhance cells killing in response to DNA damaging agents that generate oxidative stress, such as daunorubicin.

The data presented in this thesis demonstrates that OGG1 is ubiquitously expressed in AML cell lines and its prognostic value in a clinical setting is inconclusive and warrants further investigation. However, given that AML is characterised by a high oxidative burden added to

the oxidative stress caused by external sources, such as anti-leukaemia treatment, these factors collectively contribute to oxidative DNA damage and upregulation of DNA damage response, including BER, which is a normal cellular response. Therefore, upregulation of OGG1 could also be used as a biomarker for high oxidative stress as well as a prognostic marker in AML. Including established AML prognostic markers such as chromosomal translocations, FLT3, RAS or NPM1 in conjunction with OGG1 may provide better prognostic information. However, validation of OGG1 as an independent prognostic marker in other datasets is required before its inclusion can be justified.

OGG1 shRNA knockdown studies undertaken here provide evidence that OGG1 might not be a promising therapeutic target in AML. The prevailing evidence suggests that OGG1 knockdown has no or negligible effect on cell proliferation and clonogenicity. Furthermore, no evidence was found suggesting potentiation of cytotoxicity induced by genotoxic chemotherapy agents by OGG1 knockdown in AML cell lines. Taken together, these data suggest that OGG1 has no role in modulation of AML cell proliferation and has no role in affecting response to DNA damaging anti-leukaemic agents.

Although 8-oxoG lesions were not directly measured in this work, these data imply that 8-oxoG lesions were either not lethal to AML cells and/or alternative mechanisms were involved in removing the damage that would otherwise have been repaired by OGG1-mediated BER. 8-oxoG lesions can be excised by other DNA glycosylases, including MUTYH and MTH1, which support the notion that alternative mechanisms are involved in their removal (Cooke *et al.*, 2003; Russo *et al.*, 2004; Russo *et al.*, 2007; Scott *et al.*, 2014). Therefore, targeting OGG1 and the alternative enzymes that deal with 8-oxoG lesions may sensitise AML cells to DNA damaging agents.

The AML1-ETO fusion oncoprotein confers a relatively favourable prognosis (Cho *et al.*, 2003; Krejci *et al.*, 2008; Liddiard *et al.*, 2010). The favourable outcome is primarily attributed to the t(8;21) chromosomal translocation and reduced DNA damage response, as demonstrated by repressed OGG1 expression in this particular patient subgroup (Krejci *et al.*, 2008; Liddiard *et al.*, 2010). Despite these reports, knocking down OGG1 in AML cells did not sensitise them to DNA damaging agents. It is possible that other factors may be responsible for the good prognosis and better treatment response in patients with the AML1-ETO fusion protein. For example, coexistence of other cytogenetic and molecular alterations could possibly contribute to favourable outcome of patients with the t(8;21) translocation (Hartmann *et al.*, 2016). Similarly, several studies demonstrated that other factors, such as KIT and FLT3-ITD mutations, may contribute to adverse prognosis in t(8;21) AML,

regardless of OGG1 expression (Baer *et al.*, 1997; Nishii *et al.*, 2003; Gustafson *et al.*, 2009; Krauth *et al.*, 2014). Furthermore, OGG1 downregulation in AML1-ETO positive cells confers a mutator phenotype by predisposing to G:C to T:A transversions, which may drive the acquisition of further mutations that are required for disease relapse. As such, complete elimination of subclones carrying this oncogenic fusion protein is essential to prevent disease relapse (Forster *et al.*, 2016). Therefore, targeting OGG1 might not be a suitable approach for AML treatment, but rather still a potential prognostic marker if confirmed in larger scale studies.

7.2. Conclusion

APE1 knockdown and inhibition has demonstrated limited benefit in AML. Specifically, the evidence suggests that there is no value in targeting APE1 as a mechanism to potentiate the cytotoxicity of established anti-leukaemic therapies. However, there may be some clinical value in using APE1 targeting strategies as monotherapy, with evidence that this approach may potentially be used to control the proliferation of leukaemic blast cells. However, this approach may be compromised by the engagement of pro-survival mechanisms. Specifically, APE1 is essential for normal cellular functions and any disruption of its function could induce compensatory mechanisms to maintain cell protection, survival and to efficiently repair mutagenic oxidative DNA damage. The involvement of BCL2, CDKN1A, EGR1, PAX5, FOXO1 and other components appear to support AML cell survival under oxidative stress and may delay cell proliferation in order to allow sufficient DNA damage repair.

In contrast, OGG1 knockdown had no discernible effect on AML cells and it therapeutic targeting may be compromised by the presence of alternative 8-oxoG repair mechanisms. Nevertheless, OGG1 may have value as a prognostic marker in AML.

7.3. Future directions

This study was limited to only three AML cell lines. Given that AML is heterogeneous disease, including additional AML cell lines representing other genetic and molecular characteristics may further clarify the potential value of targeting APE1 in AML. Although challenging, including CD34+ cells as positive control to confirm the presence of a therapeutic window would be essential. Furthermore, including primary AML cells is essential to assess APE1 expression and to determine sensitivity to APE1 inhibitor alone or in combination with anti-leukaemia chemotherapy.

Investigating the effects of double strand break inducing drugs, such as Mylotarg®, and differentiating therapies, such as ATRA, after APE1 knockdown/inhibition is also important. Data from RNA sequencing showed upregulation of XRCC6, which binds DNA double strand breaks and recruits /activates DNA protein kinases, which govern the response to DNA damage. APE1 knockdown/inhibition (in particular inhibition of redox function) may also enhance ATRA-induced differentiation and apoptosis (Fishel *et al.*, 2010). The current study used constitutively expressed shRNA to knockdown APE1 and OGG1 in AML cells. This system has some limitations including re-expression of target protein after 10 to 14 weeks of transduction, and the inability to resuscitate the majority of knockdown cell clones after cryopreservation in liquid nitrogen. As both enzymes are critical for DNA repair, it could be possible that DNA damage induced during the freezing/thawing process was beyond repair and triggered apoptosis. Therefore, it would be prudent to explore the use of inducible shRNA transduction systems, which enable switching the target gene on/off in a controllable manner.

