Antioxidant Pathways and Human Leukaemia Chemotherapeutic Sensitivity

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Contents

Declaration

I declare all work presented in this thesis was undertaken and completed by myself, except where indicated and acknowledged, and that this has not been previously submitted for a degree. All sources of information have been fully acknowledged.

Sally-Anne Heasman
January 2013

Abstract

Cancer is uncontrolled cellular proliferation devoid of normal biological regulatory mechanisms. Therapeutic treatment of cancers relies on overcoming such proliferation, to allow cytotoxic destruction of cancer cells. Many of the recent therapeutic breakthroughs have been in the targeting of various blood cancers: eg imatinib has effectively eradicated chronic myeloid leukaemia (CML) morbidity. However, acute myeloid leukaemia (AML) remains essentially incurable for the vast majority of patients. The current gold-standard treatment for AML has remained relatively unchanged for decades, consisting of the antimetabolite cytarabine (ara-C) and the anthracycline cytotoxic antibiotic daunorubicin (DNR). The cytoprotective gene haem oxygenase-1 (HO-1) has been found regulated in various forms of cancers (including AML) and implicated in chemotherapeutic drug-resistance. HO-1 protects AML cells from induced apoptosis via its transcription factor nuclear factor erythroid-derived 2-like 2 (Nrf2). We show a role for HO-1 in regulating apoptosis in AML cells in response to ara-C and DNR. HO-1 expression was increased in response to both cytotoxic agents. Upon micro RNA (miRNA) silencing of HO-1 expression, both ara-C and DNR stimulated greater apoptotic responses in these silenced AML cells. A concurrent induction in reactive oxygen species (ROS) generation was observed. Studies with ara-C-resistant AML cell lines (THP1araC(1)) showed there to be significantly suppressed levels of Nrf2 and HO-1. A miRNA screen in these resistant cells revealed reduced basal miR-196a expression. One of miR-196a's targets is the Nrf2-inhibitory protein Bach1 (BTB and CNC homology 1), which was found to be elevated in these cells. Exogenous replacement of miR-196a with the introduction of a miR-mimic, suppressed the Bach1 overexpression, elevated HO-1 expression, and reintroduced sensitivity towards ara-C. These findings suggest HO-1 inhibition in conjunction with chemotherapy would improve the number of cases who reach complete remission (CR).

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List of Abbreviations

AP-1	Activator protein-1
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ara-C	Cytarabine, cytosine arabinoside
ara-CMP	Cytarabine monophosphate
ara-CTP	Cytarabine triphosphate
ara-U	Uracil arabinoside
APL	Acute Promyelocytic Leukaemia
luminal	5-amino-2,3-dihydro-1,4pralazinedione
APS	Ammonium Persulphate
β-actin	Beta-actin
Bach1	BTB and CNC homology 1
Bcl-2	B-cell Lymphoma-2
Bcl-xl	B-cell Lymphoma-xl
BIM	Bcl2-like 11
CDA	Cytidine deaminase
CLL	Chronic Lymphoblastic Leukaemia
CML	Chronic Myeloid Leukaemia
CFC	Colony Forming Cell assay
CR	Complete Remission
dCMPDA	deoxycytidylate deaminase
DNR	Daunorubicin
dCK	Deoxycytidine kinase
dCTP	Deoxycytidine Triphosphate
DFS	Disease Free Survival
DMSO	Dimethyl Sulfoxide
dH_2O	distilled water
ddH_2O	double distilled water
dTTP	Deoxythymidine triphosphate
EGF	Epidermal growth factor
FLIP	FLICE-inhibitory protein
FBS	Foetal Bovine Serum

FLT3	*Fms*-Like Tyrosine Kinease mutation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GCL	Glutamate Cysteine Ligase
GCLC	Glutamate Cysteine Ligase, Catalytic subunit
GCLM	Glutamate Cysteine Ligase, Modifier subunit
H_2DCFDA	2',7-dichlorodihydrofluorescein diacetate
hENTI	Human Equilibrative Nucleoside Transporter 1
HL60	Human Promyelocytic Leukemia cell line
HO-1	Haem Oxygenase-1
HSC	haematopoietic stem cell
Hsp32	Heat shock protein 32
IGF-1	Insulin-like growth factor 1
IRAK1	Interleukin-1 receptor- associated kinase 1
IL-1β	Interleukin-1β
K562	Human Erythromyeloblastoid Leukaemia
Keap1	Kelch-like ECH-associated protein 1
miRNA	Micro RNA
MM	Multiple Myeloma
NAC	N-acetyl cysteine
Novidet p-40	nonylphenyloplyethylene glycol
NQO1	NAD(P)H-quinone oxidoreductase 1
Nrf2	Nuclear factor [erythroid-derived 2]-like 2
5NT	5-'nucleoidase
P-gp	P-glycoprotein
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PDCD4	Programmed Cell Death 4
PI	Propidium Iodide
PMA	Phorbol 12-myristate 13-acetate
PTEN	Phosphatase and Tensin Homologue
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
r.c.f.	Relative Centrifugal Force
RIPA	Radioimmunoprecipitation assay buffer

ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
RTP	Room Temperature / pressure
RISC	RNA- Induced Silencing Complex
SEM	Standard Error of the Mean
SDS	Sodium Dodecyl Sulphate
siRNA	Small interfering RNA
SIRT1	Sirtuin 1
THP1	Human Acute Monocytic leukaemia cell line
THP1ara-C(1)	Human Acute Monocytic leukaemia cell line resistant to cytarabine 1μM
THP1ara-C(1) TW	Human Acute Monocytic leukaemia cell line resistant to cytarabine 1μM Treatment Withdrawn
TNF	Tumour Necrosis Factor-α
TRAF6	TNF Receptor-Associated Factor 6
U967	Human Leukemic Monocyte Lymphoma cell line
UTR	Untranslated region
wt	Wild-Type
wt1	Wilms' tumour gene 1
293 FT	Primary embryonal human kidney transformed with sheared human adenovirus type 5 DNA.

Chapter 1: Introduction

1.1 Cancer Biology

Cancer affects approximately one in three people living in the United Kingdom (UK) at some point during their life time. There are approximately 200 different types, of which breast, lung, colorectal and prostate account for over half of all cases reported in the clinic [1]. It is a genetic disease which results from a number of genetic alternations leading to uncontrollable cell proliferation.

1.2 Blood cancer

There are three main general types of blood cancer, namely leukaemia, lymphoma and myeloma – each of these depends on the stage of development of the haematopoietic stem cell (HSC) at which the cancerous phenotype emerges [2]. Lymphomas are characterised by the lymphoid cancerous cell residing in the lymphatic system and lymph glands. Myelomas are characterised as cancerous white blood cells that are plasma cells in nature. These plasma cells are the active B-cells which are the antibody-producing cells found in the normal immune system. Leukaemia's are the remainder of blood cancers where the primary cancerous tumour resides in the bone marrow stem cell niche environment. Leukaemia's are divided into myeloid and lymphoid types (see Figure 1.1), of which both are further subdivided into subtypes dependent on the aggressiveness of the condition. There are acute and chronic types which indicate the indolence of the disease progression and the severity and aggressiveness of the cancerous cell type. The general symptom profile of these blood cancers is that the cancerous cell phenotype, overwhelms the normal immune system profile, and leads to gradually reduced immune system function. The inability to elicit a normal physiological immune response means that tissue damage remains unrepaired and infection becomes far more life threatening.

1.2.1 Lymphoma

Lymphomas are a large range of individual cancers that affect the lymphatic system and resultant immune responses. The lymphoid nature of the cancer cell usually resides in the lymph nodes, but can affect other parts of the body including the spleen and bone marrow. Fever, lethargy, weight loss and night sweats are common symptoms of lymphoma diseases. The major classes of lymphomas are Hodgkin's lymphoma and Non-Hodgkin's lymphoma, although follicular, mantle cell, and diffuse large B-cell lymphomas are other major types. The treatment of lymphomas has improved of late by

the introduction of the CD20 antigenic monoclonal antibody, rituximab. Lymphomas generally respond well to chemotherapies.

Pluripotent Hematopoietic Stem Cell

Myeloid Stem Cell

Lymphoid Stem Cell

Lymphocyte

Erythrocyte   Neutrophil   Monocyte   Esoinophil   Basophil   Megakaryocyte

Platelet
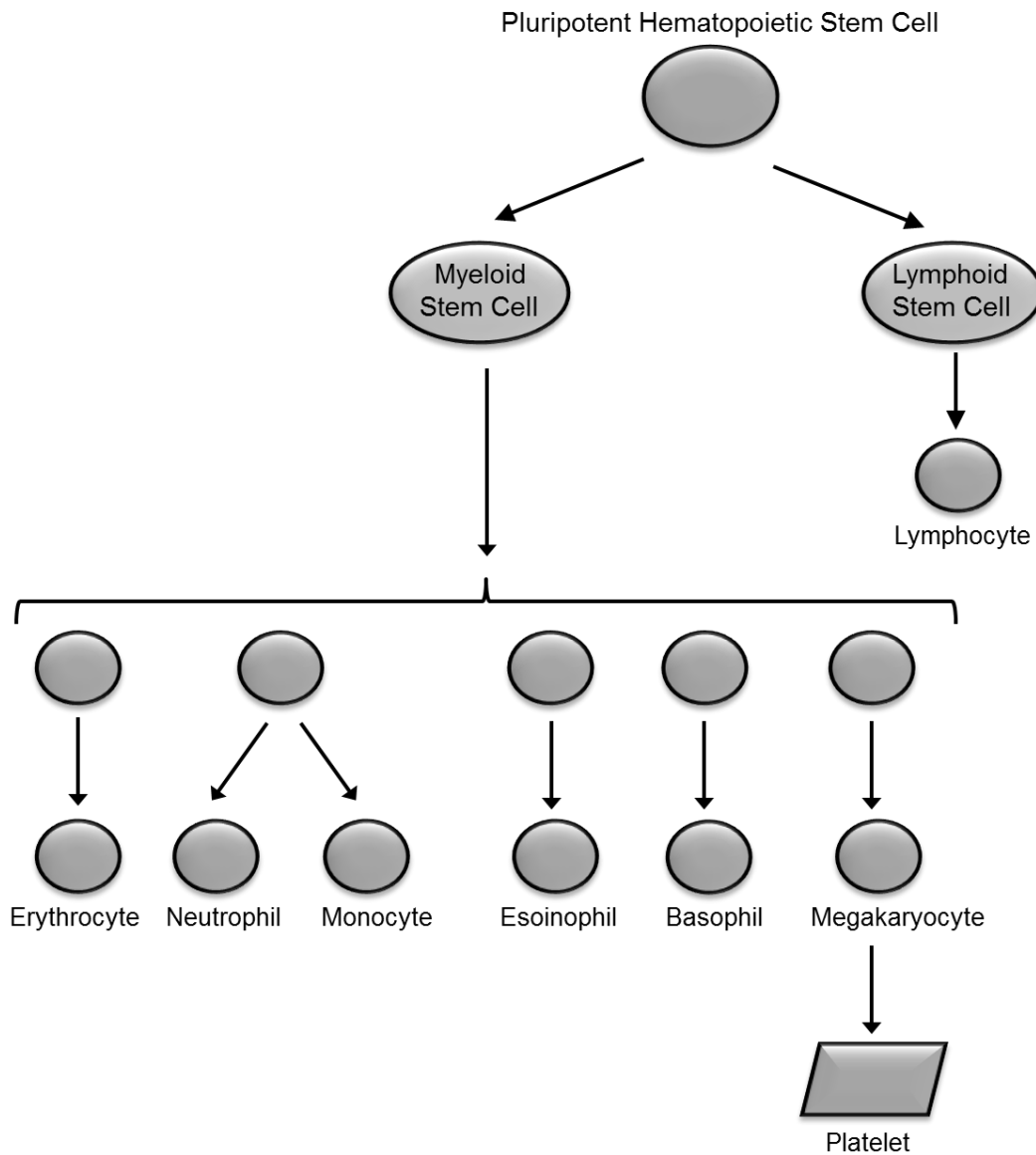
Figure 1.1. Production of blood cells by the bone marrow. The appearance of each precursor is the same for simplicity. The undifferentiated Pluripotent Hematopoietic Stem Cells are able to give rise to precursors of each of the blood cells. The first division results in either Lymphoid or Myeloid Stem Cells. Which become further differentiated upon division. Diagram adapted from Widmaier et al (2006) [3].

1.2.2 Myeloma

Myeloma starts in the bone marrow, but ultimately affects the plasma B-cells responsible for antibody generation. The main types of myeloma are plasmacytoma, localised myeloma and extramedullary myeloma, however over 90% of all myeloma cases are of multiple myeloma (MM), meaning that it affects more than one location in the body.

1.2.2.1 Multiple Myeloma

Multiple Myeloma (MM) is a B-cell malignancy which is the second biggest blood cancer accounting for 1% of all cancers diagnosed. Currently there is no treatment that can cure MM. It is characterised by the presence of excess monotypic plasma cells in the bone marrow and excess monoclonal protein in the patients' serum or urine. Even with high dose chemotherapy, MM remains relatively incurable due to acquired drug resistance [4]. When high dose chemotherapy or stem cell transplantation is deemed inappropriate, an immunomdulating drug such as thalidomide or its newer analogue lenalidomide, in combination with an alkylating agent for example melphalan and a corticosteroid usually dexamethasone (which suppress the corticotrophin secretions [5] are first-line treatment options. If thalidomide is not appropriate then the proteasome inhibitor bortezomib can be used instead [6].

1.2.3 Leukaemia

Leukaemia is a term used to encompasses both cancers of the bone marrow and blood which can be characterised by an increase in leukocyte cell proliferation and failed pro-apoptotic responses to abnormal cells [7]. It is then clinically divided into 'acute' and 'chronic'. Further sub division is dependent on the hematopoietic lineage affected usually myeloid or lymphoblastic [8]. The four key types of leukaemia are Chronic Lymphoblastic Leukaemia, Acute Lymphoblastic Leukaemia, Chronic Myeloid Leukaemia and Acute Myeloid Leukaemia.

1.2.3.1 Chronic Lymphoblastic Leukaemia

Chronic Lymphoblastic Leukaemia (CLL) is the most common type of leukaemia although it is highly indolent and often sufferers remain without severe symptoms for many years. Many of those with CLL will be monitored without treatment. However, if the disease progresses, first-line treatment consists of rituximab in combination with both fludarabine and cyclophosphamide if deemed appropriate. Rituximab a chimeric

monoclonal antibody binds to the CD20 antigen expressed on the cell surface of mature B lymphocytes and tumour cells [9]. Rituximab is also used in relapsed or refractory CLL [10].

1.2.3.2. Acute Lymphoblastic Leukaemia

Acute Lymphoblastic Leukaemia (ALL) is the most common form of cancer found in children. The majority of people with ALL are juveniles and as such, can tolerate high dose chemotherapeutic regimes that lead to good disease eradication. Many of the most dangerous types of childhood cancer cases are of AML type origin or of mixed lineage. Adults with ALL fair worse than juveniles with ALL, purely due to a juvenile's ability to withstand very toxic high dose treatments.

1.2.3.3 Chronic Myeloid Leukaemia

95% of Chronic Myeloid Leukaemia (CML) cases are characterised by the presence of the BCR-ABL fusion gene which is due to a reciprocal translocation between chromosomes 9 and 22, producing the cytogenetically shortened chromosome 22 also known as the Philadelphia chromosome [11]. The Philadelphia chromosome is present in 95% of CML patients, a third of ALL patients and occasionally present in AML patients [12]. The resultant BCR-ABL protein possess increased tyrosine kinase activity which results in either increased proliferation or decreased apoptosis of haematopoietic stem cells or progenitor cells which eventually results in increased myeloid cells, premature release of immature myeloid cells and genetic instability which results in CML disease progression. The Tyrosine Kinase Inhibitor (TKI) imatinib, inhibits the BCR-ABL tyrosine kinase. Imatinib has significantly improved long term survival in CML patients and is used as a first line treatment for chronic phase Philadelphia-chromosome positive CML or an option for patients presenting with either accelerated or blast phase CML [13]. However a number of patients have developed resistant to imatinib thus paving the way for more potent TKI development [14]. Even though the mechanisms of action behind imatinib resistance are not fully understood, 66% of imatinib-resistant patients possess mutated BCR-ABL sequences [15]. The BCR-ABL Philadelphia chromosome is composed of an ATP-binding P-loop and another larger activating loop. Two thirds of imatinib resistant patients possess mutations in the ATP binding portion of the BCR-ABL Philadelphia chromosome [15]. Treatment options include an allogeneic haematopoietic

stem cell transplant [16] or a secondary generation TKI for example dasatinib or nilotinib depending on the mutation present [14].
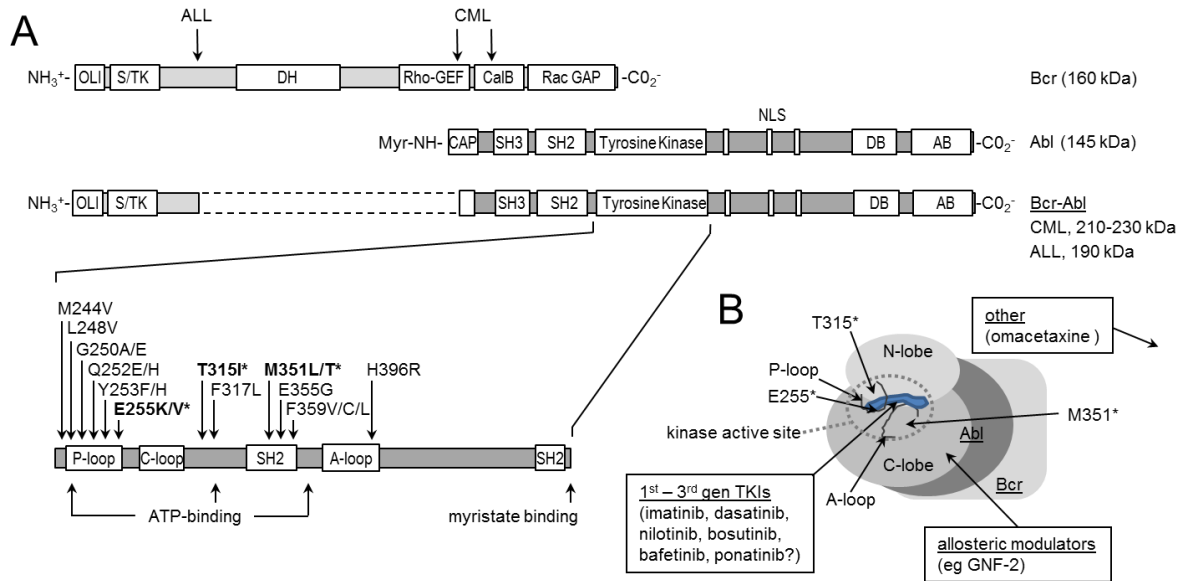
Figure 1.2. Philadelphia chromosome (Bcr-Abl). (A) Diagrammatic representation of the domains of BCR-ABL including the domains: OLI (oligomerization); S/TK (serine/threonine kinase); CAP (myristoylated (Myr) site found in normal ABL that binds the kinase domain to confer auto-inhibition – this region is truncated and replaced by BCR in the Philadelphia chromosome); SH2 (phosphotyrosine-binding); SH3 (Pro-X-X-Pro –binding motif for protein-protein interactions); DNA- and actin-binding motifs (DB and AB respectively) and nuclear localization sequences (NLS). Expanded region shows the P-, C- and A (activation)-loops and SH2-binding motifs. Indicated by the arrows are the commonly found mutations of BCR-ABL identified in the clinic; the most common of which are highlighted in bold (*) and their position is also indicated on the 3-dimensional representation (B). This diagram also shows the kinase active site and the molecular sites on BCR-ABL that are targeted by CML treatments.