AP site quantification studies in APE1 knockdown and APE1 inhibited cells (chapters 4 and 5) were limited in this thesis. Further quantification and validation of the initial results presented here are warranted in order to draw strong conclusions from these data. Furthermore, the use of assays for specific structural types of DNA damage following APE1 knockdown or inhibition using comet assay will help determine the type of the DNA damage induced.

The results of RNA sequencing analysis of APE1 knockdown cells are promising and provided evidence of the mechanisms that may be responsible for the observed phenotype (attenuated cell proliferation) in APE1 knockdown cell clones. Nevertheless, further validation of these results at the protein levels is required. Furthermore, studying a possible role for APE1 in the regulation of FOXO1, CUX1 and LAIR1 may help to further elucidate these mechanisms.

Having demonstrated that APE1 knockdown or inhibition antagonises the anti-proliferative effects of cytotoxic chemotherapy, it will be useful to explore the mechanisms responsible for mediating this phenotype, and whether this is entirely mediated by the compromised proliferation of APE1 knockdown cells or whether other mechanisms may be involved. CDKN1A and other transcription factors may have a putative role in the observed phenotype.

As OGG1 is a potential prognostic marker in AML, it is important to demonstrate the regulatory mechanisms of this protein and study its exact role in AML.

Appendix A

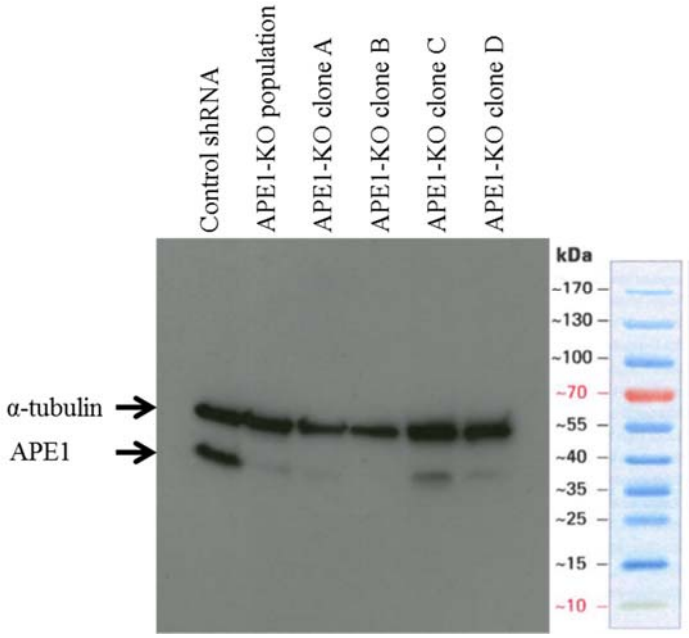


Figure shows AML3 cells with APE1 knockdown. The APE1 antibody used in this project was a mouse monoclonal (clone 13B8E5C2) specific to human APE/ref-1 protein with a molecular weight of approximately 37 kDa according to manufacturer.

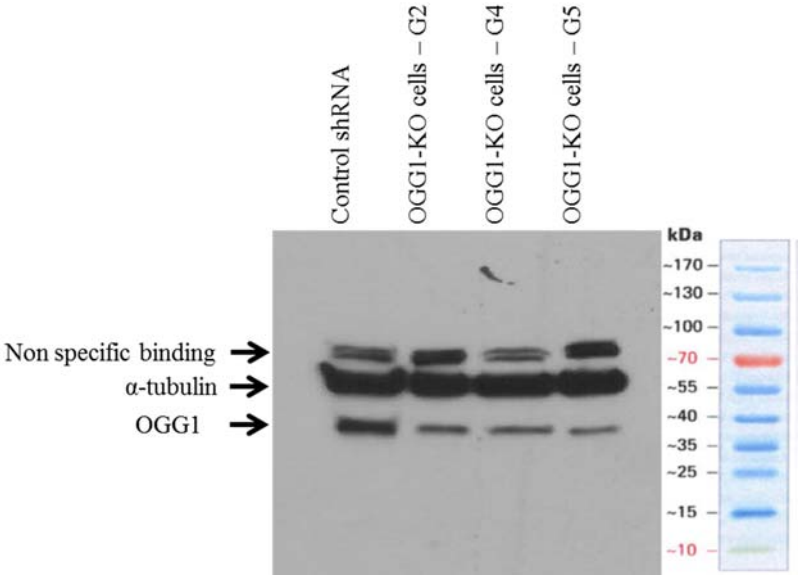
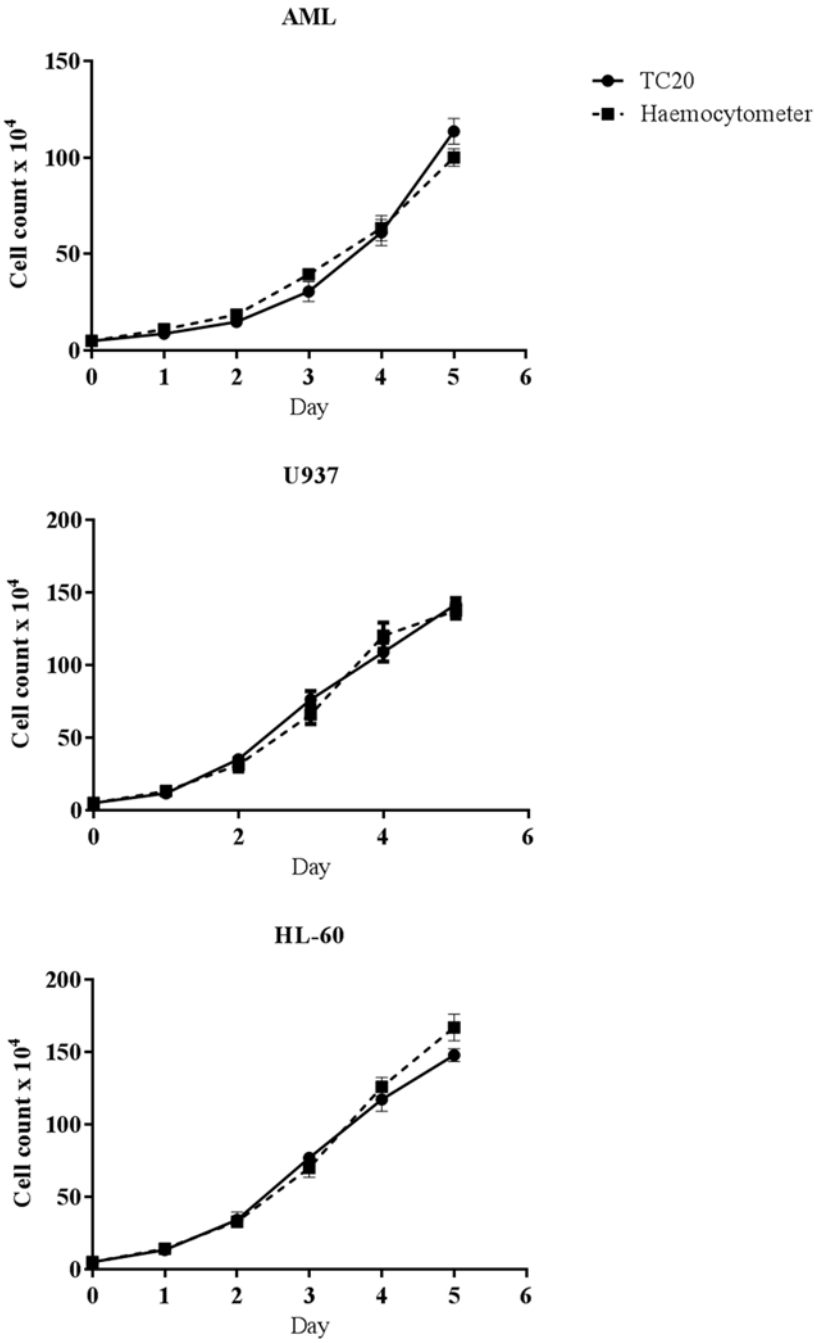