1.2.3.4 Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) accounts for approximately 1% of all cancer deaths, it is a malignancy of the haematopoietic progenitor cells [7]. AML incidence is increasing with age, particularly with an aging population therefore the number of new cases is expected to increase. The median age at diagnosis is 66 years [17]. Risk factors for AML

include exposure to ionising radiation, benzene and cytotoxic chemotherapy. Between 10 and 15 % of AML patients have previously received cytotoxic chemotherapy [7]. However less than 10% of patients over 60 are expected to survive the intense treatment regime.

An induction regime aims to reduce the tumour burden thus allowing the patient to reach Complete Remission (CR) [18]. CR is defined as less than 5% leukemic blast cells in the patient's bone marrow and normalisation of peripheral blood counts [18]. Currently the standard induction treatment for younger fitter patients consists of the antimetabolite cytarabine (ara-C; 100mg/m^2) administered via intravenous infusion over seven days plus the anthracycline antibiotic daunorubicin (DNR; 45-60mg/m^2/d) administered intravenously for three days [5, 19, 20].

Patients who go into remission would normally receive consolidation therapy with either high dose ara-C or an allogeneic stem cell transplant dependant on their clinicopathological characteristics. Despite these intensive treatment strategies, a significant number of patients relapse and only approximately 50% of younger fitter patients can be cured. Patient treatment outcomes are dependent on a variety of clinical and biological factors including cytogenetics, age and drug-resistance [19, 21-23].

Table 1.1: Treatment strategies used in AML treatment. Table adapted from Tallman et al (2005) [19]

Stage		Treatment Options
Induction		• Ara-C 100mg/m^2 infused over 7 days + • DNR 45-60mg/m^2 daily for three days
Remission	Consolidation Therapy	• High dose ara-C • Allogeneic stem cell transplant
Relapsed or Refractory AML		• Chemotherapy • Allogeneic transplantation • Autologous transplantation • Palliative care

Palliative care is provided when advanced progressive disease is present. It encompasses management of a patients pain and any other symptoms which they may be experiencing for example constipation, depression, xerostomia or nausea and vomiting and also providing psychological, social and spiritual support [24].

1.2.3.4.1 Acute Promyelocytic Leukaemia

The AML subtype, Acute Promyelocytic leukaemia (APL) is at current the most curable form of AML. It is identified by a t(15;17) translocation causing a fusion transcript, known as the promyelocytic leukaemia retinoic acid receptor α (PML-RARX). Current treatment for APL consists of targeting the above genetic mutation using all-*trans* retinoic acid [19, 25, 26].

1.3.1 AML Treatments

AML 17 trial sponsored by Cardiff University is suitable for the inclusion of AML and high risk Myelodysplastic syndrome patients between the ages of 18 and 60 years. Patients over the age of 60 can also be included if intensive therapy is deemed appropriate. AML 17 is a randomised, controlled, open label phase III trial. There are two distinct sections; (A) Patients with AML or high risk myelodysplasia and (B) Patients with APL. The trial aims to explore (A) Five different induction chemotherapy schedules (B) CEP-701 a FLT3 inhibitor and its benefit in treating patients with *Fms*-Like Tyrosine Kinease (FLT3) activating mutation (C) mTOR inhibition in patients negative for either the FLT3 mutation or Core Binding Leukaemia (CBF) And high risk patients (D) Comparing the number of treatment course (three verse four) and (E) High risk patients (1) benefit of DNR/ Clofarabine versus standard FLAG-ida; composed of idarubicin, fludarabine, ara-C and granulocyte-colony stimulating factor (2) allogeneic stem cell transplantation [27].

There are two type of FLT3 activating mutation present which have been identified. CBF leukaemia possess either a t(8;21) or inv(16)/t(16;16) balance chromosomal re-arrangement which results in the production of a fusion transcript either called AML1-ETO or CBFβ-MYHII [27].

1.3.1.1 Cytarabine

Cytarabine (ara-C) its chemical name 1-β-$_D$-arabinofuranosylcytosine and also known as cytosine arabinoside, cytarabine hydrochloride and arabinofuranosyl. It is usually administrated alongside the anthracycline antibiotic, daunorubicin (DNR) in AML treatment. It is administered to the patient either subcutaneously, intravenously or intrathecally. As with any drug, cytarabine has a number of associated side effects which are also common to other chemotherapeutic agents. These are oral mucositis, tumour lysis syndrome, hyperuricaemia, local tissue necrosis if ara-C leaks into the extravascular compartment whilst being administered, nausea and vomiting, alopecia, bone marrow suppression and thromboembolism [5].

After rapid IV injection ara-C plasma concentration declines in a biphasic manner. During the initial phase its half-life is 10 mins and then increases to between 1-3 h in the terminal phase. However, when ara-C is administered intrathecally its half-life is increased to approximately 2 h. It is predominately excreted by the liver and to a lesser extent the kidneys, gastro intestinal mucosa and granulocytes where it is converted into ara-U and eventually excreted in the urine of which 10% remains unchanged. Between 70 and 80% of the original ara-C dose is excreted within 24 hours of administration [28, 29].

Ara-C is a antimetabolite analogue of cytidine, which possess an arabinose sugar moiety. Allowing it to compete with cytidine, for incorporation into DNA. Due to the sterical hindrance from the arabinose sugar, DNA replication is inhibited during S phase [30] thus it is known as a S phase specific drug,[31] which eventually leads to chromatid breakage [32]. Ara-C is phosphorylated into ara-C triphosphate (ara-CTP) its active metabolite, by deoxycytidine kinase (dCK) [33]. Which goes onto to inhibit DNA replication [34] by inhibiting cytidine disphosphate [35]. ara-C monophosphate (ara-CMP) is further phosphorylated by pyrimidine nucleoside monophosphate into ara-CDP and then subsequently phosphorylated into ara-CTP by diphosphate kinases [36]. ara-CTP is metabolised into its inactive form, uracil arabinoside (ara-U) by cytidine deaminase (CDA) [37, 38].
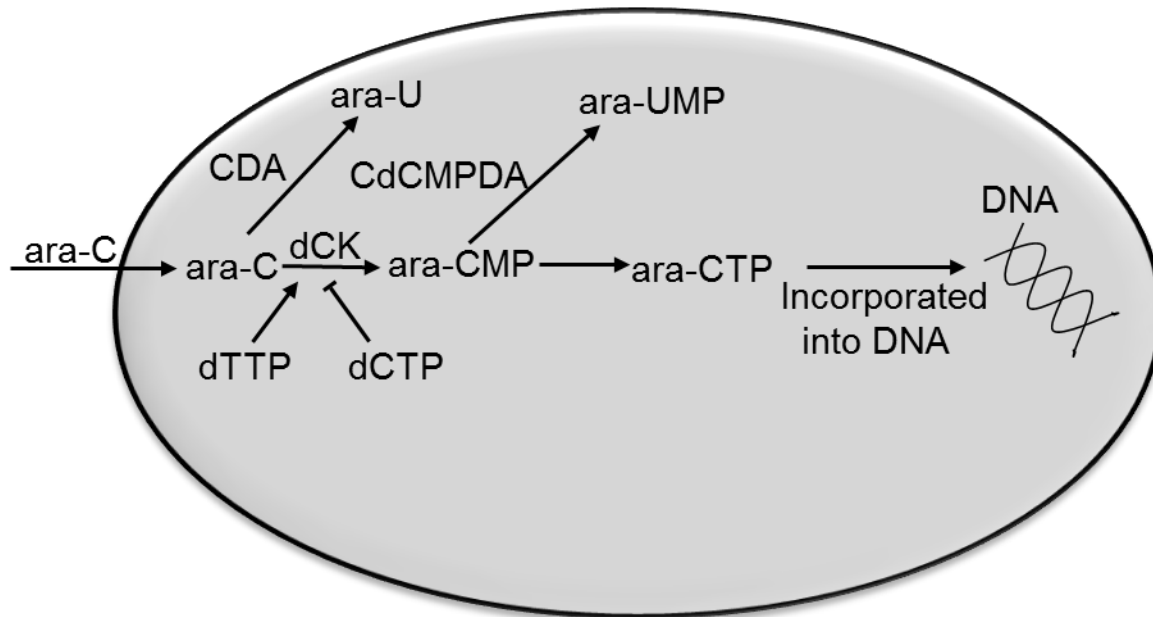
Figure 1.3. Cytarabine (ara-C) metabolism. Initally ara-C is transported into the cell, dCK phosphorylates ara-C into ara-CMP. dCK is controlled by a negative feed back mechanism involing dTTP and dCTP. Ara-CMP is subsequently phosphorylated into ara-CTP and then incorprated into the cellular DNA. Ara-C is also deaminated by CDA into ara-U, whilst ara-CMP is deaminated by dCMPDA into ara-UMP. Diagram adapted from Y. Ge. et al (2004) [39]. Ara-CMP = cytarabine monophosphate, ara-CTP = cyarabine-triphosphate, ara-U = uracil arabinoside, CDA = cytidine deaminase, dCK = deoxycytidine kinase, dTTP = deoxythymidine triphosphate, dCTP = deoxycytidine triphosphae and CdCMPDA = deoxycytidylate deaminase.

1.3.1.2 Ara-C Resistance

A number of mechanisms have been proposed to contribute towards drug resistance in AML patients. Which include the cell failing to undergo apoptosis in response to the chemotherapy agent, drugs failing to reach their intracellular targets or being removed by the ABC membrane transport protein, P-glycoprotein (Pgp). Pgp is an efflux transporter, present within the cells plasma membrane, its expression in AML patients has been reported to be relatively low, however an increase in its expression after drug treatment and during relapse have been noted [40]. Galmarini et al have shown that high levels of 5'-nucleotidase (5NT) [41], which is involved in DNA repair and membrane

transport, is related to the poor prognosis and patient outcomes in AML patients. Both expression and enzyme activity of 5NT has been shown to be higher in cytarabine resistant cells compared to that of non-resistant cells. Human equilibrative nucleoside transporter 1 (hENTI) expression was found to be widely expressed by patients suffering from AML at the point of diagnosis. hENTI is also known to be the main transporter of ara-C. Suggesting 5NT expression and hENTI deficiency could be involved in ara-C resistance in AML patients [42]. These studies suggest that AML cells have evolved to regulate pathways that provide protection against toxic chemotherapeutic agents.

A number of ara-C, DNR and Bortezomib resistant cells lines have been established over the last 10 years by various groups [43]. Funato developed a ara-C resistant K562 (K562/AC) cell line by increasing ara-C doses over a 3 month period to examine the differences between them and ara-C sensitive K562 cells [44].

1.3.1.3 Daunorubicin

Daunorubicin (DNR) part of the anthracycline antibiotics family and is indicated in the treatment of acute leukaemia and is administered via intravenous infusion. It is also licensed for use in AIDs-related Kaposi's sarcoma. Side effects include bone marrow suppression, hyperuricaemia, alopecia, extravasation of intravenous drugs, oral mucositis, tumour lysis syndrome and nausea and vomiting [5]. DNR functions by forming a complex with cellular DNA and thus intercalating between the base pairs which allows it to inhibit Topoisomerase II by stabilising the complex between the enzyme and DNA. Which results in both single and double DNA stranded breaks [45].

1.3.1.4 Etoposide

Etoposide an anti-neoplastic, is indicated for the treatment of small cell carcinoma of the bronchus, lymphomas and testicular cancer; it is either administered orally or via slow intravenous infusion. The same side effects as for DNR can be expected [5].

1.3.1.5 Amsacrine

Amsacrine is occasionally used to treat AML, it is administered via intravenous infusion, patients could experience myelosuppression along with the side effects listed for DNR [5].

1.3.1.6 Proteasome Inhibitors

Bortezomib is a proteasome inhibitor licensed to treat multiple myeloma which has progressed even though it has been treated with at least one other chemotherapeutic agent, the patient has already had or is unable tolerate a bone marrow transplant [5]. We have previously shown HO-1 up-regulation in response to bortezomib and lenalidomide exposure in MM cell lines. HO-1 expression was also explored in the lenalidomide resistant MM cell line and shown to be up regulated [46]. Bortezomib use in AML treatment has revealed disappointing results. We have previously shown AML cell lines are less sensitive to bortezomib than their control non-cancerous cells. [47]

1.3.1.7 Mitoxantrone

Mitoxantrone an anthracycline derivative is licensed for use to treat metastatic Breast cancer, Non-Hodgkin's Lymphoma, Adult Non-Lymphocytic Leukaemia and Non-Resectable Primary Hepatocellular Carcinoma treatment. It is administered intravenously however both myelosuppression and dose-related cardio toxicity can occur [5].

1.4 World Health Organisation (WHO)

WHO AML classification of AML subtypes supersedes the previous classification based around the French-American-British (FAB) system based on HSC differentiation status. The above classification takes into account genetics, both biological and clinical features as well as morphological results during AML subtype diagnosis.

Table 1.2 WHO Classifications of acute leukaemia's. Adopted from Vardiman et al (2009) and Dohner et al (2010) [22, 48].

Categories
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22)or t(16;16)(p13.1;q22); CBFB-MYH11
AML with t(15;17)(q22;q12); PML-RARA
AML with t(9;11)(p22;q23);MLLT3-MLL
AML with t(6:9)(p23;q34); DEK-NUP214
AML with inv(3) (q21q26.2) or t(3,3)(q21;q26.2);RPNI-EVI1
AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
Provisional etity: AML with mutated NPMI
Provisional entity: AML with mutated CEBPA
AML with myelodysplasia- related changes
Therapy- related myeloid neoplasm
AML, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/ monocytic leukemia
Acute erythroid leukaemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma (syn; extramedullary myeloid tumor; granulocytic sarcoma; chloroma)
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemia of ambiguous lineage
Acute undifferentiated leukaemia
Mixed phenotype acute leukaemia with t99;2209q34;q11.2);BCR-ABL1
Mixed phenotype acute leukaemia with t(v;11q23);MLL rearranged
Mixed phenotype acute leukaemia, B/myeloid, not otherwise specified
Mixed phenotype acute leukaemia, T/myeloid, not otherwise specified

1.5 Cytoprotection

Cellular cytoprotection and resistance towards chemotherapeutics is governed by a number of transcription factors; Bach1, NF-κB and Nrf2 and their genes; HO-1, NQO1, GCLM and GCLC of which, each will be discussed in detail during the course of this chapter.

1.5.1 NF-κB

The mammalian NF-κB family is composed of 5 members, class 1 proteins NF-κB1 (p105 → p50), NF-κB2 (p100 → p52), class II proteins RelA (p65), RelB and c-Rel [49]. Its activation is governed by a number of stimuli including pro-inflammatory cytokines; tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and epidermal growth factor (EGF) [50, 51]. NF-κB signalling occurs via two main pathways the Classical and Alternative pathway [52, 53]. In non-stimulated cells, NF-κB remains inactivate within the cytoplasm, upon activation NF-κB subunits move into the nucleus.

NF-κB represents a major anti-apoptotic pathway that must be overcome if cancer cell cytotoxicity is to be achieved. Many cytotoxic agents have been shown to inhibit NF-κB pathways as part of their cell killing mechanisms. The relationship between NF-κB and Nrf2 pathways was recently shown by our group to be pivotal to understanding better cytotoxic efficacy in cancerous versus non-cancerous cells [54-56].