Figure shows AML3 cells with OGG1 knockdown using 3 different shRNA particles (G2, G4 and G5). The OGG1 antibody used in this project was a rabbit IgG polyclonal reactive to mouse and human OGG1 protein with a molecular weight of approximately 39 kDa according to manufacturer. The antibody was raised against a synthetic peptide corresponding to aa 318-345 of the C terminal of human OGG1 protein

Appendix B

Comparison between cell count using TC20 automated cell counter and manual counting using haemocytometer to validate using TC20 for routine cell counting. AML3, U937 and HL-60 cells were seeded into cell density of $5x10^4$ cells/ml in the first day and counted every 24 hours for 5 days using TC20 cell counter and haemocytometer. Data presented in each case represent the mean of three independent experiments and error bars of standard deviation



Appendix C

Comparison between counting using TC20 automated cell counter and manual counting using haemocytometer. AML3, U937 and HL-60 cells were seeded into cell density of $5x10^4$ in the first day and counted every 24 hours for 5 days using TC20 cell counter and haemocytometer. Cell count represent the mean of three independent experiments.

AML3 cell line

	TC20 cell counter		Haemocytometer	
Day	Cell count x 10^4	Standard deviation	Cell count x 10^4	Standard deviation
0	5.0	0.0	5.0	0.0
1	8.8	1.6	11.0	2.6
2	14.9	1.3	18.7	2.3
3	30.7	5.2	39.5	2.3
4	61.4	6.9	63.5	6.6
5	113.7	6.7	100.2	4.5

U937 cell line

	TC20 cell counter		Haemocytometer	
Day	Cell count x 10^4	Standard deviation	Cell count x 10^4	Standard deviation
0	5.0	0.0	5.0	0.0
1	11.7	2.4	13.3	1.5
2	35.2	2.4	31.3	4.7
3	76.4	5.9	66.0	6.6
4	109.0	6.6	120.3	9.0
5	142.0	4.4	137.7	5.5

HL-60 cell line

	TC20 cell counter		Haemocytometer	
Day	Cell count x 10^4	Standard deviation	Cell count x 10^4	Standard deviation
0	5.0	0.0	5.0	0.0
1	13.5	2.4	14.2	1.8
2	34.2	5.5	33.0	3.0
3	77.1	3.5	70.2	6.4
4	125.0	5.6	126.0	6.6
5	148.0	4.6	160.7	2.5