We have previously shown under NF-κB inhibition and TNF treatment, HO-1 regulation was increased in AML cell lines but not primary white blood cells. Increased HO-1 regulation conferred protection towards cell death in AML cell lines whilst the primary white blood cells underwent cell death. Next, HO-1 expression was blocked using HO-1 targeted siRNA, the cells were yet again exposed to both TNF treatment and NF-κB inhibition and increased cell death was seen. ROS generation within the AML cell lines was also shown to be up-regulated in response to TNF treatment and NF-κB inhibition. Which induced Nrf2 activation resulting in increased HO-1 regulation thus providing cellular resistance to TNF-induced cell death [57]. Moreover, HO-1 appears to be supressed by NF-κB activation, of which NF-κB subunits p50 and NF-κB1 an p65 subunit appear to be responsible for HO-1 suppression in AML cell lines [49].
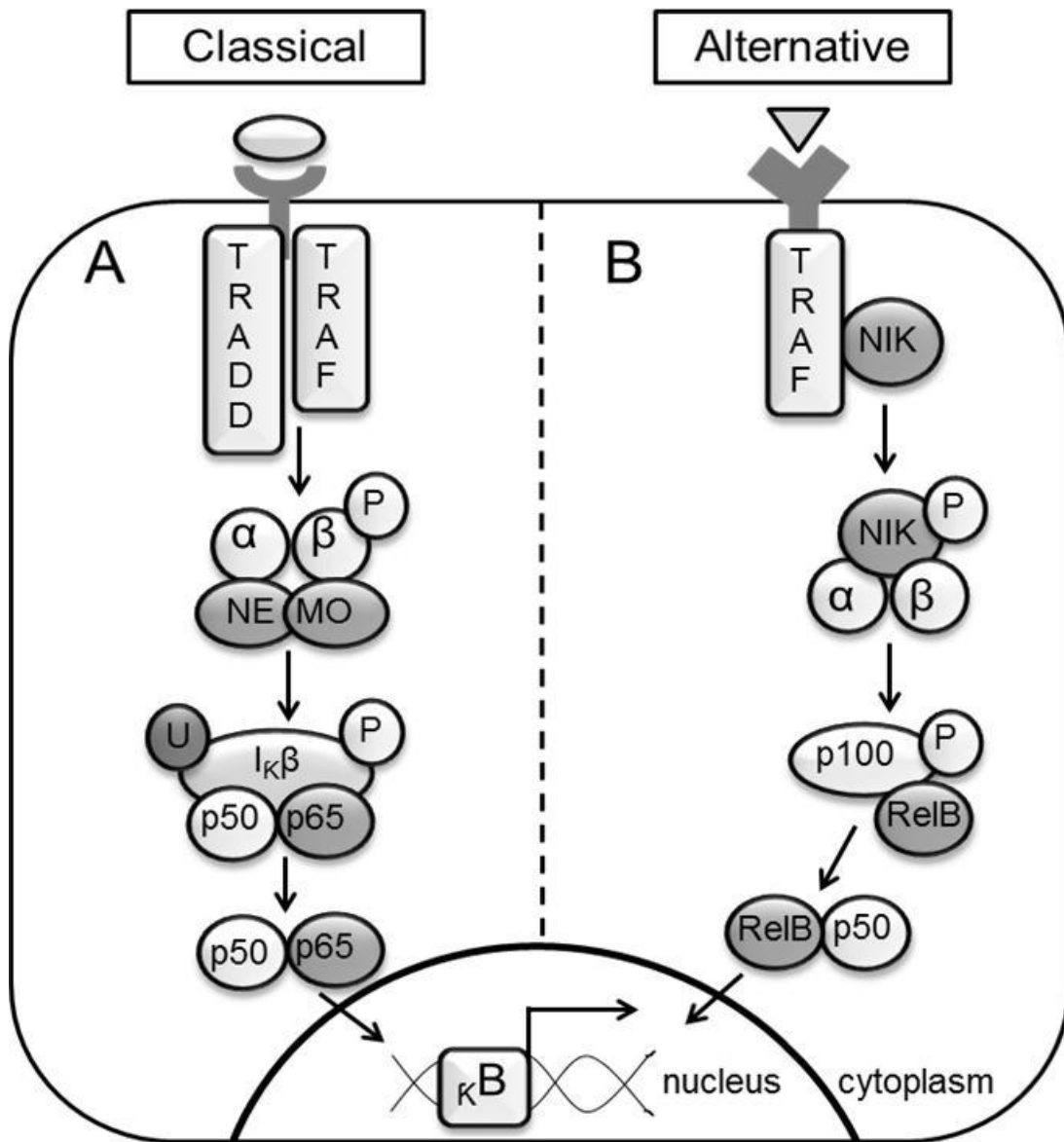
Figure 1.4. The classical and Alternative NF-κB pathways utilised during cytoprotective responses within all cells. Figure adapted from [58].

1.5.2 Nuclear factor erythroid-derived 2 related factor 2

The Cap 'N' Collar transcription factor family member, Nuclear factor erythroid-derived 2 related factor 2 (Nrf2) is localised in the cytoplasm and bound to Keap1 in its inactive state [59]. In response to either oxidative stress or electrophilic stress, Nrf2 is released from Keap1 and translocates into the nucleus where it heterodimerizes with a b-zip partner and then goes on to bind to the anti-oxidant response element (ARE) within its target genes promoters [60] [59]. Nrf2 is responsible for the expression of phase II detoxifying and oxidative stress enzymes for example HO-1, NQO1 and GCLM.

1.5.3 Kelch-like ECH-Associated Protein 1

Kelch-like ECH-Associated Protein 1 (Keap1) a member of the Kelch family, which possess Kelch repeats at the C terminus and an BTB/POZ domain near the N terminus [61]. Keap1 consists of two structural domains; the DGR moiety also known as the Kelch motif and the BTB protein interaction domain. It is localised within the cytoplasm. It binds to the amino-terminal domain on Nrf2 forming an interaction between 'Neh-DGR' which represses Nrf2 transcriptional activity [60]. Both the Neh2-DGR interaction and Keap1's cellular location lead Itoh et al to suggest Keap1 is involved in bridging Nrf2 with the cells actin cytoskeleton [60]. There is inhibitory relationship between Keap1 and Nrf2 as well as Bach1 and Nrf2 (see Figure 1.5). In the cytoplasm, Keap1 binds Nrf2 and keeps it inactive. Upon oxidative stress of the cell, Keap1 dissociates from Nrf2 where it translocates to the nucleus, where it can replace Bach1 at the anti-oxidant response elements (ARE) for cytoprotective genes. Nrf2 is now free to act as the transcription factor at the ARE to elicit gene expression of many different cytoprotective proteins.
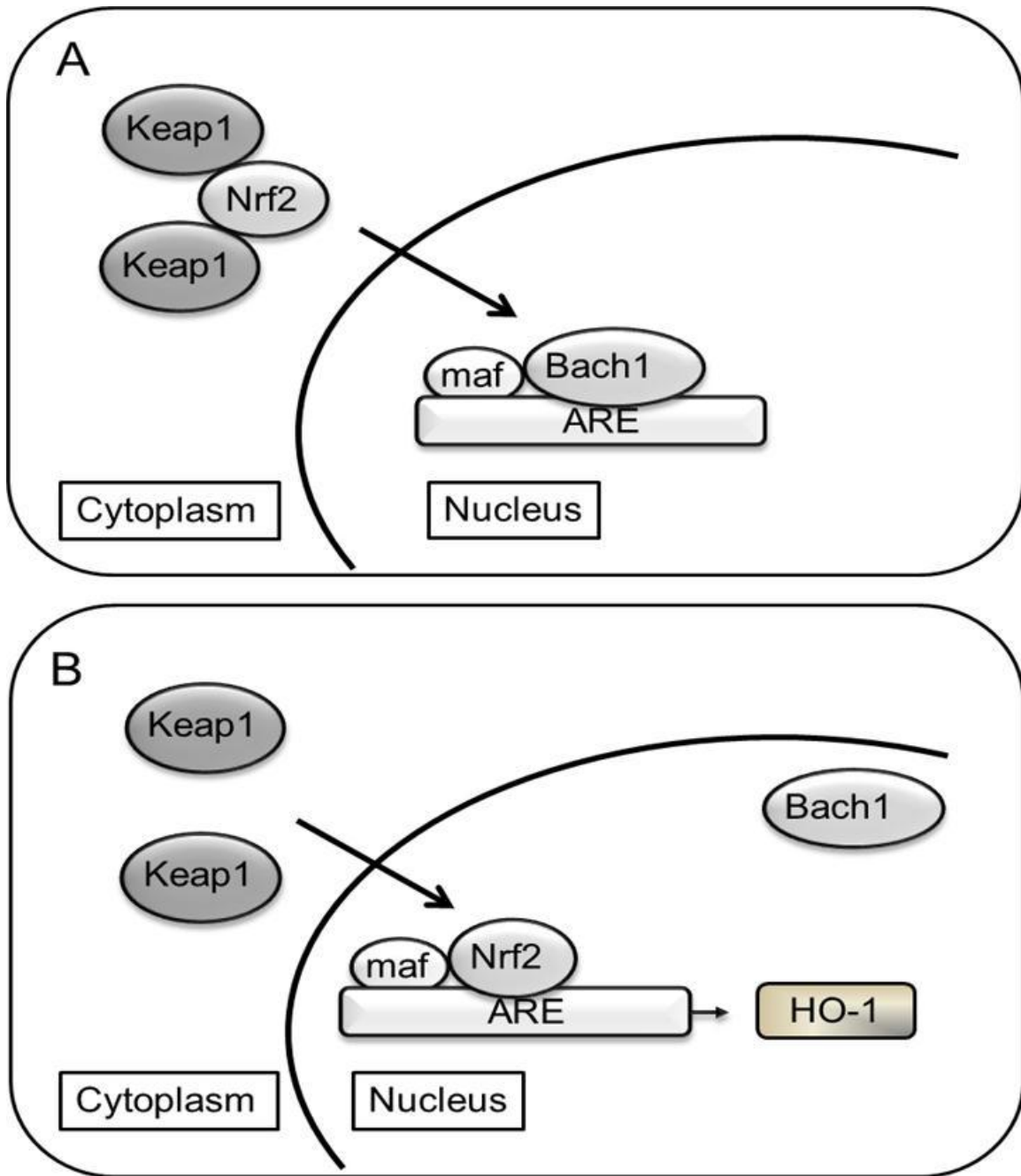
Figure 1.5. Keap1 and Nrf2s relationship. (A) Inactive Nrf2 is bound to Keap1 within the cytoplasm. In response to either oxidative or electrophilic stress, Nrf2 is activated and released by Keap1 where it translocates into the nucleus. (B) Nrf2 binds to the ARE complex resulting in phase II detoxifying and oxidative stress enzyme expression. Diagram adapted from [62].

1.5.4 Haem Oxygenase -1

Haem Oxygenase-1 (HO-1) is encoded by the HMOX1 gene, its expression is induced in response to oxidative stress [63]. To date, three isoforms of haem oxygenase (HO-1) have been identified, HO-1, HO-2 and HO-3 [64]. HO-2 is encoded for by HMOX2, its expression is constitutive [63]. Haem is a protoporphyrin IX ring which contains a Fe^{2+} atom. HO-1 provides protection against cellular stress, regulates cellular haem levels and converts intracellular haem into carbon monoxide, free iron and biliverdin [64]. Biliverdin is further reduced into the potent antioxidant bilirubin [65, 66] by biliverdin reductase [67]. Depletion of the cyto-protectant bilirubin by RNA interference towards biliverdin reductase, has been shown to increase ROS levels in tissues and thus causes cellular apoptosis. However, bilirubin is oxidised back into biliverdin, via a 'redox cycle' and can be reduced back into bilirubin via biliverdin reductase, thus explaining how bilirubin can act as an antioxidant [66]. However, decreased glutathione levels, results in a reduced increase in ROS and cellular apoptosis [66]. Florczyk et al (2011) explored the relationship between both HO-1 and biliverdin reductase in genetically modified NIH 3T3 mouse fibroblasts. They demonstrated the over expression of biliverdin reductase resulted in increased HO-1 expression which conferred protection against chemotherapeutics; cisplatin and doxorubicin [68].

HO-1 possesses the following cytoprotective properties anti-inflammatory, anti-oxidative and anti-apoptosis [69, 70]. HO-1 belongs to the heat shock protein family (Hsp-32), its expression is triggered by a variety of stress inducing stimuli including UV radiation, hyperthermia, inflammatory cytokines, bacterial endotoxins and heavy metals [71-75]. HO-1 regulation is under the control of signalling components [76, 77] and a number of transcription factors such as nuclear factor-κB (NF-κB), NF-E2-related factor 2 (Nrf2) and activator protein-1 (AP-1) [78-80].
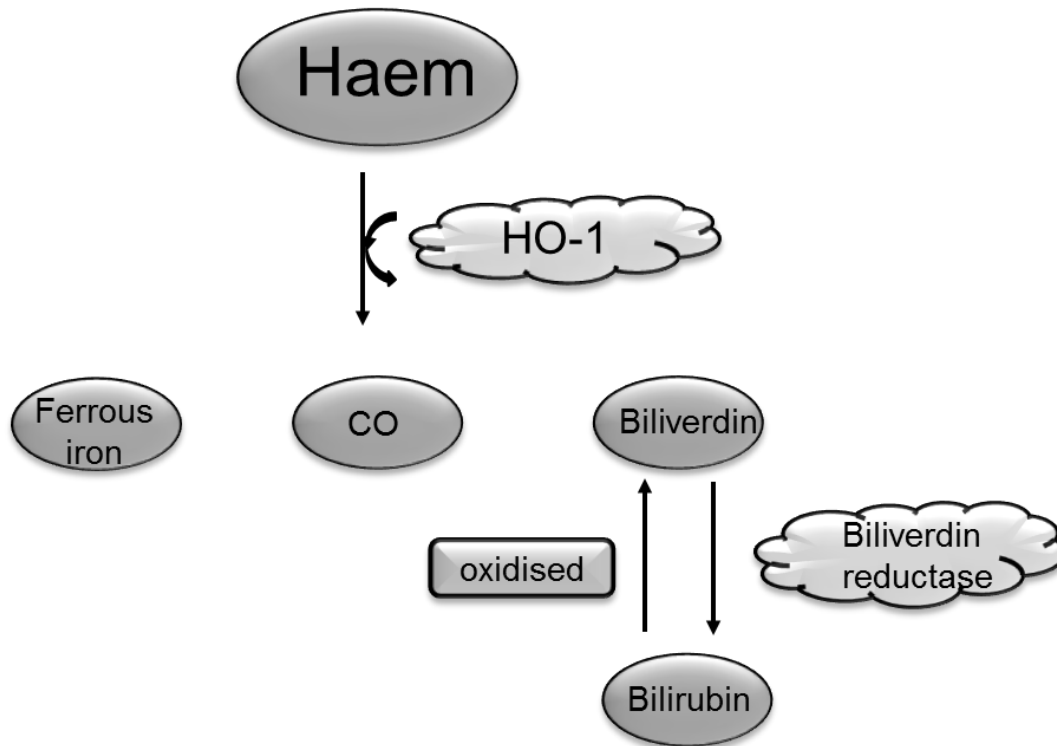
Figure 1.6. Haem is converted into ferrous iron, CO and biliverdin. Heam is cleaved by HO-1 into three products; ferrous iron, CO and biliverdin. Biliverdin is further coverted into bilirubiin by biliverdin reductase, which can be oxidised back into biliverdin.

1.5.5 NAD(P)H:quinine oxidoreductase-1

NAD(P)H:quinine oxidoreductase-1 (NQO1) is an FAD-binding protein that is involved in the electron transfer chain, and is a cytoplasmic electron reductase enzyme. NQO1 mutations have been implicated in breast cancers, as well as in Alzheimer's disease and in models of hepatotoxicity.

1.5.6 Glutathione Synthesis

The antioxidant, Glutathione (GSH) a non-protein thiol is involved in cellular protection against oxidative stress. Glutathione synthesis is governed by the availability of cysteine, the sulphur amino acid precursor and Glutamate Cysteine Ligase, the rate limiting enzyme. Glutamate Cysteine Ligase (GCL) is composed of two subunits; Glutamate Cysteine Ligase Catalytic (GCLC) and Glutamate Cysteine Ligase Modifier (GCLM) subunit [75].

1.6 MicroRNA

During the last decade the involvement of microRNAs (miRNAs) within cancer diagnosis, progression and treatment has started to be extensively researched. They have been shown to regulate a number of transcription factors and in turn control cellular cytoprotective gene expression. Possibly indicating their potential as future therapeutic targets or biomarkers in AML treatment and diagnosis. Therefore we have decided to explore miRNAs expression in response to chemotherapeutics exposure in AML treatment.

1.6.1 miRNA Biogenesis

miRNAs are small pieces of non-coding conserved RNA usually between 18 and 25 nucleotides in length thus act as expression regulators of genes involved in development, differentiation, proliferation, survival and cell death [81]. miRNA biogenesis occurs in the nucleus, where long primary miRNA (pri-miRNA) are transcribed from non-coding/ non- messenger DNA by RNA polymerase II [82]. These transcripts are then cleaved by RNA polymerase III (drosha) producing the precursor miRNA (pre-miRNA) which is approximately 70 nucleotides long [83]. This is subsequently transported via Exportin 5 (Nuclear export factor) out of the nucleus and into the cytoplasm. Whilst in the cytoplasm another ribonuclease, Dicer complexed with a double stranded RNA binding protein (TRBP) [84] processes the pre- miRNA into a mature double stranded miRNA of approximately 22 nucleotides in length [85]. The duplex then separates into two separate strands, the passenger and the functional strand. The functional strand is incorporated into the RNA-induced silencing complex (RISC). Once incorporated into the RISC complex the functional strand is unwound and involved in target recognition (3'UTR) and incorporating specific target mRNAs into the RISC complex. Whilst the passenger strand, is degraded. [84, 86, 87]. It is thought that miRNAs control gene expression by binding to the 3'-untranslated region (3'-UTR) of the targeted mRNA. RISC is guided towards the target mRNA via the functional strand. The mechanism of silencing the target mRNA expression employed is dependent on the degree of complementarily dictated by the functional strand for the target mRNA. The mRNA will be cleaved if full complementarily for the target mRNA is present if not then translation, expression or alterations to the target mRNA will take place.
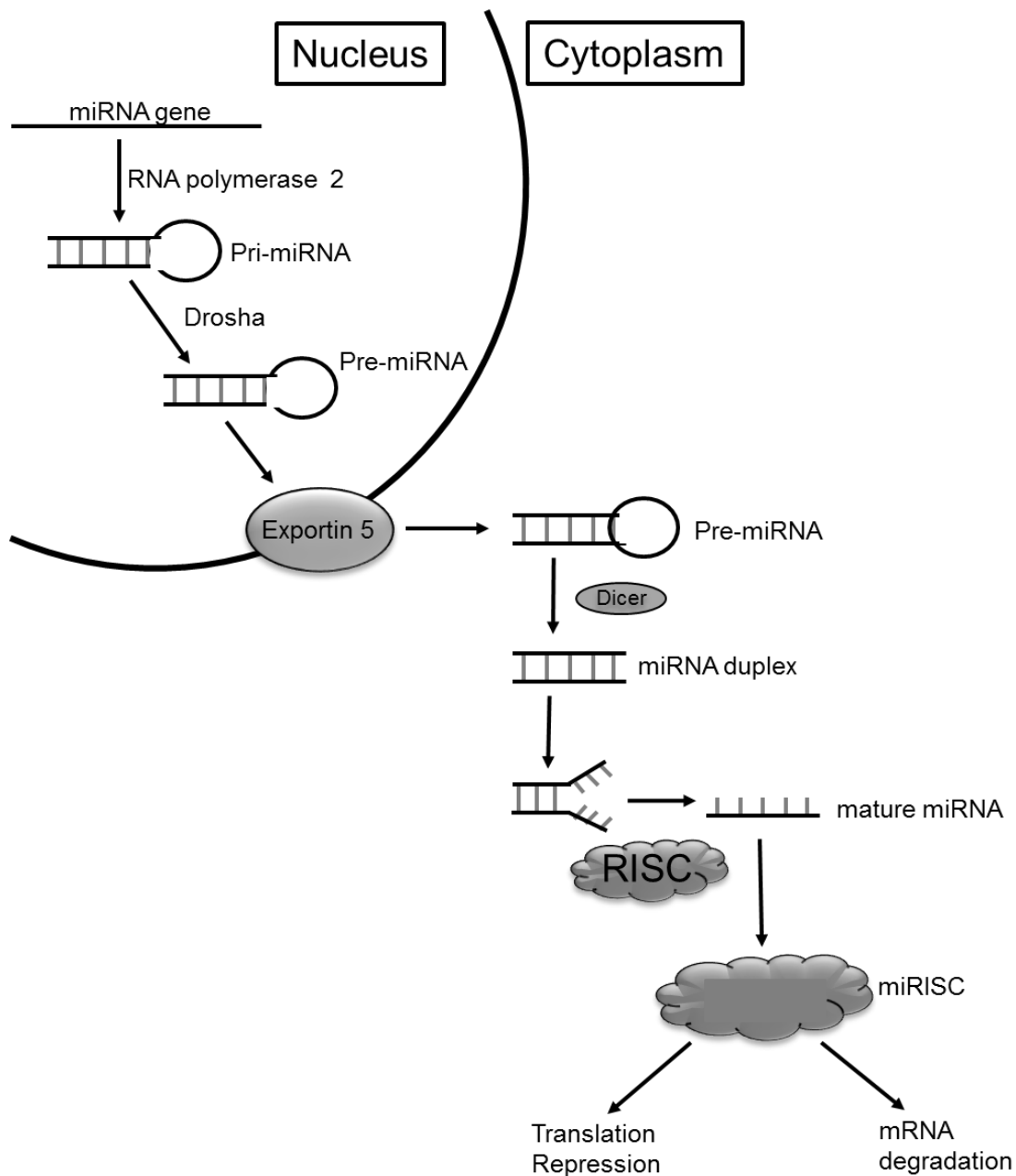
Figure 1.7. miRNA Biogenesis. Initially occurs in the nucleus, RNA Polymerase 11 transcribes miRNAs into pri-miRNA, long primary miRNA transcripts. RNA Polymerase 111 also known as Drosha, cleaves Pri-miRNA into pre-miRNA which is transported into the cytoplasm via exportin 5. Pre-miRNA is further processed by Dicer, another RNase enzyme into a miRNA duplex, of 19-25 nucleotides in length. The miRNA duplex separates, the mature strand is incorporated in to RNA-induced silencing complex (RISC). Diagram adapted from R. Garzon. 2010 [88] and R. Lima et al. 2011 [89].