Appendix D

Commonly upregulated genes in AML3 and HL-60 cells following APE1 knockdown

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
01	*ABCA1*	2.55	1.01E-003	2.16	1.29E-002
02	*AC003090.1*	2.46	2.13E-003	4.19	2.63E-002
03	*ACOT11*	1.91	1.49E-004	1.80	1.73E-003
04	*ACPP*	1.83	8.26E-007	1.98	3.72E-004
05	*ADCY1*	3.23	6.71E-005	5.07	1.24E-003
06	*AGBL3*	1.76	2.27E-002	1.78	5.42E-003
07	*ALDH3B1*	1.52	5.24E-004	1.70	4.22E-005
08	*ANPEP*	2.30	8.82E-006	1.59	2.29E-002
09	*AOAH*	1.81	1.72E-002	2.08	1.35E-003
10	*AP5B1*	1.58	4.86E-004	1.58	4.82E-004
11	*APOBR*	1.54	4.83E-002	1.75	1.47E-002
12	*APOLD1*	1.54	4.61E-004	1.66	1.13E-003
13	*ARHGAP10*	5.70	7.84E-003	37.85	5.65E-009
14	*ATP10D*	1.88	9.45E-010	4.63	6.87E-011
15	*ATP8B4*	1.78	9.64E-004	1.50	4.58E-002
16	*ATXN1*	1.60	1.99E-003	1.50	1.17E-002
17	*BARX1*	2.48	3.39E-007	3.22	2.59E-002
18	*BCL2A1*	2.30	2.53E-005	1.54	4.66E-002
19	*BCL2L11*	1.78	3.38E-003	1.67	1.52E-002
20	*BEST1*	1.79	2.10E-002	2.20	1.90E-002
21	*BMX*	2.30	1.12E-003	8.07	2.18E-026
22	*BTG2*	1.93	2.54E-004	3.28	1.67E-010
23	*C1orf186*	3.75	6.20E-041	1.39	8.09E-003
24	*C1RL*	1.39	6.68E-005	1.77	1.10E-009
25	*C5*	1.39	2.49E-002	1.75	1.41E-002
26	*CARD6*	1.30	1.73E-002	1.32	4.38E-002
27	*CASP8*	1.26	1.45E-003	1.27	8.82E-004
28	*CD38*	1.54	9.72E-004	1.42	1.38E-002
29	*CD47*	1.19	1.25E-002	1.21	2.03E-003
30	*CDKN1A*	2.89	1.31E-006	2.17	8.59E-003
31	*CFAP44*	1.67	9.68E-003	1.89	7.14E-004
32	*CLTCL1*	1.23	4.67E-002	1.82	7.31E-011
33	*CNTRL*	1.32	4.52E-004	1.25	7.23E-003
34	*COL4A1*	2.55	2.25E-002	2.31	1.80E-003
35	*CPNE4*	5.07	3.12E-007	2.53	1.70E-002
36	*CPQ*	1.76	7.28E-004	3.21	1.48E-005
37	*CRISPLD2*	1.71	6.20E-003	2.98	6.20E-003
38	*CRTC1*	1.62	2.37E-002	1.59	3.45E-002
39	*CST3*	1.50	8.53E-005	1.54	6.72E-003
40	*CTD-2231E14.8*	1.69	1.85E-009	2.59	1.37E-003

Appendix D

Continue: Commonly upregulated genes in AML3 and HL-60 cells following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
41	*CTD-2542L18.1*	3.92	1.59E-003	2.28	4.52E-002
42	*CTSK*	2.25	2.04E-003	2.04	9.33E-003
43	*CUX1*	1.40	4.14E-002	1.46	2.39E-002
44	*CYP4V2*	1.33	4.15E-002	2.42	1.50E-007
45	*CYTIP*	2.31	5.29E-005	2.78	9.40E-010
46	*DENND1B*	1.39	7.63E-004	1.25	4.19E-002
47	*DGKG*	1.87	3.04E-005	1.65	1.17E-003
48	*DHRS2*	7.45	9.26E-003	4.07	2.06E-003
49	*DNASE1*	1.35	5.00E-003	1.55	2.24E-005
50	*DPY19L4*	1.27	4.89E-003	1.24	2.50E-002
51	*DSE*	1.67	1.77E-002	1.68	1.63E-002
52	*DTX3L*	1.75	4.59E-004	1.74	2.29E-003
53	*E2F7*	2.21	4.81E-006	2.62	2.94E-009
54	*EGR1*	2.29	6.14E-006	1.81	2.41E-003
55	*EMR4P*	1.95	1.82E-002	2.31	2.74E-003
56	*EOGT*	1.28	1.82E-002	1.56	2.72E-004
57	*EPHB6*	30.30	7.64E-034	1.93	1.50E-003
58	*ERAP1*	1.47	1.89E-003	1.34	2.99E-002
59	*EXOC6B*	1.93	3.46E-003	1.77	4.83E-002
60	*FAM110B*	2.04	9.01E-003	2.23	1.13E-002
61	*FAM20C*	1.40	8.97E-003	4.67	6.26E-004
62	*FAM65B*	3.34	9.62E-004	12.01	3.11E-004
63	*FBP1*	1.35	2.05E-002	2.33	4.94E-014
64	*FBXL17*	1.61	8.46E-005	1.54	1.11E-003
65	*FNDC3B*	1.65	1.12E-005	1.66	1.03E-005
66	*FOXO1*	2.05	1.72E-003	2.03	4.40E-003
67	*FRS2*	1.37	3.12E-002	1.47	1.00E-002
68	*GALNS*	1.26	3.20E-002	1.30	2.72E-002
69	*GATA2-AS1*	3.92	1.17E-007	1.77	1.12E-002
70	*GFI1*	1.39	7.55E-010	1.22	8.82E-004
71	*GLCE*	1.59	6.75E-004	2.64	3.81E-012
72	*GPR137B*	1.33	2.30E-002	2.22	1.20E-009
73	*GTF2IRD2B*	1.48	4.44E-002	2.03	2.14E-004
74	*HGSNAT*	1.35	3.60E-002	1.88	6.29E-005
75	*IGIP*	2.11	2.78E-006	1.71	5.82E-003
76	*ITGB2*	1.62	1.52E-006	1.34	1.10E-002
77	*ITGB2-AS1*	1.65	4.91E-005	2.11	1.83E-005
78	*KANK1*	1.40	1.19E-002	1.53	8.22E-004
79	*KIAA0825*	1.80	3.08E-002	2.19	3.12E-002
80	*KIAA1211L*	1.87	2.56E-002	3.53	1.28E-003

Appendix D

Continue: Commonly upregulated genes in AML3 and HL-60 cells following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
81	*KITLG*	5.52	6.09E-005	11.27	1.26E-003
82	*LAIR1*	1.22	4.15E-002	1.54	4.69E-007
83	*LAMB3*	1.59	2.06E-002	3.92	5.11E-003
84	*LINC00638*	1.75	3.62E-002	2.35	6.49E-003
85	*LMO7*	1.61	9.21E-006	1.38	1.26E-002
86	*LPP*	2.39	2.76E-009	2.41	3.46E-009
87	*LRCH3*	1.36	1.75E-002	1.34	2.98E-002
88	*LRPAP1*	1.27	1.24E-002	1.42	5.44E-005
89	*MAN2B2*	1.69	1.46E-006	1.40	4.11E-003
90	*MAPRE3*	2.31	1.61E-003	2.60	1.76E-002
91	*MB21D2*	2.43	3.27E-004	9.72	1.02E-007
92	*MED12L*	2.87	4.78E-002	5.30	4.11E-006
93	*MFSD6*	1.42	4.01E-002	3.64	3.78E-011
94	*MR1*	1.34	1.19E-002	1.45	2.13E-003
95	*MVB12B*	1.71	3.83E-003	2.20	2.38E-008
96	*MYO10*	1.95	5.70E-004	2.20	1.44E-002
97	*NCEH1*	1.75	1.26E-002	1.79	1.12E-002
98	*NCF1*	1.64	6.49E-004	2.50	3.13E-012
99	*NCF1C*	1.62	3.26E-004	2.99	3.71E-019
100	*NEB*	3.68	5.39E-004	3.61	6.99E-006
101	*NEDD9*	2.00	7.25E-006	2.99	4.49E-010
102	*NIPAL2*	1.69	4.54E-006	1.66	1.38E-003
103	*NPL*	1.79	3.50E-003	1.88	6.24E-004
104	*ODF2L*	1.31	7.64E-003	1.28	1.93E-002
105	*OSTM1*	1.29	7.15E-004	1.43	4.10E-007
106	*P2RX1*	1.47	1.79E-002	1.94	1.09E-005
107	*PAX5*	3.61	2.80E-004	2.48	1.91E-004
108	*PCGF5*	1.71	2.08E-007	4.07	4.05E-041
109	*PCSK6*	1.77	1.68E-005	1.60	9.15E-004
110	*PDE4B*	3.00	3.38E-009	1.99	6.99E-005
111	*PFN2*	2.04	2.82E-002	2.17	1.60E-002
112	*PHC3*	1.55	1.95E-003	1.62	5.35E-004
113	*PHLDA1*	1.31	2.84E-002	1.99	4.71E-011
114	*PHTF1*	1.24	4.54E-002	1.27	2.61E-002
115	*PILRA*	2.24	4.52E-004	2.02	1.59E-002
116	*PIWIL4*	1.71	4.19E-006	1.81	2.67E-006
117	*PPM1H*	1.77	3.69E-003	5.93	2.66E-019
118	*PRKCE*	2.24	6.31E-005	3.01	5.31E-007
119	*PTAFR*	1.94	3.72E-003	1.95	3.02E-003
120	*PTGFRN*	1.38	1.25E-002	48.29	1.47E-056

Appendix D

Continue: Commonly upregulated genes in AML3 and HL-60 cells following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
121	*PTPRC*	1.38	2.70E-002	1.52	2.99E-003
122	*RAB37*	1.41	1.44E-003	1.53	2.10E-004
123	*RAB7B*	3.02	8.11E-018	1.62	1.37E-003
124	*RAC2*	1.51	1.42E-003	1.47	4.98E-003
125	*RASAL2*	2.47	4.91E-008	2.21	5.54E-003
126	*RN7SL141P*	3.58	3.46E-003	2.28	2.50E-002
127	*RNASEL*	1.41	4.88E-004	1.46	4.26E-003
128	*RNF165*	1.90	6.54E-003	2.24	5.59E-004
129	*RP11-37B2.1*	2.16	5.40E-004	1.71	3.78E-002
130	*RP11-443B7.1*	1.51	2.59E-002	2.42	8.18E-006
131	*RP11-53B2.2*	2.34	3.24E-002	4.72	4.29E-002
132	*RP11-556H2.3*	2.24	2.45E-002	4.64	4.93E-002
133	*SCAMP2*	1.42	3.32E-005	1.28	1.17E-002
134	*SERPINA1*	2.17	3.11E-002	5.28	3.62E-007
135	*SESN3*	2.20	6.06E-004	11.71	9.19E-027
136	*SIPA1L2*	1.72	4.28E-002	4.47	1.39E-005
137	*SIRPB2*	1.36	4.08E-002	2.58	1.40E-002
138	*SKOR1*	3.88	1.79E-002	1.76	2.57E-002
139	*SLC38A10*	1.90	1.00E-008	1.56	3.02E-004
140	*SLC46A3*	1.74	2.98E-003	2.62	3.55E-006
141	*SLFN5*	2.01	4.88E-002	3.32	4.30E-003
142	*SPPL2A*	1.30	2.70E-003	1.44	1.48E-005
143	*SQSTM1*	1.45	1.33E-003	1.32	3.00E-002
144	*SRGN*	1.63	9.74E-005	2.15	6.72E-011
145	*ST7L*	1.31	3.48E-003	1.28	2.91E-002
146	*STK17B*	1.17	3.32E-002	1.40	1.41E-007
147	*STXBP5*	1.22	3.51E-002	1.77	4.04E-022
148	*STXBP5-AS1*	4.02	2.58E-002	2.75	1.03E-002
149	*SUCNR1*	3.72	1.47E-011	1.92	1.36E-009
150	*SYNE1*	2.57	8.33E-005	5.23	2.59E-006
151	*SYTL2*	2.44	1.95E-002	2.58	1.56E-002
152	*TBCEL*	1.34	7.39E-003	0.84	1.30E-002
153	*TGFA*	3.52	2.57E-005	3.88	1.65E-003
154	*THBS4*	1.33	7.17E-003	1.22	1.51E-003
155	*TLR1*	2.27	7.97E-004	3.50	8.92E-006
156	*TLR2*	1.52	1.18E-002	1.82	3.14E-003
157	*TNFRSF14*	1.65	4.55E-003	2.28	8.44E-005
158	*TRGC1*	2.66	3.37E-005	4.41	6.19E-014
159	*TRGC2*	2.29	1.72E-002	4.81	6.47E-010
160	*TSPAN5*	3.32	2.76E-009	4.52	7.08E-007