1.6.2 miRNAs can target a number of genes and proteins

It is thought that one miRNA can target and block a number of target mRNAs being transcribed. One mRNA can also be targeted by a number of miRNAs, for example PTEN which is mediated by both miR-21 and 222.

1.6.3 miRNAs involvement in leukaemia progression

A number of studies have shown that miRNAs are involved in cancer generation. Mi et al discovered that miRNA can be used to differentiate ALL from AML by looking at 27 miRNAs which were either over or under expressed in the opposite condition. miRNAs can act as either oncogenes or tumour suppressor genes. The following miRNAs might possess oncogenic activity as they have been shown to be up-regulated in tumour cells. These are miR-21, miR-155, miR-221, miR-222 and mir-17-92 [90].

1.6.4 miRNAs, cell proliferation and chemotherapy- induced resistance

More recently, attention has turned to the role that miRNAs play not only in the progression of AML disease, but also in the development of a chemotherapy-resistance phenotype. Cell lines studies have highlighted a potential role of some miRNAs involved in acquired resistance. Some of these more recent examples are outlined below [8].

1.6.4.1 miR-21 is highly expressed in DNR resistant CML cell line, K562

miR-21 has a number of targets of which both phosphatase and tensin homologue (PTEN) [91] and programmed cell 4 death (PDCD4) [92] are activated in leukaemia. Bai et al have shown miR-21 is highly expressed in the DNR resistant leukaemia cell line, K562. miR-21 contributes towards DNR resistance by regulating PTEN expression in K562 cells. Thus the authors go on to suggest the up regulation of miR-21 and the sequential down regulation of PTEN could potentially be a novel DNR resistance mechanism in leukaemia cells [91]. When miR-21 was over expressed the P13K/Akt pathway was activated leading to a decrease in PTEN, indicating PTEN is a target for miR-21 [91]. An anti-miR-21 oligonucleotide was used to down regulate miR-21 expression in HL60 cells which were subsequently treated with ara-C resulting in cellular apoptosis. Suggesting miR-21 is playing a part in the cells increased sensitivity towards ara-C. miR-21 directly targets PDCD4, which was up-regulated in response to miR-21 down regulation. Therefore the authors suggest the down regulation of miR-21 results in ara-C induced apoptosis which could be partially due to the up regulation of PDCD4 [93].

1.6.4.2 Cell proliferation is inhibited when miR-222 expression is suppressed

Zhang and his colleagues have demonstrated both miR-221 and miR-222 target PTEN in SGC7901 cells, a gastric cancer cell line via a luciferase construct. When miR-221 and miR-222 expression is suppressed, both cell proliferation and invasion is inhibited whilst cell apoptosis is induced. PTEN up regulation results in increased radio sensitivity within the cell line [94, 95].

1.6.4.3 miR-34a has been shown to inhibit cell proliferation in the CML cell line, K562. miR-34a a tumour suppressor gene, inhibits SIRT1 expression, however p53 regulates miR-34a expression [96]. Yamakuchi et al have suggested p53 induces miR-34a expression which in turn increases p53 activity via SIRT1 in a positive feedback loop [96]. miR-34a targets SIRT1, p53 targets miR-34a. miR-34 family 3 miRs which are coded for by two different genes.

Ichimura et al explored miRNA regulation in K562 cells during megakaryocytic differentiation induced by Phorbol 12-myristate 13-acetate (PMA). PMA activates the extracellular signal-regulated protein kinase (ERK) which in turn induces miR-34a expression. Interestingly the group found over expression of miR-34a significantly reduced K562 proliferation [97].
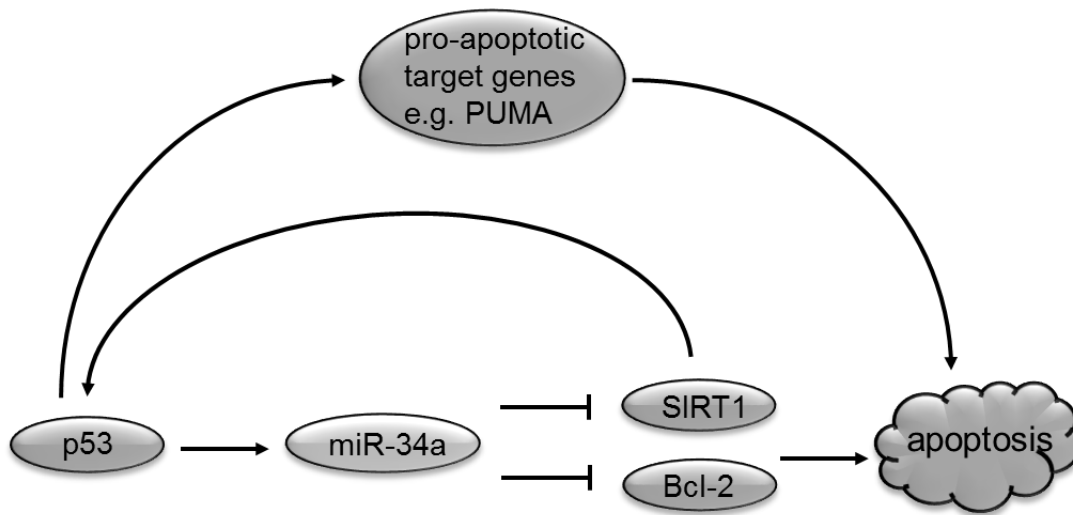
Figure 1.8. p53 and miR-34a relationship regarding apoptosis. p53 induces miR-34a expression which subsequently inhibits SIRT1 and Bcl-2 expression promoting apoptosis. However SIRT1 activates p53 transcription and in turn p53 targets e.g. PUMA [98]. Diagram adapted from H. Hermeking. 2009 [98].

1.6.4.4 miR-181a targets Bcl-2 and has been shown to be involved in acquired ara-C resistance in the AML cell line HL60s

The Nrf2 pathway is regulated and repressed by the presence and absence of Keap1.

Bai et al concluded miR-181a directly targets Bcl-2, via both reporter gene assay and immunoblot analysis. They investigated miR-181a role within resistance in a cytarabine resistant AML cell line; HL60 (HL60-ara-C). miR-181a was found to be down regulated whilst Bcl-2 was up-regulated in these cells compared to their expression in the wild type HL60 cells. However when the authors overexpressed miR-181a in the HL60-ara-C cell, they became more sensitive to the presence of ara-C thus indicating miR-181a is involved in ara-C resistance [99]. Interestingly, Bcl2 expression could be significantly up-regulated in the THP1ara-C(1) suggesting miR-181a and Bcl2 are not involved in ara-C resistance in THP1ara-C(1).

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1.7 Project aims

The present studies were undertaken to investigate the following points:

- The role of HO-1 in regulating cellular cytoprotective responses in response to ara-C and DNR, the two front-line chemotherapeutic agents for the treatment of AML.

- Explore acquired ara-C resistance in a ara-C resistant AML cell line, THP1ara-C(1).

- Investigate miRNA expression in both wt THP1 cells and ara-C resistant THP1ara-C(1) cells.

- Explore miR-196a and Bach1s relationship in response to continual ara-C exposure and subsequent generated resistance in wt THP1 cells.

The current MacEwan laboratory focus revolves around AML cytoprotection and acquired cellular resistance. Previously, the laboratory has investigated the effects of TNF, NF-κB and bortezomib (Figure 1.9: 1.2.3.4.) on HO-1 expression in AML cells. So far, little work has been performed on investigating the effect of front-line chemotherapeutic agents or miRNA expression in relation to cytoprotection and cellular resistance. Therefore, the studies undertaken during the course of this thesis will aim to shed light on these important clinical points.
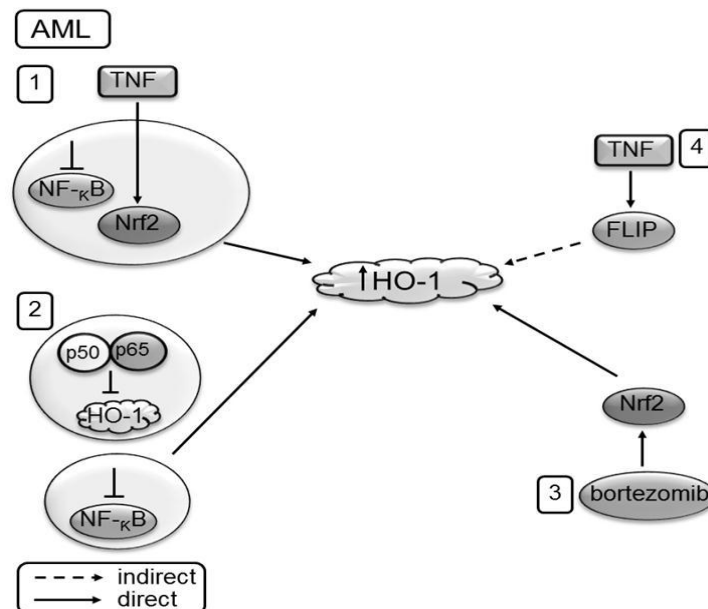
Figure 1.9. Diagramatic representation of the presently understood points of knowledge from within our laboratory.

Chapter 2: Materials and Methods

2.1 Materials

Unless otherwise stated all reagents were purchased from Sigma Aldrich inc.

2.2 Cell Culture

All cell lines, except 293FT, were cultured in RPMI 1640 Medium (Invitrogen Gibco) supplemented with 10% (v/v) heat inactivated FBS (Fetal Bovine Serum; Biosera) and 1% (v/v) L-Glutamine (Invitrogen Gibco) in 75 cm^2 flasks (Corning) and incubated at 37 °C and 5% CO_2. 293FT were cultured in DMEM High Glucose (Invitrogen ;Gibco) supplemented with 10% heat inactivated FBS, 6 mM L-Glutamine, 0.1 mM MEM Non-Essential Amino Acid solutions (Invitrogen Gibco) and 1 mM Sodium Pyruvate (Invitrogen Gibco). Cells were incubated in media until confluent. When splitting cells the dilution used was dependent on the cell line being passaged (Table 2:1).

Table 2.1: Cell lines.

Cell line	Cell type	Passage dilution	Source
U937	Human leukemic monocyte lymphoma cell line	1:9 every three days.	European Collection of cell cultures
THP-1	Human acute monocytic leukaemia	1:1 every four days.	European Collection of cell cultures
THP1ara-C(1)	Ara-C resistant human acute monocytic leukaemia	1:1 every four days.	European Collection of cell cultures
THP1ara-C(1) TW	Ara-C resistant human acute monocytic leukaemia	1:1 every four days.	European Collection of cell cultures
HL60	Human promyelocytic Leukaemia	1:9 every three days.	European Collection of cell cultures
K562	Human erythromyeloblastoid leukaemia	1:1 every four days.	European Collection of cell cultures
293 FT	Primary embryonic human kidney transformed with sheared human adenovirus type 5 DNA.	1:5 every four days	Invitrogen

2.2.2 Developing ara-C resistant U937 and THP-1 cells.

Parent THP-1 and U937 cells were treated with 0.5 μM of ara-C (Sigma Aldrich) weekly until the cells had developed resistance towards ara-C [44]. Resistance was confirmed by exposing the cells to various doses of ara-C and assessing cell death over 72 hours using the Promega Celltitre 96 AQ$_{ueous}$ One Solution Reagent MTS assay. 1 μM of ara-C is a clinically achievable concentration obtained in situations where a standard dose of ara-C therapy is administered to a patient [42]. Thus a reduced dose was selected to treat the resistant cells, which would simulate the clinical environment without killing the entire population. The resistant cells were cultured like the parent cells and thus split normally.

2.2.3 Freezing and thawing Cells

5 x10^6 cells were centrifuged at 1200 r.c.f. for 5 min before the supernatant was removed to leave the exposed pellet. This was subsequently re-suspended in 1.5 mL of freezing solution (90% (v/v%) RPMI 1640 supplemented medium and 10% DMSO [Sigma Aldrich] and transferred to a CryoTube^{TM} vial (Nunc). A 5100 Cryo 1°C Freezing Container (Nalogene) was used during the first 24 h to prevent potential damage to the cells caused by freezing. Samples were stored at -80 ˚C.

To thaw cells CryoTubes^{TM} were removed from -80˚C and placed into an incubator (37°C and 5% CO$_2$) for 2 min. The contents of the CryoTubes^{TM} was re-suspended in 10 mL of fresh RPMI 1640 supplemented medium and centrifuged for 5 min at 1200 r.c.f. The supernatant discarded; the exposed pellet was re-suspended in 10 mL of fresh RPMI 1640 supplemented medium and transferred to a 25 cm^3 flask (Nunc).

2.2.4 AML sample preparation

Donor blood or bone marrow aspirate was slowly pipette onto 10 mL of Histopaque®1077 (Sigma-Aldrich) to create two distinct layers. The mixture was centrifuged for 20 min at 1200 r.c.f, and 21˚C to create a density gradient (Figure 2.1). The autologous plasma layer was removed and filtered through a 0.2 μM pore Minisart® high flow syringe filter (Sartorius). 20 mL of serum free RPMI 1640 medium was added to the filtered plasma. The white blood cells (WBC) layer was removed and washed in 1x

Hanks Balanced Salts Solution (HBSS; Gibco). WBC were centrifuged for 10 min at 1200 r.c.f. The supernatant was discarded to leave the exposed pellet that was then re-suspended in the RPMI 1640 medium supplemented with the previously filtered autologous plasma.
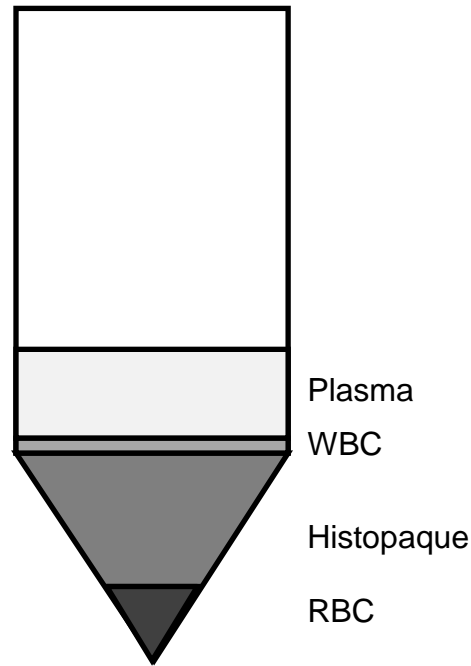
Figure 2.1: Density gradient separation of donor blood or bone marrow aspirate.

2.2.5 Trypan Blue Exclusion assay of cell viability

20 μL of cell suspension was diluted 1:1 with Trypan Blue Solution (Sigma Aldrich) . The sample was transferred to the haemocytometer and viewed under the microscope. The number of cells present were counted and recorded to establish the number of viable cells present within the population under investigation. Dead and dying cells present within the suspension take up the Trypan Blue through their damaged cell membranes thus appearing blue under the microscope whilst the live cells remain uncoloured because their membranes are still intact.