Appendix D

Continue: Commonly upregulated genes in AML3 and HL-60 cells following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
161	*TTC14*	1.50	1.63E-003	0.91	2.97E-002
162	*TTLL7*	2.26	3.85E-003	2.76	5.82E-006
163	*TXNIP*	1.49	6.28E-003	1.46	3.06E-004
164	*TYROBP*	2.28	5.63E-004	2.15	3.39E-003
165	*UACA*	1.85	1.28E-002	6.14	4.03E-005
166	*UBA7*	1.70	1.02E-003	1.51	5.91E-003
167	*USP18*	1.64	3.46E-002	2.02	1.57E-002
168	*VPS8*	1.75	4.08E-012	0.92	3.12E-004
169	*WAS*	1.56	1.12E-002	1.26	1.49E-002
170	*WSB2*	1.33	4.59E-004	0.80	4.43E-003
171	*WWOX*	1.68	4.17E-005	1.05	1.57E-002
172	*ZCWPW1*	1.37	4.16E-002	1.45	2.12E-002
173	*ZMAT3*	2.39	1.65E-025	1.09	7.12E-005
174	*ZNF438*	1.44	9.08E-003	1.16	1.63E-002
175	*ZNF641*	1.29	4.47E-002	1.06	5.09E-003
176	*ZNF75D*	1.48	1.52E-003	1.38	2.21E-002

Appendix E

Commonly downregulated genes in AML3 and HL-60 following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
01	*ABCA3*	-1.9	3.81E-006	-1.4	9.83E-003
02	*ABCF1*	-1.6	1.52E-005	-1.3	9.86E-003
03	*ACP1*	-1.3	5.21E-003	-1.3	4.37E-002
04	*ALDH18A1*	-1.4	2.02E-004	-1.5	1.40E-006
05	*ALKBH2*	-1.5	1.26E-002	-1.5	6.44E-003
06	*APEX1*	-8.6	6.33E-162	-12.6	1.40E-233
07	*ARF4*	-1.2	1.87E-002	-1.3	3.68E-003
08	*ARMCX5*	-16.7	3.92E-045	-1.3	1.55E-002
09	*ASCC2*	-2.1	3.33E-022	-2.1	3.33E-022
10	*ASF1A*	-1.3	8.22E-003	-1.2	2.71E-002
11	*ATF5*	-1.4	3.22E-002	-1.5	1.09E-002
12	*ATP5G2*	-1.4	1.45E-002	-1.4	8.69E-003
13	*BCAT1*	-1.5	7.42E-004	-1.8	3.96E-007
14	*BFAR*	-1.4	9.42E-004	-1.3	9.40E-003
15	*BIVM*	-1.8	4.36E-004	-1.6	1.15E-005
16	*BLMH*	-1.3	1.63E-002	-1.3	1.39E-002
17	*BTK*	-1.2	7.79E-003	-1.2	1.16E-002
18	*BZRAP1*	-1.3	4.88E-002	-1.4	3.23E-003
19	*C12orf75*	-2.9	1.60E-025	-2.0	1.81E-008
20	*CACNB1*	-2.0	1.88E-005	-2.9	1.31E-012
21	*CAMSAP2*	-1.4	3.41E-004	-1.4	1.41E-002
22	*CCBL2*	-1.3	6.60E-003	-1.6	8.72E-009
23	*CDC42*	-1.6	9.02E-005	-1.6	9.37E-005
24	*CDK16*	-1.5	2.29E-003	-1.4	8.07E-003
25	*CENPV*	-1.4	1.57E-002	-1.4	1.48E-002
26	*CFDP1*	-1.5	1.70E-002	-1.7	9.66E-004
27	*CGN*	-4.1	2.93E-002	-2.3	3.49E-003
28	*CIAPIN1*	-1.3	7.17E-004	-1.2	3.64E-002
29	*CNNM4*	-1.5	7.77E-004	-1.6	1.73E-004
30	*CPOX*	-1.7	3.70E-005	-1.5	1.45E-003
31	*CSE1L*	-1.4	2.26E-006	-1.3	3.81E-004
32	*CUTC*	-1.4	6.65E-005	-1.3	1.82E-003
33	*DANCR*	-1.7	6.25E-008	-1.7	2.16E-007
34	*DDN*	-5.5	8.11E-033	-1.7	2.64E-007
35	*DDX21*	-1.3	8.68E-007	-1.2	3.38E-002
36	*DFFA*	-1.4	4.72E-003	-1.4	1.28E-002
37	*DNAJC24*	-1.5	1.28E-002	-1.8	2.14E-005
38	*DNMT3B*	-1.6	1.03E-003	-1.5	6.03E-003
39	*EFCAB2*	-1.4	1.17E-002	-1.4	9.27E-003
40	*EIF4B*	-1.4	1.88E-002	-1.5	6.89E-003