2.3 Virus construction and infection

Virus construct were performed by Dr. Lyubov Zaitseva (University of East Anglia). Briefly; microRNA (miRNA) sequence miRNA-HO-1-166 (5'-TCCTCATGAACTCAGCATTCT-3') targeting human HO-1 was selected with Block-iT

RNAi Designer software (Invitrogen) and pcDNA^{TM}6.2-GW/EmGFP-miR-neg plasmid (Invitrogen) (Paisley, UK) was used as the source for the negative control. miRNA-encoding viruses were produced with Block-iT Lentiviral Pol II miRNAi Expression system purchased from Invitrogen (Paisley, UK) in 293FT cells. Lentiviral stocks were concentrated using the Lenti-X^{TM} Concentrator and titres were obtained with the Lenti-X^{TM} qRT-PCR Titration kit (Clontech). For transduction, 2.5 x 10^5 U937 cells were infected with lentiviral stock at an MOI of 10 in presence of Polybrene (hexadimethrine bromide; Sigma Aldrich) and analysed by flow cytometry, qRT-PCR and Western Blot 48 h after infection. Stably transduced cells were selected using Blasticidin (8 µg/ml, 2 weeks).

2.4 Nucleofection and Lipofection

Suspension cells: 2 x 10^6 cells were centrifuged at 1200 r.c.f. for 5 min before supernatant was discarded and samples were re-suspended in non-supplemented RPMI 1640 medium and centrifuged for a further 5 min. Supernatant was discarded and cells were again re-suspended in non-supplemented 1640 medium before cells were transferred into Amaxa^{TM} certified 100 µL Aluminium Electrode Curvettes (Lonza) with 25 µM of transfection reagent (Table 2.2) and transfected using the Amaxa Biosystems Nucleofector$^®$ II (Lonza), programme T 001. Following transfection cells were re-suspended in 2 mL of fresh supplemented RPMI 1640 medium.

Adherent cells: DNA vectors/ transfection reagents (Table 2.2) and Lipofectamine 2000$^®$ (Life Technologies) were diluted in OPTI-MEM GlutaMax^{TM} (Invitrogen Gibco) separately and incubated at room temperature for 5 min before being combined. The mixture was incubated for a further 20 min at room temperature before being added to 3.5-4 x 10^5 cells, drop wise. Cells were incubated for a further 48 h at 37°C and 5% CO_2.

Table 2.2: Transfection Reagents

	Mature miRNA sequence	Manufacture
Silencer® negative control # 1		Ambion
miR-21	5-'UAGCUUAUCAGACUGAUGUUGA-3'	Qiagen
miR-145	5-'GUCCAGUUUUCCCAGGAAUCCCU-'3	Qiagen
miR-196a	5'-UAGGUAGUUUCAUGUUGUUGGG-3'	Qiagen
miR-196a	5'-UAGGUAGUUUCAUGUUGUUGGG-3'	Ambion
miR-222	5'-AGCUACAUCUGGCUACUGGGU-3'	Qiagen

2.5 Luciferase Assay

Suspension cells: 2×10^5 cells were transfected via nucleofection with the desired transfection reagent (Table 2.2), 400 ng pMIR-REPORT™-β-galactosidase control plasmid (pMIR-REPORT™ System, Applied Biosystems) and 400 ng of the desired DNA construct in a pMIR-REPORT™ plasmid as previously described in section 2:2.7.1 Cells were incubated at 37˚C, 5% CO_2 for 48 h.

Adherent cells: $3.5 - 4 \times 10^4$ cells were transfected via Lipofectamine2000™, with the desired reagent (Table 2.2), 400 ng pMIR-REPORT™-β-galactosidase control plasmid and 400 ng of the desired DNA construct in a pMIR-REPORT™ plasmid as previously described in section 2:2.7.1. Cells were incubated at 37˚C, 5% CO_2 for 48 h.

Both pMIR-REPORT™ plasmid and pMIR-REPORT™-β-galactosidase control plasmid activities were assessed using the Dual-Light® Combined Reporter Gene Assay System for Detection of Luciferase and β-galactosidase (Applied Biosystems). Briefly, both suspension and adherent cells were washed twice with sterile PBS, cells were centrifuged at 400 r.c.f, 4˚C for 5 mins before supernatant was removed to leave the exposed pellet. Either 25 μL (suspension cell lines) or 75 μL (adherent cell lines) of Lysis Solution was added to each sample before being centrifuged at maximum speed for 2 min to pellet debris. The supernatant was then transferred to a fresh tube and stored at -70˚C until analysis.

10 µL of sample extract was added to a 96 well plate (Corning), Buffer A and Buffer B; Galacton-Plus (100:1) were added according to the manufactures instructions. After 1-2 seconds delay the luciferase signal was read using the Envision 2103 Multilabel Reader and Wallac EnVision Software (PerkinElmer). Samples were left to incubate at room temperature for a further 60 min, before Accelerator-II was added to each well. β-galactosidase signal was analysed again on EnVision 2103 Multilabel Raeder.

2.6 Flow Cytometry

2.6.1 Annexin V-FITC Apoptosis Detection

Approximately 1×10^5 cells were centrifuged at 4,000 r.c.f. for 1 min, before supernatant was discarded. The cell pellet was re-suspended in Annexin V binding buffer containing 0.005% (w/v) Propidium Iodide (PI) and 0.02% (w/v) Annexin-FITC Annexin V-FITC Apoptosis Detection Kit; Abcam) and each sample was incubated for 5 min at room temperature. Cell death was then evaluated by flow cytometery using the BD Accuri™ C6 Flow Cytometer (BD bioscience). 2×10^4 events per sample were recorded at medium velocity.

2.6.2 ROS quenching assay

5 µM NAC (N-Acetyl Cysteine; Sigma Aldrich) was added to 1×10^6 cells and incubated for 20 min at 37ºC and 5% CO_2. Cells were then treated with either ara-C (0.5 µM) or DNR (0.2 µM; Sigma-Aldrich), or a combination thereof, and incubated for a further 24 h at 37ºC and 5 % CO_2. Cells were centrifuged at 4000 r.c.f. before supernatant was removed and discarded and the pellet was re-suspended in PBS. Cell death in the presence of the ROS-quenching NAC was analysed by flow cytometry as detailed in 2:2.9.1.

2.6.3 ROS generation H_2DCFDA assay

Following any treatment, 1 µM or 10 µM H_2DCFDA (2',7-dichlorodihydrofluorescein diacetate; Invitrogen) was added to 1×10^6 cells and incubated for 15 min at 37˚C and 5% CO_2. Cells were centrifuged at 4000 r.c.f. for 1 min before supernatant was discarded and the pellet re-suspended in PBS. Fluorescence intensity was examined using the BD Accuri™ C6 Flow Cytometer. The mean channel fluorescence of each

sample is expressed as a percentage of the untreated control. The relative fluorescence intensity of H_2DCFDA detected represents the relative steady state of ROS generation.

2.6.4 Cell cycle analysis

1×10^6 cells were centrifuged at 4,00 r.c.f. for 1 min before supernatant was discarded and the pellet was re-suspended in PBS, Ethanol was added to a final concentration of 70% (v/v) to fix cells, and the cells were incubated for 20 mins on ice. Fixed cells were centrifuged at 4,000 r.c.f for a further 5 min before supernatant was removed and the pellet was re-suspended in PBS containing 0.005% (w/v) PI, 0.1 mg/mL RNase A (Sigma-Aldrich) and 0.05% Triton-X^{TM} 100 (Sigma-Aldrich) before being analysed on the BD Accuri^{TM} C6 Flow Cytometer.

2.7 Cell Viability assays

2.7.1 Promega CellTiter 96 AQ$_{ueous}$ One Solution Reagent MTS cell viability assay

CellTiter 96 AQ$_{ueous}$ One Solution Reagent (Promega) was added to 1×10^5 cells in a 1:9 dilution and incubated at 37°C, 5% CO_2 for between 2 and 4 h depending on the cell line and cell type. CellTiter 96 AQ$_{ueous}$ One Solution Reagent contains a novel tetrazolium compound which is reduced to a formazan product. The concentration of the formazan product present within the well is directly proportional to the number of living cells present within the culture. The absorbance was read at 490 nM using the FLUOstar OMEGA micro plate reader (BMG Labtech).

2.7.2 CellTitre-Glo® Luminescent Cell Viability Assay

CellTitre-Glo® Luminescent Cell Viability Assay reagent (Promega) was added to 5×10^4 cells in a 1:1 dilution and incubated at room temperature for 30 mins before luminescence was read using the FLUOstar OMEGA micro plate reader.

2.8 qRT-PCR

2.8.1 Total RNA extraction

1×10^6 cells were lysed in Total RNA Lysis Solution (Applied Biosystems), diluted 1:1 in PBS, then vortexed. Cell lysates were stored at -20°C until use.

Total RNA was extracted from cell lysates using the 6100 Nucleic Acid Prep Station (Applied Biosciences) and Total RNA Purification Tray (Applied Biosystems) according

to the manufacturer's instructions. Briefly, after cultured cell lysates were disrupted and stabilised by Total RNA Lysis Solution (Applied Biosystems) and transferred to the 6100 Nucleic Acid Prep Station (Applied Biosciences), samples were washed to remove cell debris and contaminates before the purified RNA was eluted. RNA was eluted into 100 µL of Nucleic acid purification solution (Applied Biosystems). Samples were used immediately or stored at -20°C until use. Total RNA yield was assessed via the ND-100 Spectrophotometer (Nanodrop Technologies) at 260 nm and 280 nm. 260/280 nm ratio ≥ 1.8 indicated good quality uncontaminated RNA.

2.8.2 Reverse Transcription

Reverse transcription of mRNA was performed using the GeneAmp® RNA PCR Core Kit (Applied Biosystems) according to the manufacturer's instructions. Roughly 350 ng of RNA was added to master mix 1 (1 µL Random Hexamers 0.1 µg/µL [Quiagen], 2.5 µL $MgCl_2$ solution 25 mM, 1 µL 10 x RT Buffer, 1 µL dNTP Mix [with dTTP] 10 mM [2.5 mM each], 0.3 µL MultiScribe Reverse Transcriptase enzyme 50 Units/µL and 0.3 µL RNase Inhibitor 20 Units/µL). Samples were mixed and centrifuged at 400 r.c.f. for 1 min then transferred to the BioRad C1000 where they were incubated initially at 42ºC for 15 min, then 95ºC for 3 min and finally 4ºC until required. cDNA was ready for qRT-PCR or stored at -80ºC. Briefly collected purified mRNA was reverse transcribed into cDNA and then amplified before qRT-PCR.

2.8.3 quantitative Real Time-PCR

cDNA from the reverse transcription reaction was diluted 1:9 in RNase-free water before 5 µL of the diluted cDNA was added to 15 µL of Master Mix 2. Master Mix 2 was composed of 10 µL 2 x SYBR, 1 µL Primer under investigation 10 nM (Invitrogen) and 4 µL RNase free water (Sigma). SYBR green technology (Roche) was used to detect amplification of DNA for the primers under investigation. After pre-amplification at 95ºC for 2 min the PCRs were amplified for 45 cycles of 95ºC for 15 s and 60ºC for 10 s and 72ºC for 10 s on the LightCycler® 480 Real-Time PCR System (Roche)). Relative mRNA expression of the gene of interest was standardised against GAPDH mRNA expression and experimental controls using the comparative C_T ($2^{-\Delta\Delta C_T}$) method and is expressed as fold change. Briefly, cDNA undergoes denaturing, annealing and finial elongation each cycle. SYBR green intercalates with the double stranded DNA and fluoresces.

Fluorescence intensity increases with increasing cycle number and PCR product accumulation..

2.8.4 Primers

All primers were designed using Invitrogen[TM] Custom DNA Oligos software and purchased from Invitrogen except GAPDH (Qiagen).

Table 2.3: Primers

Gene	Primer sequence: F (5'-3')	Primer sequence: R (5'-3')	Base pair position	Amplicon size
GAPDH	ACCAGCCTCAAGATCATCAGC	TGCTAAGCAGTTGGTGGTGC	624-672	48 bp
Annexin1	CAGGTCACCTTGAGGAGGTTGT	CAGCACGAAGTTCATCAGCATC	376-450	74 bp
Bach-1	CCAGAAGAGGTGACAGTTAAAGG	TTGCACACTTCATCCACATTC	370-467	97 bp
Bcl-2	CACCTGTGGTCCACCTGAC	AGCTGGCTGGACATCTCG	762-850	88 bp
BIM	AGCGATACAGGTCTCGTTCC	TCTGTAATCGCGACGATAATCT	348-433	85 bp
Fas L	TGGGGATGTTTCAGCTCTTC	GTGTGCATCTGGCTGGTAGA	453-525	72 bp
GCLC	GGCGATGAGGTGGAATACAT	GTCCTTTCCCCCTTCTCTTG	626-739	113 bp
GCLM	GCGAGGAGCTTCATGATTGT	CTGGAAACTCCCTGACCAAA	412-496	84 bp
HO-1	ATGGCCTCCCTGTACCACATC	TGTTGCGCTCAATCTCCTCCT	258-312	54 bp
IKKa	CTGTACCAGCATCGGGAACT	ATGGCACCATCGTTCTCTGT	146-235	89 bp
IRAK1	CTCCAGCCCCTTCTTCTACC	CTGCAGGAGGGACACTGAG	597-654	57 bp
NQO1	GCCGCAGACCTTGTGATATT	TTTCAGAATGGCAGGGACTC	471-533	63 bp
Nrf2	AACCACCCTGAAAGCACAGC	TGAAATGCCGGAGTCAGAATC	1546-1596	50 bp
P65	GGCGAGAGGAGCACAGATAC	CCTGGTCCTGTGTAGCCATT	282-350	68 bp
PgP (MDR1)	GATGAAGCCACGTCAGCTCT	CCTTCTCTGGCTTTGTCCAG	4091-4164	73 bp
PUMA	GACGACCTCAACGCACAGTA	GAGATTGTACAGGACCCTCCA	717-803	86 bp
TRAF 6	TGGCATTACGAGAAGCAGTG	TGGACATTTGTGACCTGAAT	609-699	94 bp

2.8.5 miRNA Reverse Transcriptase

Naturally miRNAs are not polyadenylated compared to mRNA which are. Therefore during reverse transcription miRNA are polyadenylated by poly(A)polymerase and then converted into cDNA. miRNA was reverse transcribed from total RNA using the Qiagen miScript Reverse Transcription kit (Qiagen) as per the manufacturer's instructions. Briefly 400 ng of template RNA was added to each tube containing the reverse-transcription master mix (2 µL 5 x miScript RT Buffer [contains Mg^{2+}, dNTPS and

primers], 0.5 µL miScript Reverse Transcriptase Mix [poly(A) polymerase and reverse transcriptase] and 2.5 µL RNase-free water). Samples were incubated for 60 min at 37˚C followed by a further incubation at 95˚C for 5 min. miRNAs are polyadenylated by poly(A) polymerase whilst the RNA is converted into cDNA by Reverse transcriptase.

2.8.6 miRNA Real time PCR

cDNA was diluted 1:9 with RNase free water, Master Mix 2 mix was prepared as outlined above (2:2.11.3) However the reverse primer was removed and replaced with 1 µL of Universal primer. PCRs were pre and amplified for 45 cycles as previously described in 2:2.11.3. miRNA expression of the primer under investigation was standardised against U6 expression, using the comparative C_T ($2^{-\Delta\Delta C_T}$) method and expressed as fold change.

Table 2.4: miRNA Primers

miRNA	Sequence (5'-3')
U6	CGCAAGGAUGACACGCAAAUUC
SN25	ACTGAGCTCCGTGAGGATAAAT
miR-196a	TAGGTAGTTTCATGTTGTTGG
miR-200a	TAACACTGTCTGGTAACGATGT
miR-21	TAGCTTATCAGACTGATGTTGA
miR-222	AGCTACATCTGGCTACTGGGTCTC
miR-145	GTCCAGTTTTCCCAGGAATCCCTT
miR-15a	TAGCAGCACATAATGGTTTGTG
miR-92a	TATTGCACTTGTCCCGGCCTG
miR-101	TACAGTACTGTGATAACTGAAG
miR142-3p	TGTAGTGTTTCCTACTTTATGGA
miR-146a	TGAGAACTGAATTCCATGGGTT
miR-149	TCTGGCTCCGTGTCTTCACTCC
miR-183	TATGGCACTGGTAGAATTCACTG
miR-186	CAAAGAATTCTCCTTTTGGGCTT
miR-34a	TGGCAGTGTCTTAGCTGGTTGT
Universal Primer	GAATCGAGCACCAGTTACGC

2.9 Western Blotting

2.9.1 Total Protein extraction

Approximately 1 x 10^6 cells were washed with PBS and lysed with Radio Immunoprecipitation Assay Buffer (RIPA; 10% [v/v] PBS, 1% [v/v] Novidet P-40 [NP-40] substitute [Fluka Biochemika], 0.5% Sodium Deoxycolate [Sigma], 0.1% [W/V] SDS [Melford], made up in dH_2O plus one cOmplete Mini, EDTA free, Protease Inhibitor Cocktail Tablet [Roche]). Samples were either stored at -20 ˚C until use otherwise on ice ready to be used. Samples were further centrifuged at 161000 r.c.f for 15 min at 4 ˚C, supernatant was retained and returned to ice.

Protein concentration was established by spectrophotometry using the Eppendorf BioPhotometer (Eppendorf). Protein concentration was by dilution with PBS. Protein was diluted 1:1 in 2 x Sample Buffer (20% [v/v] Glycerol, 10% [v/v] β-mercaptoethanol, 5% [w/v] SDS, 20% [v/v] 1.0 M Tris HCL pH 6.7 and 2 mg Bromophenol blue made up with dH_2O) and heated at 100°C for 5 min to denature proteins before being returned to ice.

2.9.2 Cytosolic and nuclear protein extraction

5 x 10^6 cells were harvested and centrifuged at 3000 r.c.f. for 5 min. Supernatant was discarded and the pellet was re-suspended in 1 mL of ice cold PBS and centrifuged at 3000 r.c.f. for a further 5 min. Supernatant was discarded and the pellet was re-suspended in 50 µL of buffer A (1% [w/v] 1M HEPES pH 7.9, 1% [w/v] 1M KCL, 30% [w/v] 1M Sucrose, 0.12% [w/v] 1M $MgCl_2$, 0.05% [v/w] 1M DTT, 0.1% [v/v] NP-40, 1 x cOmplete Mini, EDTA free, Protease Inhibitor Cocktail Tablet made up with dH_2O), which lysesd the cell membranes therefore releasing the cytosolic proteins. Samples were returned to ice for a further 5 min before beginning pulse centrifuged for 5 s at 134000 r.c.f. The supernatant was transferred to a fresh tube (cytosolic fraction), whilst the pellet (nuclear fraction) was re-suspended in 50 µL of Buffer B (2% [w/v] 1M HEPES pH 7.9, 10% [w/v] 1M KCL, 2% [w/v] 5M NaCl, 0.05% [v/w] 1M DTT, 20% [w/v] Glycerol, 1 x cOmplete Mini, EDTA free, Protease Inhibitor Cocktail Tablet made up in dH_2O) and vortexed. Protein samples were diluted 1:1 with 2 x Sample Buffer and stored as previously described in 2:2.13.1.