Appendix E

Continue: Commonly downregulated genes in AML3 and HL-60 cells following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
41	*EIF4E*	-1.4	6.39E-003	-1.3	4.56E-002
42	*ELP5*	-1.4	3.90E-004	-1.4	5.76E-004
43	*FARP2*	-2.0	1.38E-019	-2.1	7.32E-021
44	*FDXACB1*	-1.5	2.09E-002	-1.7	1.26E-003
45	*FIRRE*	-4.6	1.15E-016	-121.4	2.62E-023
46	*FSD1*	-2.1	2.91E-004	-1.9	4.97E-002
47	*G3BP1*	-1.3	2.94E-005	-1.2	9.13E-004
48	*G3BP2*	-1.2	3.47E-002	-1.4	9.19E-010
49	*GJA3*	-5.1	5.75E-015	-2.6	1.25E-012
50	*GPD1L*	-1.4	3.19E-002	-1.4	2.32E-002
51	*GPR63*	-2.5	1.68E-007	-3.1	1.45E-014
52	*GRSF1*	-1.2	7.22E-003	-1.2	1.53E-002
53	*GSTP1*	-1.6	1.88E-005	-1.4	6.52E-003
54	*GTF2H1*	-1.2	4.79E-002	-1.5	1.18E-005
55	*GTF2H2C*	-1.2	4.24E-002	-1.2	4.01E-002
56	*GTF2H3*	-1.3	7.64E-003	-1.4	6.20E-004
57	*GTSF1*	-1.4	3.15E-003	-1.5	4.72E-005
58	*HMMR*	-2.0	1.04E-004	-2.3	3.84E-006
59	*HN1L*	-1.6	2.05E-004	-1.4	2.20E-002
60	*HNRNPDL*	-1.4	1.35E-004	-1.3	2.08E-002
61	*HNRNPH3*	-1.2	3.79E-002	-1.3	1.41E-002
62	*ILF2*	-1.2	1.87E-002	-1.2	4.24E-002
63	*IMPA1*	-1.8	7.37E-015	-2.2	4.93E-025
64	*IMPDH2*	-1.3	1.44E-004	-1.3	5.06E-004
65	*IPO5*	-1.3	1.59E-002	-1.5	5.95E-005
66	*ITGB1BP1*	-1.4	8.28E-004	-1.3	3.87E-002
67	*JOSD1*	-1.7	8.88E-008	-1.6	5.57E-005
68	*KCTD3*	-1.4	9.64E-003	-1.4	7.66E-003
69	*KIF3C*	-1.9	2.93E-006	-2.4	9.47E-006
70	*LEPREL2*	-1.6	3.46E-003	-6.6	2.03E-048
71	*LMO4*	-1.4	3.90E-002	-1.4	2.46E-002
72	*LPPR2*	-2.5	1.86E-022	-3.0	6.28E-018
73	*LRRC8B*	-1.8	4.72E-006	-1.4	1.40E-002
74	*LRRC8C*	-1.5	1.52E-002	-1.5	4.33E-002
75	*MAK16*	-1.3	1.12E-002	-1.3	6.89E-003
76	*MAP4K1*	-1.9	4.04E-006	-2.7	1.31E-014
77	*MAPK3*	-1.3	2.92E-002	-1.5	2.05E-003
78	*MAPK6*	-1.5	2.12E-014	-1.5	1.79E-015
79	*MARCKS*	-1.6	2.67E-002	-3.5	7.04E-005
80	*MARS*	-1.2	1.45E-002	-1.5	1.62E-010

Appendix E

Continue: Commonly downregulated genes in AML3 and HL-60 cells following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
81	*MCCC2*	-1.3	3.21E-002	-1.7	1.91E-007
82	*ME1*	-1.5	6.11E-003	-2.4	2.05E-018
83	*ME2*	-1.2	3.03E-002	-1.3	3.02E-003
84	*MED6*	-1.8	1.25E-009	-1.6	5.00E-007
85	*METTL2A*	-1.2	2.00E-002	-1.2	2.58E-002
86	*METTL8*	-1.3	7.06E-003	-1.2	4.88E-002
87	*MOGS*	-1.3	4.47E-002	-1.3	4.21E-002
88	*MPZL1*	-1.8	5.64E-007	-1.4	3.35E-003
89	*MRPL9*	-1.9	2.41E-018	-2.0	2.40E-023
90	*MRPS2*	-1.4	1.73E-003	-1.4	1.28E-002
91	*MRPS27*	-1.2	2.48E-002	-1.2	1.39E-002
92	*MTCH2*	-1.4	1.59E-003	-1.7	8.11E-009
93	*MTHFD1*	-2.4	8.64E-050	-2.1	8.64E-038
94	*MTOR*	-1.3	2.01E-002	-1.3	3.02E-003
95	*NAP1L1*	-1.3	1.58E-002	-1.3	2.24E-002
96	*NAP1L4*	-1.1	3.00E-002	-1.3	1.15E-005
97	*NARS2*	-1.2	2.63E-002	-1.2	4.50E-002
98	*NCR3LG1*	-1.5	8.70E-003	-1.7	2.65E-004
99	*NIPA2*	-1.5	2.17E-005	-1.4	2.67E-003
100	*NLE1*	-1.3	4.52E-003	-1.2	4.68E-002
101	*NPM1*	-1.4	7.11E-004	-1.3	1.12E-002
102	*NPM3*	-1.5	4.61E-002	-1.8	6.10E-004
103	*NRARP*	-3.1	9.44E-003	-1.5	4.28E-002
104	*NUP54*	-1.2	2.27E-002	-1.2	1.73E-003
105	*PALD1*	-1.8	3.21E-005	-1.8	5.58E-005
106	*PBX2*	-1.5	6.87E-004	-1.4	1.56E-002
107	*PCID2*	-1.2	2.35E-002	-1.4	6.89E-005
108	*PFAS*	-1.4	1.15E-004	-1.3	1.73E-002
109	*PFKM*	-2.2	9.12E-020	-1.8	5.65E-013
110	*POLE4*	-1.6	8.88E-004	-1.4	3.22E-002
111	*PPFIA3*	-1.5	1.05E-002	-1.4	2.23E-002
112	*PTK7*	-1.7	6.76E-003	-16.5	8.84E-022
113	*RAB29*	-1.2	4.86E-003	-1.4	9.20E-007
114	*RAB31*	-1.4	1.53E-002	-1.9	7.70E-007
115	*RAB4A*	-1.4	3.81E-006	-1.5	2.84E-009
116	*RABEP1*	-1.6	2.29E-006	-1.5	1.49E-004
117	*RAP2A*	-2.1	2.67E-011	-1.5	1.25E-003
118	*RASSF3*	-1.3	5.87E-004	-1.3	1.07E-003
119	*RBM12*	-1.2	4.10E-002	-1.2	8.16E-003
120	*RBM19*	-1.4	2.15E-002	-1.3	3.93E-002