2.9.3 SDS-PAGE and Transfer

Approximately 50 μg of protein sample and 10 μL of BioRad Precision Plus Protein Ladder Dual Colour (BioRad) were loaded into a 5% Poly acrylamide Stacker gel (16.9% Acryl amide 30% [Bis solution, 37.5:1,2.6%] mix [BioRad], 12.64% 1.0 M TRIS Base pH 8.8 [Fisher Scientific],10% SDS [Melford],10% APS [Sigma], 0.10% TEMMED [BioRad] in d_2H_2O) and resolved on a 12% Poly acrylamide Resolving Gel (40% Acryl amide 30% [Bis solution, 37.5:1,2.6%] mix [BioRad], 25% 1.5 M TRIS Base pH 8.8 [Fisher Scientific], 1% SDS [Melford] 1% APS [Sigma], 0.004%TEMMED [BioRad] in d_2H_2O) (selected due to the molecular weight of the proteins under investigation). For 40 min at 200 v in 1 x Running Buffer (Glycine 1.5% [w/v], TRIS 0.3% [w/v], SDS 2.4% [w/v] in dH_2O).

Immun-Blot PVDF Membranes (BioRad) were activated with methanol (Sigma) for 10 s and then washed with 1 x Transfer buffer (Glycine 1.5% [w/v], TRIS 0.3% [w/v] in dH_2O) for 5 min. Protein from the resolving gel was transferred onto the PVDF membrane for 60 min at 100 V and 4°C. After protein transfer the PDVF membranes were blocked with 5% (w/v) non-fat milk powder (Marvel) in PBS for 60 min. The Primary antibody was diluted in 5% (w/v) non-fat milk powder in PBST (1% [v/v] PBS with 0.1% [v/v] Tween *20 [Fisher Scientific]) and incubated with the membrane either over night at 4°C or for 1 h at room temperature. Membranes were washed with PBST before incubation with secondary antibody (in 5% [w/v] non-fat milk powder in PBST) for 60 min at room temperature. The membranes were washed with PBST 5 x 10 min before storage in PBS at 4°C until required.

Table 2.1: Primary Antibodies

Antibodies	Manufacturer	Molecular weight (kDa)	Dilution used	Species
HO-1	AbCam	32	1:500	mouse
HO-1	R & D Systems	32	1:2000	goat
Nrf2	AbCam	68	1:1000	rabbit
Nrf2	Santa Cruz Biotechnology	57	1:200	rabbit
β-Actin	Sigma-Aldrich	40	1:100,000	mouse
Bach-1	Santa Cruz Biotechnology	92	1:200	goat
NQO1	Santa Cruz Biotechnology	31	1:200	mouse

Table 2.2: Secondary Antibody

Antibodies	Manufacturer	Dilution
Anti -mouse HRP	Abcam	1:1000
Anti -goat HRP	Abcam	1:1000
Anti -rabbit HRP	Abcam	1:1000

2.9.4 Protein Detection

Membranes were incubated in ECL solution, composed of 50`µL Reagent A (68 mM p-couraric acid [Fluka] in DMSO), 5 mL of Reagent B (1.25 mM luminal [Sigma] in 0.1 M Tris, pH 8.5) and 1.5 µL of hydrogen peroxide (Sigma) for approximately 1 min. Exposure time required is dependent on the primary antibody being detected.

2.9.5 Stripping Membranes

Membranes were stripped with 10 x Re Blot Plus Strong Solution (Millipore) diluted 1:10 with dH$_2$O. Membranes were then washed with PBST 3 x for 10 min before being re blocked with 5% [w/v] non-fat milk and PBST for a further h at room temperature.

2.10 Immunohistochemistry Protocol

Approximately 1 x 10^5 cells were collected and cytospined at 35 r.c.f. for 5 min before being fixed with 4 % paraformaldehyde (Sigma) dissolved in PBS, pH 7.4 for 15 min. Samples were washed with PBS and blocked for a further 15 min in 30 % Goat Quench (30 % goat serum: 70 % gelatine quench). Cells were then permeabilised with 0.05 % Triton x-100 (Acros Organics) in 30 % goat quench for 20 min. Primary antibody diluted according to manufacturers instruction (Table 2.: Primary antibody dilutions) in 30 % goat quench and incubated for either 1 h at room temperature or 4°C over night. Samples were washed 3 x with PBS every 5 min. The secondary antibody was diluted in 30 % goat quench according to the manufactures instructions and incubated for a further 60 min at room temperature. The samples were washed again 3 x with PBS every 5 min. Samples were stained and incubated with DAPI (Sigma) diluted with PBS (1:2000) for a further 20 min before being mounted with 1.5 µL of Fluoromount G (Southern Biotech) and stored at 4°C until examination.

Table 13: Immunohistochemistry Antibodies

Primary antibody	Manufacture	Molecular weight/ kDa	Dilution	Species
Keap1	Santa cruz biotechnology	69	1:50	goat
Nrf-2	Santa cruz biotechnology	57	1:50	rabbit
NQO1	Santa cruz biotechnology	31	1:50	mouse
HO-1	R and D Biosystems	32	1:50	goat

2.11 Colony forming cell assay

Approximately 2 x10^4 cells were re-suspended in RPMI supplemented media and mixed slowly into 500 µL of methylcellulose media (HSC003, R & D Systems). Samples were left to incubate for 20 min allowing any air bubbles a chance to escape. Samples were then transferred to a 24 well plate (Nunc) and slowly decanted into their designated well. The gaps between the wells were filled with sterile water to ensure the required humidity was maintained throughout the experiment for colony development. Optimal cell plating concentrations varies and is dependent on the cell line being studied. The plates were incubated for 14 days at 37ºC and 5 %CO_2. Colony Scoring was performed at the end of the experiment.

Chapter 3: Does Haem Oxygenase-1 protect Acute Myeloid Leukaemia cells from front-line chemotherapeutic agents?

3.1 Introduction

The standard induction treatment for either young or fitter AML patients consists of the antimetabolite, ara-C and the anthracycline antibiotic DNR [5, 19]. Depending on the clinicopathological characteristics, patients who go into remission would commonly receive consolidation therapy with either high dose ara-C or allogeneic stem cell transplant [22]. Even with these intensive treatment strategies a significant number of patients relapse and only 50% of younger fitter patients can be cured. Treatment outcomes are dependent on a number of clinical and biological factors including cytogenetics, age and drug-resistance [19, 21].

To date three isoforms of haem oxygenase have been identified, HO-1, HO-2 and HO-3 [64]. HO-1, which is the most intriguing in terms of providing cell protection against cellular stresses, regulates the intracellular haem levels, and converts intracellular haem into carbon monoxide, free iron and biliverdin [64]. Biliverdin is further reduced into the potent antioxidant bilirubin [65, 66] by biliverdin reductase [67]. This metabolite possesses cytoprotective properties including anti-inflammatory, anti-oxidative and anti-apoptosis [69, 70]. HO-2 is constitutively expressed and HO-3 is not catalytically active and thought to be involved in oxygen-sensing. HO-1 belongs to the heat shock protein family (Hsp-32), thus its expression is triggered by a variety of stress inducing stimuli including, ultraviolet irradiation, hyperthermia, inflammatory cytokines, bacterial endotoxins and heavy metals [71-74, 100]. The regulation of HO-1 is under the control of many transcription factors including NF-κB, Nrf2 and activator protein-1 (AP-1) [78, 79].

Increased regulation of the cytoprotective gene HO-1 has been shown in response to a number of chemotherapeutic agents in various cancer cell lines and patient samples. We have previously shown, HO-1 expression is increased in response to bortezomib exposure in both the multiple myeloma (MM) cell line RPMI8226 and patient samples over an 8 h time course. An increase in basal HO-1 expression was also seen in response to Bortezomib acquired resistance, potentially indicating HO-1s involvement in acquired resistance [46]. Bortezomib is usually used in MM treatment [5] however it has been shown to induce the expression of the following cytoprotective genes; HO-1, ferritin, GSR, GCLM and NQO1 in both AML cell lines (HL60 and wt THP1 cells) and primary AML samples [47].

We have also investigated NF-κB expression in relation to HO-1 activation. In AML cells high NF-κB basal expression is seen. However when NF-κB expression is inhibited, induced HO-1 expression is reported which enables HO-1 to act as a silent anti-apoptotic factor therefore preventing apoptosis. The reverse was seen in control non-malignant cells. Furthermore, when both NF-κB and HO-1 expression was inhibited in AML cells, induced cell death was seen, however the same response was not seen in the control non-malignant cells. This inhibitory mechanism involving both HO-1 and NF-κB expression could potentially be utilised in the treatment of resistant AML [49].

3.2 Aims

HO-1 regulation in response to both ara-C and DNR has not been explored in the following AML cell lines; U937 and wt THP1 cells. Therefore we wanted to examine the role of this cytoprotective gene and determine its involvement in the regulation of cytoprotection in response to the chemotherapeutics agents; ara-C and DNR, which are both widely used in treating patients presenting with AML.

Table 3:1 Differential HO-1 regulation in response to induced cell death or chemotherapeutic exposure.

Leukaemia	Cell line/ patient cells	Comments	Reference
AML	wt THP1 and U937 primary AML cells	Induced HO-1 expression in response to ara-C and DNR exposure, however cell viability decreased when HO-1 expression was silenced.	[101]
AML	wt THP1 primary AML cells	NF-κB activation supresses HO-1 expression in AML cells, however upon NF-κB inhibition, HO-1 expression is induced providing secondary protection to chemotherapeutics.	[49]
AML	HL60, wt THP1 and U937 primary AML cells	NF-κB inhibition leads to Nrf2 activation by TNF leading to HO-1 induction thus inhibiting TNF induced cell death.	[57]
AML	HL60, wt THP1 and primary AML cells	Induced HO-1, ferritin, GSR, GCLM and NQO1 in response to bortezomib exposure.	[47]
CML	K562 cells either expressing wt BCR/ABL or imatinib-resistant mutants of BCR/ABL.	When Hsp32/HO-1 is targeted, growth is inhibited in both imatinib sensitive and resistant CML cells. Thus drugs targeting Hsp32/HO-1 inhibit growth and induce apoptosis in these cells.	[102]
MM	RPMI8226	Induced HO-1 in response to bortezomib exposure. Increased basal HO-1 expression in response to bortezomib acquired resistance.	[46]

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Table 3.2: Characteristics of study patient samples. AML disease characteristics including world health organisation (WHO) diagnosis and cytogenetics. Percent blast denotes percentage of AML blasts after purification using density gradient centrifugation (* denotes % of blasts and promyelocytes). Previous treatments are as outlined [101] .

	Age	Gender	WHO diagnosis	Cytogenetics	% Blasts	Previous treatment
AML 8	40	male	Acute promyelocytic leukaemia with t(15;17)(q22:q12) PML-RARA	t(15;17)	95 *	1999 DAT, DAT, MACE, MiDAC
AML 9	49	male	AML with maturation	normal	80	not known
AML 10	84	male	Acute monoblastic and monocytic leukaemia	not available	n/a	not known
AML 11	46	female	AML with maturation	+4,+8, t(9;22)	70	not known
AML 12	78	male	AML with myelodysplasia related changes	not available	85	not known
AML 13	27	male	AML with t(8;21)(q22;q22) RUNX1-RUNX1T1	t(8;21)	n/a	not known
AML 14	28	female	Acute myelomonocytic leukaemia	normal	n/a	not known
AML 15	51	female	AML with maturation	normal	75	not known
AML 16	66	female	Therpay related myeloid neoplasm	complex	85	1999 DAT, DAT, MACE, MiDAC
AML 17	82	female	AML with myelodysplasia	deletion 13	85	not known
AML 18	77	male	AML with myelodysplasia related changes	complex	95	not known
AML 19	31	female	AML with minimsl differtiation	normal	n/a	not known
AML 20	40	male	AML monoblastic and monocytic leukaemia	constitutional XYY only	90	not known

3.3 Results

3.3.1 AML resistance to ara-C and DNR. To better understand the mechanisms underlying chemo resistance in AML, levels of cell-death in AML cell lines and primary AML patient samples were examined in response to both ara-C and DNR at physiologically relevant concentrations. Concentrations were selected and based on previous studies [103, 104]. For example; 1 µM of ara-C is a clinically achievable plasma concentration, which can be obtained when a standard dose of ara-C is administered to a patient [105].

Figure 3.1 A shows a dose-dependent significant decrease in percentage viable cells in response to ara-C (0.5, 1 µM) and DNR (0.2, 0.5 µM) 24 h post treatment in all AML cells lines and 6 AML samples analysed. However cell viability in both AML-9 and AML-20 was significantly increased when they were treated with ara-C (0.5 µM). Cell viability was also significantly increased when AML-8 was treated with DNR (0.2 µM). Potentially due to experimental variation. To further investigate the effect of ara-C and DNR on AML cells we utilised the AML cell lines wt THP1 and U937. Figure 3.1 B shows cell-death induced by ara-C (0.5 µM), DNR (0.2 µM) or a combination thereof in these cells lines using annexin-V, PI staining and flow cytometry. Interestingly when the chemotherapeutic agents are used alone DNR elicits a greater response than ara-C. However, when ara-C and DNR are combined, the percentage of cells undergoing both early and late apoptosis is actually less (U937 58.4%, wt THP1 22.9%) than when the AML cells are treated individually with DNR (U937 82.2%, wt THP1 33.3%).

3.3.2. Primary AML cell response to classic AML chemotherapeutics. To further explore the response of primary AML cells to classic chemotherapies, one AML patient sample was chosen (AML-11) for further investigation. Figure 3.2 shows primary AML cell response to varying concentrations of key chemotherapeutics used in the clinic; amsacrine, bortezomib, DNR, etoposide and mitoxantrone, over a 48 h time course. With all five agents the percentage of viable cells present within the population decreased with increasing dose and duration of drug exposure. With all chemotherapeutic agents except etoposide there was significant decrease in viability with the four highest concentrations. Figure 3.3 A shows cell-death induced by bortezomib, DNR and mitoxantrone in AML-17 using annexin-V, PI staining and flow cytometry. The percentage of cells undergoing both early and late apoptosis is increased

with increasing chemotherapeutic concentration. AML-17 was diagnosed with AML with myelodysplasia using the WHO diagnosis, previous treatments used prior to sample collection was not supplied.
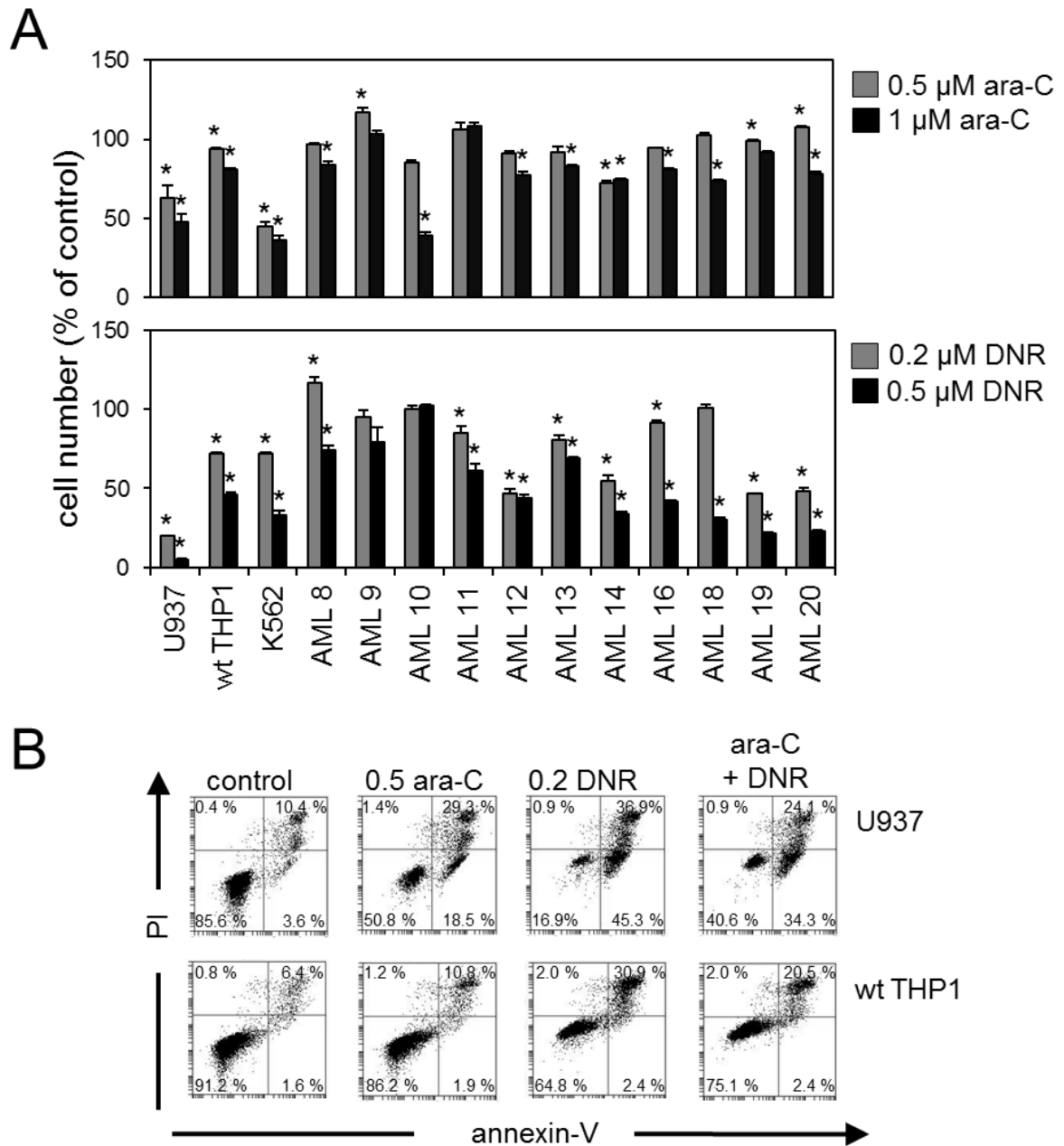
Figure 3.1: AML cell resistance to ara-C and DNR. (A) AML cell lines and patient samples were treated with ara-C (0.5 µM or 1 µM, top panel) or DNR (0.2 µM or 0.5 µM, bottom panel) for 24 h before cells were stained with annexin-V and PI and percentage of viable cells was established using flow cytometry. Bars represent mean ± SEM from three independent experiments (n = 3). (B) U937 and wt THP1 cells were treated with ara-C (0.5 µM) or DNR (0.2 µM) or combination thereof for 24 h. Samples were prepared for flow cytometry as described above (n = 1). Statistical significance was calculated by Student's t-test; *p≤ 0.05.
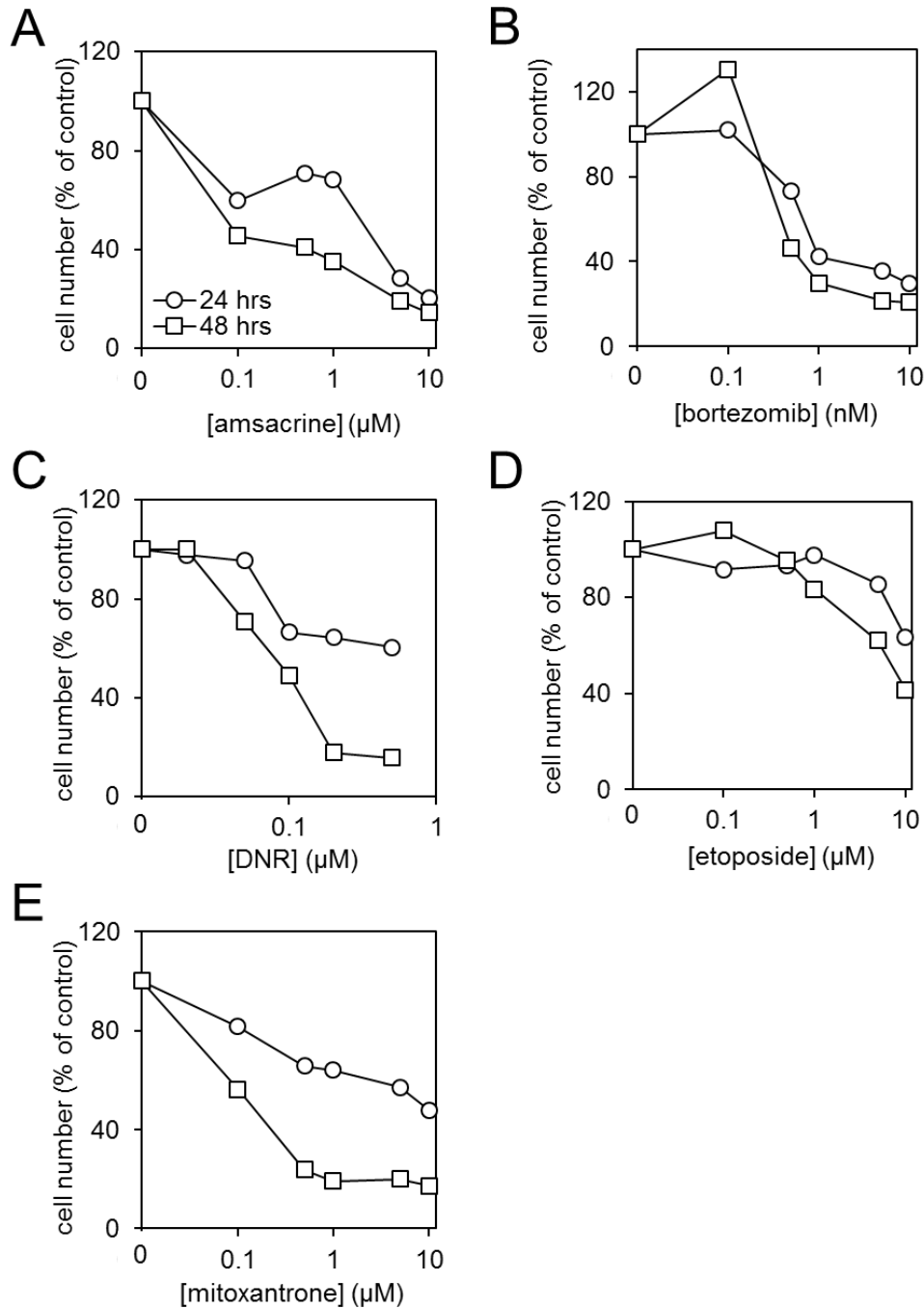
Figure 3.2: Primary AML cell response to varying concentrations of classic chemotherapeutics. Patient sample AML-11 was treated with varying concentrations of (A) amsacrine (0, 0.1, 0.5, 1, 5, 10 μM), (B) bortezomib (0, 0.1, 0.5, 1, 5, 10 nM), (C) DNR (0, 0.02, 0.05, 0.1, 0.2, 0.5 μM), (D) etoposide (0, 0.1, 0.5, 1, 5, 10 μM), or (E) mitoxantrone (0, 0.1, 0.5, 1, 5, 10 μM). Cell viability 24 and 48 h post treatment was examined by MTS assay. (Biological replicates = 1: technical replicates = 3).
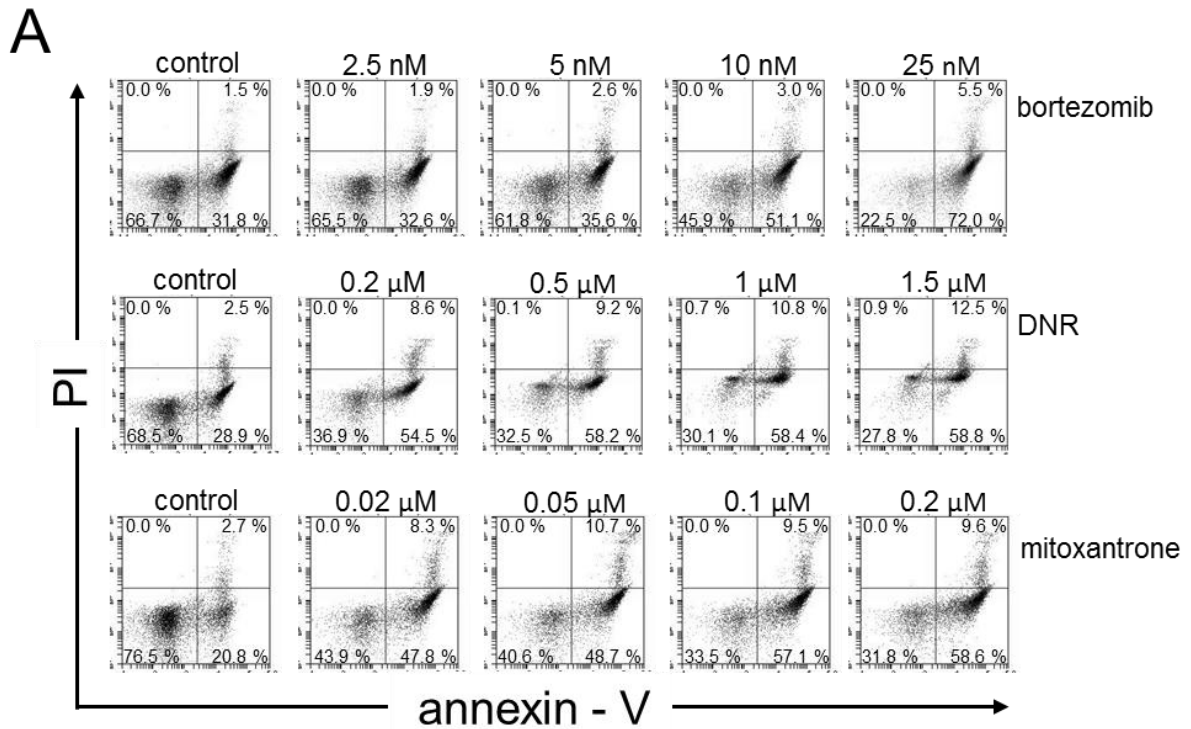
Figure 3.3: Patient sample AML-17 resistance to various chemotherapeutics. (A) Patient sample AML-17 was treated with bortezomib (0, 2.5, 5, 10, 25 nM), DNR (0, 0.2, 0.5, 1, 1.5 μM) and mitoxantrone (0, 0.02, 0.05, 0.1, 0.2 μM). Cells were stained with annexin-V and P1, number of viable cells was established using flow cytometry, (n = 1).

3.3.3 Ara-C and DNR induce HO-1 expression in AML cells. Previous work from our laboratory has shown that HO-1 protects AML cells from apoptotic stimuli, such as exposure to cytotoxic chemotherapeutics [57]. We wanted to determine whether ara-C or DNR are also able to induce HO-1 expression in AML cells. Figure 3.4 A shows a significant increase in HO-1 mRNA expression in both AML cell lines and ten out of eleven patient samples in response to either ara-C or DNR treatment for 24 h. In general, HO-1 expression is induced in a dose-dependent manner. To explore this induction of HO-1 mRNA expression, AML cell lines were treated for 24 h with either ara-C, DNR or a combination thereof. HO-1 mRNA expression in response to these drugs is significantly increased when compared to the control, when both AML cell lines are treated with a combination of both chemotherapeutic agents. However previously, we didn't see an addictive effect regarding cell viability when both agents where used in combination. HO-1 mRNA fold increase is greater in U937s treated with ara-C compared to wt THP1 cells. However the percentage of viable cells present within the same population of U937 cells was less than that of the equally treated wt THP1 cells (Figure 3.1 B). Possibly, suggesting HO-1 mRNA expression is involved in a cellular protection mechanism. Figure 3.5 A shows a Western blot indicating total HO-1 protein expression in both U937 and wt THP1 cells treated with either ara-C or DNR for 24 h. HO-1 protein expression is increased in both cell lines after ara-C or DNR exposure, however wt THP1 cells show higher levels of HO-1 in response to DNR compared with U937 cells.

HO-1 induction in response to ara-C, DNR or a combination of both agents was further investigated in patient sample AML-15. Cells were treated with ara-C, DNR or a combination thereof for 24 h, before HO-1 mRNA expression was examined using qRT-PCR. Figure 3.6 A shows a significant increase in HO-1 mRNA expression in response to ara-C, DNR or a combination thereof, therefore reiterating the above findings in Figure 3.4.
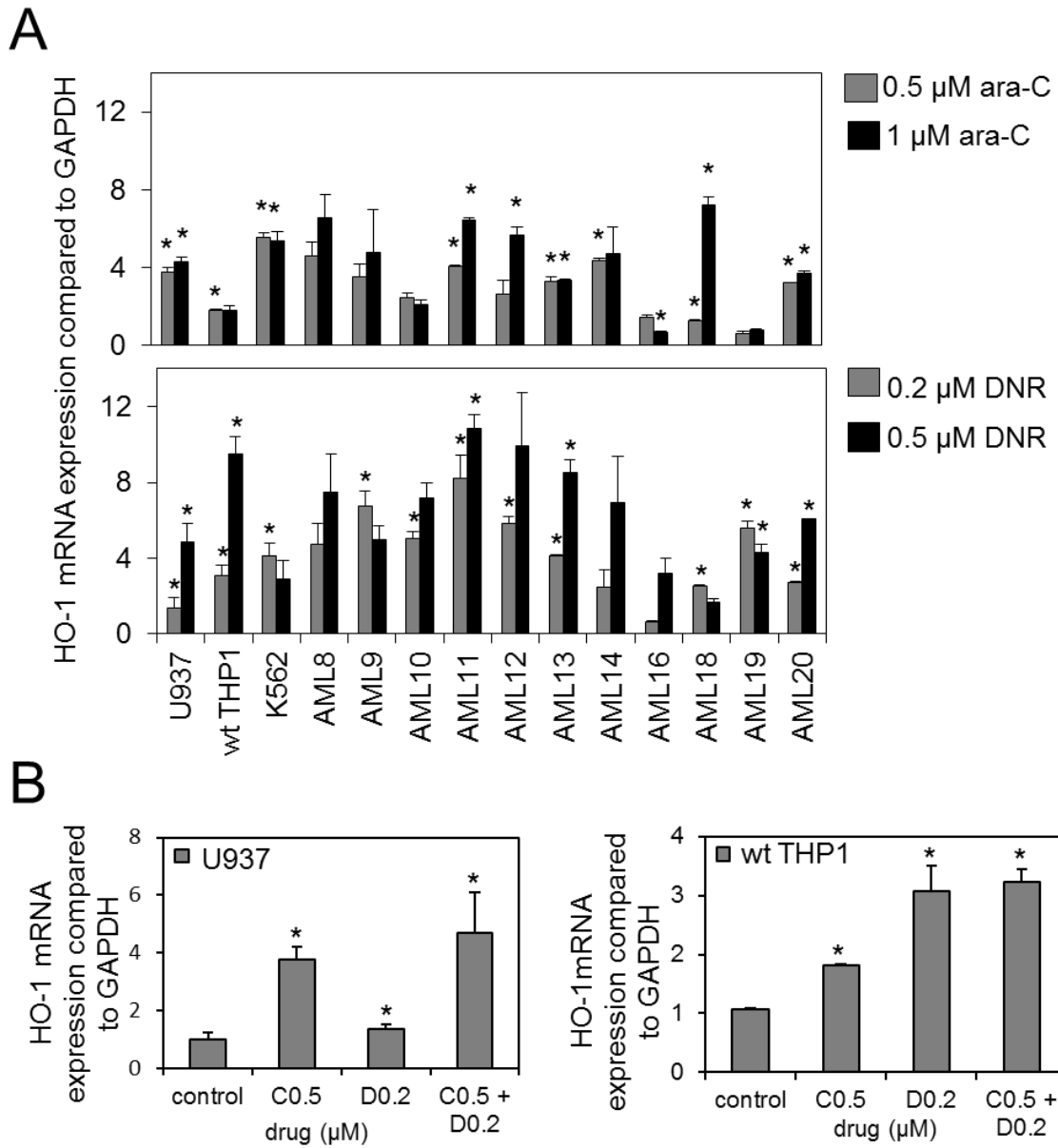
**Figure 3.4: HO-1 mRNA expression is up-regulated in primary AML cells and AML cell lines in response to ara-C and DNR.** (A) AML cell lines (U937, wt THP1 and K562) and AML patient samples were treated with either ara-C (0.5μM or 1μM) or DNR (0.2 μM or 0.5 μM) for 24 h before HO-1 mRNA expression was measured via qRT-PCR. (Cell lines n = 3, primary patient AML samples n = 1) (B) AML cell lines (U937 and wt THP-1) were treated with ara-C (0.5 μM), DNR (0.2 μM) or in combination, for 24 h and HO-1 mRNA expression was analysed using qRT-PCR. Bars represents the mean ± SEM (n = 3). Statistical significance was calculated by Student's t-test; *p≤ 0.05.

Figure 3.5: Total HO-1 protein expression is up-regulated in AML cell lines in response to ara-C and DNR. (A) Both U937 and wt THP1 cells were treated with either ara-C (0.5 μM or 1 μM) or DNR (0.2 μM or 0.5 μM) for 24 h before whole cell protein extracts were collected and separated using SDS-PAGE and then investigated using Western immunoblotting analysis of HO-1 expression. Equal sample loading across the membrane was assessed by probing for β-actin. Blots are representative of three separate experiments.
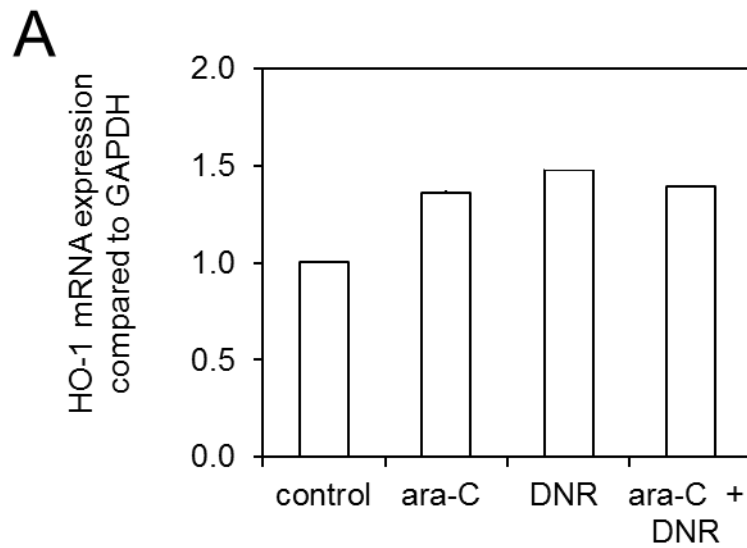
Figure 3.6: HO-1 mRNA expression is up-regulated in patient sample AML-15 in response to ara-C and DNR. AML-15 was treated with ara-C (0.5 μM), DNR (0.2 μM) or a combination thereof for 24 h before HO-1 mRNA was analysed using qRT-PCR. (n = 2).

3.3.4 Silencing HO-1 expression increases apoptosis to ara-C and DNR in wt THP1 and U937 cells. A lentiviral-based miRNA delivery systems based on a GFP backbone was developed by the group to target HO-1 mRNA. Therefore reducing HO-1 expression in targeted cells. Figure 3.7 A shows a 97% transfection efficiency achieved using this system and measured via flow cytometry. To confirm knock-down both HO-1 mRNA and protein expression were investigated in wt THP1 and U937 cells transfected with either HO-1 targeting miRNA (HO-1 miRNA) or non-targeting negative miRNA (negative miRNA). Figure 3.7 B indicates HO-1 expression in both wt THP-1 and U937 HO-1 silenced cells is less than 5% of the negative miRNA control. Figure 3.7 C shows a Western blot indicating reduced HO-1 protein expression in U937 cells transfected with HO-1 miRNA compared to un-transfected and negative miRNA control samples.