Appendix E

Continue: Commonly downregulated genes in AML3 and HL-60 cells following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
121	*RCC1*	-1.9	9.86E-007	-1.7	1.39E-004
122	*RDH11*	-1.3	2.34E-002	-1.4	2.22E-002
123	*RENBP*	-3.0	7.99E-004	-2.7	1.56E-002
124	*RHOF*	-1.4	1.18E-003	-1.3	6.00E-003
125	*RHOQ*	-1.3	3.04E-002	-1.7	8.83E-008
126	*RHPN2*	-1.9	7.51E-007	-1.5	7.28E-003
127	*RIC8A*	-1.2	4.61E-002	-1.5	6.43E-005
128	*RNU12*	-1.5	4.22E-002	-1.8	1.21E-003
129	*RP11-1082L8.4*	-2.4	3.49E-004	-3.1	4.20E-003
130	*RP11-253E3.3*	-2.0	4.36E-003	-12.5	2.01E-006
131	*RP11-391M1.4*	-1.9	3.54E-003	-1.6	3.79E-002
132	*RP1-239B22.5*	-1.9	1.13E-005	-1.7	2.23E-004
133	*RPL14*	-1.3	3.73E-002	-1.3	3.78E-002
134	*RPL19*	-1.3	1.58E-002	-1.4	4.98E-003
135	*RPLP0P6*	-1.8	1.08E-003	-1.5	5.00E-002
136	*RRM1*	-1.4	1.12E-004	-1.3	1.26E-003
137	*RUVBL1*	-1.3	3.48E-002	-1.3	1.43E-002
138	*RWDD4*	-1.5	4.53E-004	-1.5	6.26E-004
139	*SAAL1*	-1.3	4.16E-002	-1.5	6.29E-005
140	*SEC13*	-1.2	2.21E-002	-1.3	1.49E-003
141	*SERP1*	-1.3	1.89E-003	-1.4	5.55E-005
142	*SLC19A2*	-1.4	1.71E-003	-1.3	2.96E-002
143	*SLC25A15*	-1.4	4.09E-003	-1.5	2.96E-004
144	*SLC25A17*	-1.3	2.16E-003	-1.3	3.25E-003
145	*SLC37A4*	-1.3	3.47E-002	-1.6	1.54E-004
146	*SLC7A2*	-1.4	2.12E-003	-1.8	1.51E-011
147	*SNHG16*	-1.5	1.88E-008	-1.3	4.32E-003
148	*SNHG8*	-1.4	3.90E-002	-1.6	9.23E-004
149	*SNORD12C*	-1.7	3.28E-003	-1.6	2.05E-002
150	*SNRNP48*	-1.5	2.07E-006	-1.2	2.51E-002
151	*SNRPA*	-1.3	3.54E-002	-1.4	2.91E-002
152	*SOBP*	-1.4	1.99E-002	-1.5	1.07E-002
153	*SORT1*	-4.6	1.31E-033	-1.4	1.13E-002
154	*SPATS2*	-1.3	1.57E-002	-1.7	7.04E-007
155	*SPIRE1*	-1.3	1.43E-002	-1.4	8.63E-003
156	*SPTBN2*	-2.5	3.48E-002	-1.5	1.74E-002
157	*SRPR*	-1.4	3.20E-003	-1.8	4.60E-010
158	*ST13*	-1.5	2.76E-005	-1.5	1.19E-004
159	*ST13P3*	-1.4	5.71E-003	-1.5	1.64E-003
160	*ST13P4*	-1.6	6.68E-004	-1.6	2.13E-003

Appendix E

Continue: Commonly downregulated genes in AML3 and HL-60 cells following APE1 knockdown.

	Gene	AML 3		HL-60	
		Fold Change	P value	Fold Change	P value
161	*ST13P5*	-1.5	4.52E-003	-1.5	4.52E-003
162	*STK32C*	-1.5	5.59E-003	-1.7	2.61E-003
163	*TBC1D4*	-1.5	1.68E-002	-1.9	5.15E-005
164	*TEAD4*	-1.4	2.81E-002	-23.5	3.69E-012
165	*TEX19*	-4.1	2.13E-002	-2.8	4.04E-003
166	*THUMPD1*	-1.2	4.82E-004	-1.2	1.56E-002
167	*TMEM14B*	-1.4	1.31E-003	-1.3	4.30E-002
168	*TMEM194A*	-1.4	3.34E-003	-1.4	1.05E-002
169	*TNNT1*	-2.0	9.36E-006	-12.8	2.36E-037
170	*TOP1MT*	-1.3	3.61E-002	-1.4	4.24E-002
171	*TRAP1*	-1.3	3.61E-002	-1.3	4.66E-002
172	*TRIM24*	-1.5	4.14E-005	-1.3	4.40E-002
173	*TUBA1C*	-1.4	3.74E-004	-1.3	1.85E-002
174	*TWISTNB*	-1.6	5.05E-013	-1.3	1.27E-003
175	*UAP1L1*	-1.3	2.12E-002	-1.6	2.07E-004
176	*UBFD1*	-1.4	2.73E-005	-1.3	1.42E-003
177	*USMG5*	-3.5	2.87E-011	-2.8	8.83E-008
178	*USP46*	-1.5	2.64E-003	-1.3	4.18E-002
179	*VASH2*	-1.6	6.33E-003	-1.7	3.33E-003
180	*VDAC2*	-1.2	1.06E-002	-1.1	4.93E-002
181	*VEGFB*	-1.3	1.74E-002	-1.4	3.59E-003
182	*VPS4A*	-1.9	8.05E-008	-1.6	2.61E-004
183	*VWA9*	-1.2	1.64E-002	-1.2	4.06E-002
184	*WNT10B*	-2.0	3.98E-005	-1.6	2.12E-003
185	*XPO6*	-1.8	7.37E-010	-1.6	3.02E-007
186	*XRCC6*	-1.2	1.80E-002	-1.2	1.03E-002
187	*YWHAQ*	-1.2	2.72E-003	-1.2	3.65E-003
188	*ZDHHC2*	-1.9	1.49E-011	-1.3	1.43E-002
189	*ZFP1*	-1.3	1.01E-002	-1.4	5.65E-003
190	*ZMAT5*	-1.4	4.61E-002	-1.8	6.94E-005
191	*ZNF343*	-1.5	3.23E-003	-1.4	5.44E-003

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