To investigate whether HO-1 expression confers protection from cell death on AML cells we utilised the lentiviral-based miRNA system characterised above. Untreated control, negative miRNA, and miRNA HO-1 transfected wt THP1 and U937 cells were treated with either ara-C or DNR for 24 h. Figure 3.8 A shows the apoptotic response in these cells after treatment measured by annexin V /PI staining and flow cytometry. The HO-1 miRNA transfected wt THP1 and U937 cells were more susceptible to apoptosis compared to their relevant controls thus suggesting HO-1 may be involved in protection against apoptosis and AML cell resistance to these chemotherapeutic agents.
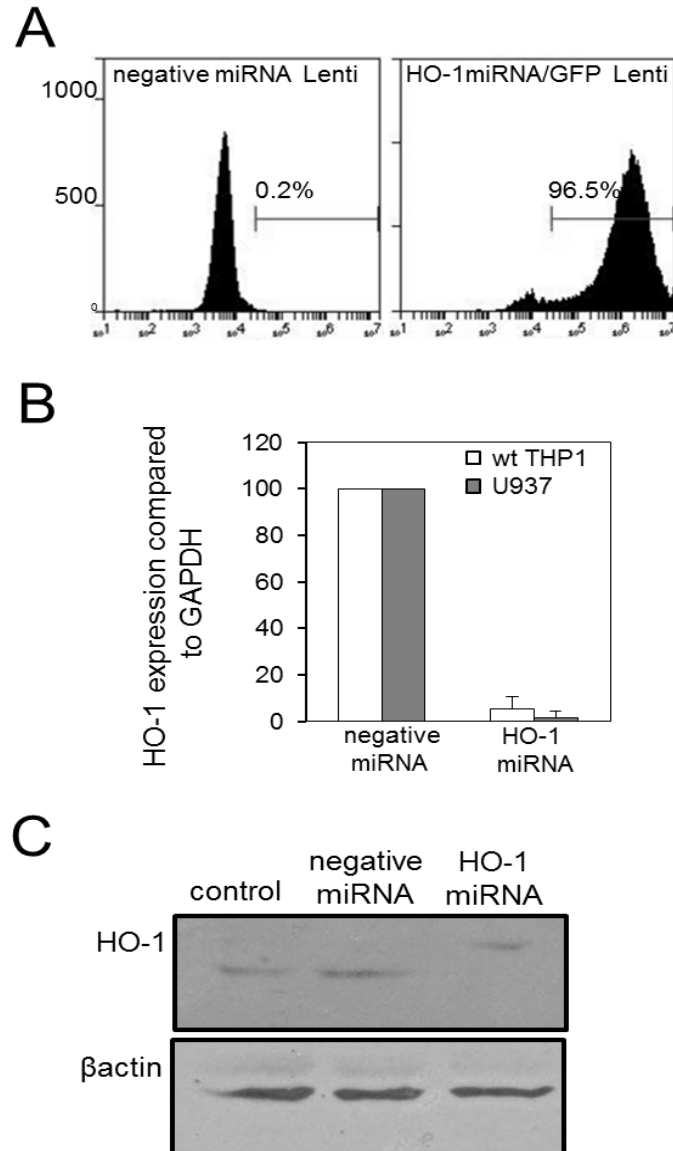
Figure 3.7: Lentiviral- based HO-1 targeting miRNA in wt THP1 and U937 cells. (A) Flow cytometry was used to assess the transfection efficiency of the lentiviral based miRNA HO-1/GFP knockdown construct. (n = 1). Both HO-1 mRNA and protein expression were assessed in HO-1 silenced and control wt THP1 and U937 cells. (B) HO-1 mRNA expression was assessed using qRT-PCR. Bars represent the mean ± SEM (n = 3). Statistica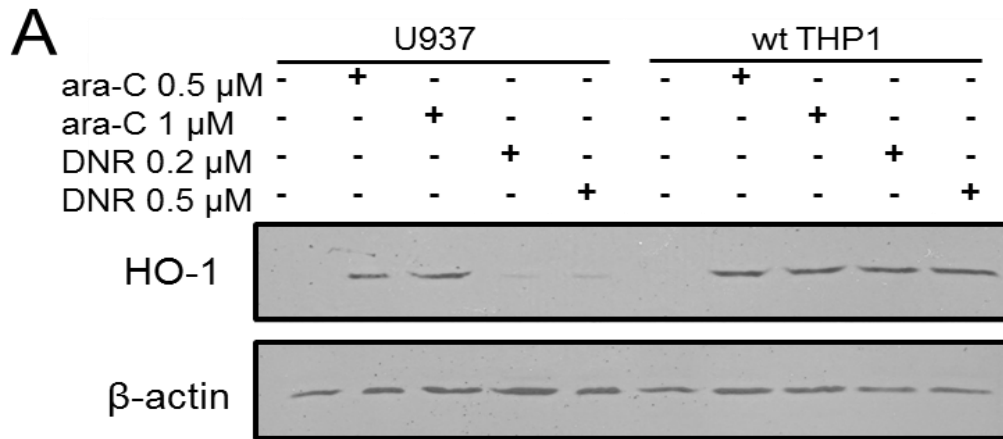l significance was calculated by Student's t-test; *p≤ 0.05. (C) Whole protein extracts were collected and separated using SDS-PAGE, and then investigated using western immunoblotting analysis of HO-1 expression. Equal sample loading across the membrane was assessed using β-actin. Blots are representative of three separate experiments.
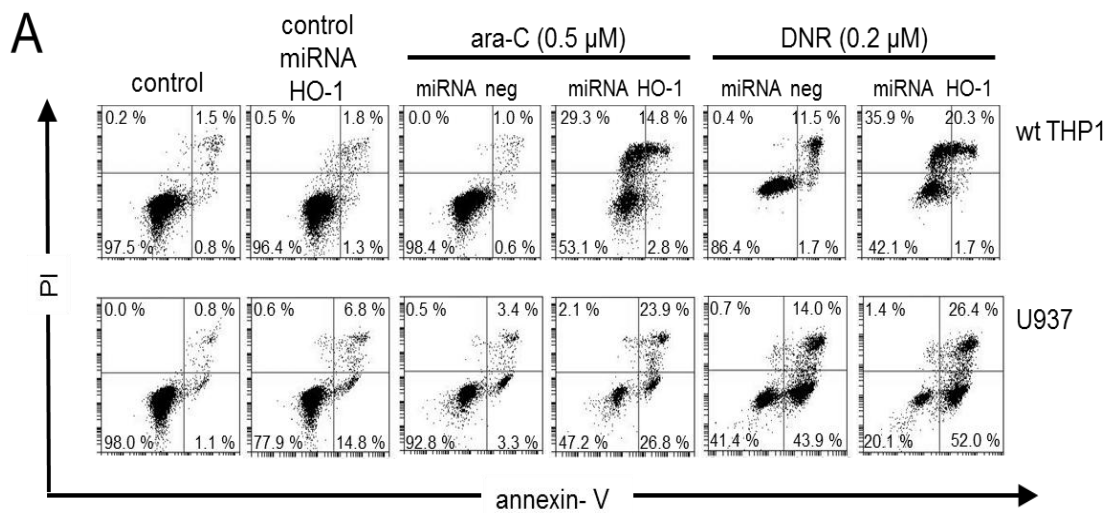
**Figure 3.8: Apoptotic response to ara-C and DNR exposure was increased in HO-1 silenced cells.** (A) HO-1 silenced and miRNA negative wt THP1 and U937 cells were treated with either ara-C (0.5 µM) or DNR (0.2 µM) for 24 h. Cells were stained with annexin-V and PI and number of viable cells was established using flow cytometry. wt THP1 (n = 1), U937 (n = 1). Abbreviations: neg = negative.

3.3.5 ROS modulate the apoptotic potential of ara-C and DNR in AML cells. In healthy cells the cytoprotective effect of HO-1 is activated via ROS. As HO-1 expression appears to confer some protection to AML cells, we wanted to determine whether the enhanced HO-1 expression in response to ara-C or DNR was being medicated through a ROS-dependent response. wt THP1 and U937 cells were treated with either ara-C or DNR for up to 6 h, before incubation with the ROS indicator fluorescent probe H_2DCFDA (1 μM). Flow cytometry was then used to examine ROS generation over time. Figure 3.9 A shows significantly enhanced H_2DCFDA oxidation in wt THP1 cells over time in response to both ara-C and DNR exposure, indicating increased ROS levels. Figure 3.9 B indicates a 'bi-phasic' response to ara-C in U937 cells, a significant decrease in ROS generation can be seen in the first 30 min of ara-C exposure, before a return to basal levels. A significant increase can be seen in response to DNR in U937 cells with levels remaining raised throughout the time course. Potentially suggesting ROS levels are affected by ara-C and DNR in AML cells and therefore may be implicated in the HO-1 protective response.

3.3.6 NAC inhibits apoptosis induced by ara-C and DNR exposure. To confirm the role of ROS in the HO-1 protective response we utilised the antioxidant NAC, which quenches ROS activity present within the cellular environment. We wanted to determine whether NAC could block ara-C or DNR induced apoptosis by inhibiting ROS dependent HO-1 induction. We treated both wt THP1 and U937 cells with ara-C or DNR with or without NAC. Figure 3.10 A shows that NAC inhibits apoptosis and (B) represses HO-1 induction in both ara-C and DNR treated wt THP1 and U937 cells suggesting that ROS is partly responsible for ara-C and DNR induced cell death in AML cells.
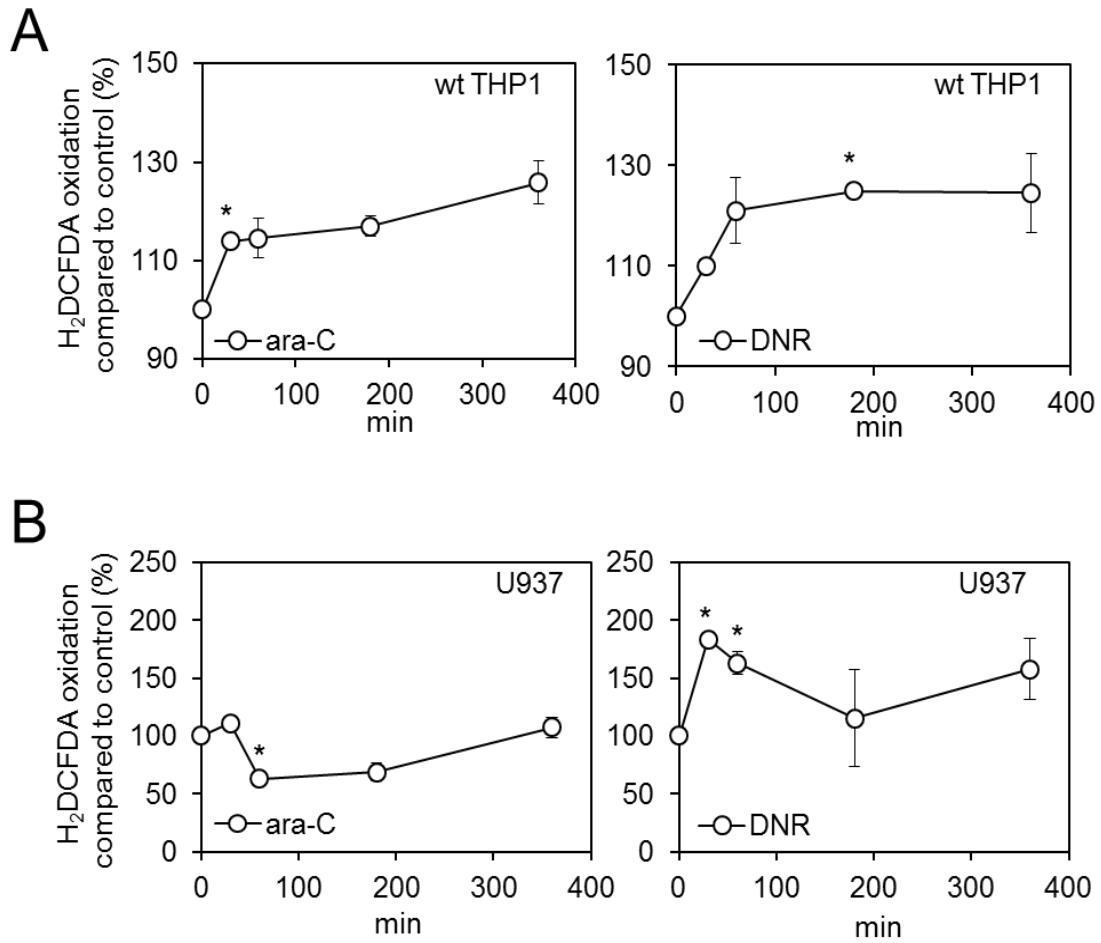
Figure 3.9: Generation of ROS in AML cells in response to ara-C and DNR. (A) wt THP1 and (B) U937 cells were treated with ara-C (0.5 µM, left panels) and DNR (0.2 µM, right panels) for up to 6 h, then washed with PBS and incubated for a further 15 min with the fluorescent probe H₂DCFDA (10 µM). Cleavage of H₂DCFDA/ ROS production in response to chemotherapeutic drug exposure was examined using flow cytometry. Circles represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.
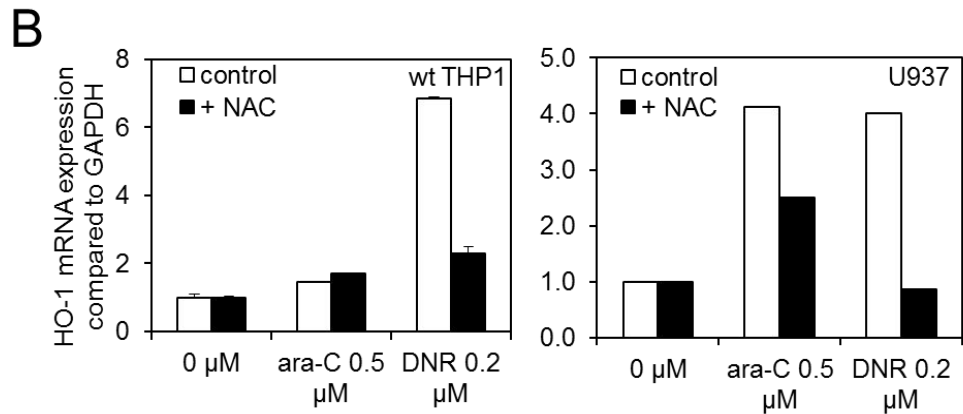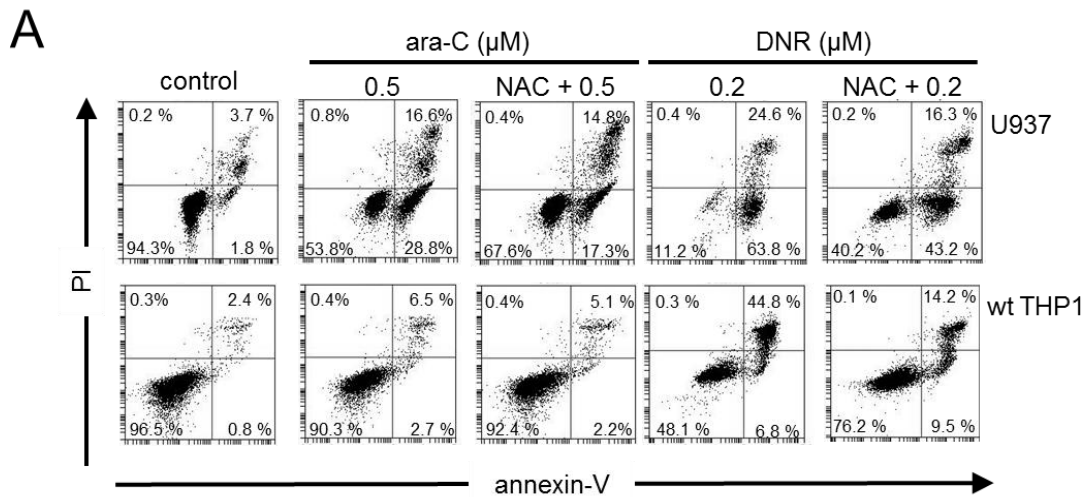
Figure 3.10: N-acetylcysteine (NAC) inhibits apoptosis induced by ara-C and DNR exposure and prevents induction of HO-1. (A) U937 and wt THP1 cells were treated with ara-C (0.5 µM) or DNR (0.2 µM) and incubated for 24 h, with or without 30 min NAC pre-treatment (10 µM for DNR treated cells and 5 µM for ara-C treated cells). Cells were washed and stained with annexin-V/ PI, before flow cytometry. (n = 1). (B) U937 and wt THP1 cells were treated as above before RNA was extracted and HO-1 mRNA expression was measured using qRT-PCR. Bars represent mean ± SEM. (n = 1).

3.4 Discussion

In this chapter we have explored the role of HO-1 within cytoprotection and cell survival in response to either ara-C or DNR treatment in both AML patient samples and AML cell lines. We have shown increased HO-1 expression in response to both ara-C and DNR in a time and dose-dependent manner. This reflects our previous work, which shows that enhanced basal levels of HO-1 protects AML cells from apoptosis in response to both NF-κB inhibition with BAY11-7082 and the proteasome inhibitor, bortezomib, another chemotherapeutic agent used in the treatment of MM. [47, 49].

Cell viability is decreased in both AML cell lines and patient samples in response to increasing chemotherapeutic agent (ara-C and DNR) concentration and exposure time (Figure 3.1 and 3.2) which would be expected. We also investigated the effect of varying concentrations of etoposide, amsacrine, bortezomib, DNR and mitoxantrone on cell viability over a 48 h time course. Yet again both increasing drug concentration and exposure time was shown to significantly reduce cell viability within the population.

HO-1 expression is consistently induced over 24 h in response to ara-C and DNR in a broad range of morphologic and cytogenetic subtypes of primary human AML cells and human AML cell lines [101]. Even though HO-1 expression is increased in response to either ara-C or DNR treatment interestingly, HO-1 mRNA expression is increased further when AML cells are treated with a combination of both agents (Figure 3.4 B). This could potentially be due to each chemotherapeutic agent utilising a different mode of action to induce apoptosis. However, the percentage of viable cells present within the combined treatment population is actually between that of with separate ara-C or DNR exposure (Figure 3.1 B).

When HO-1 expression is silenced in both wt THP1 and U937 cells in combination with either ara-C or DNR treatment a greater apoptotic response is induced. This in combination with the enhanced ROS generation observed reinforces the chemo-protective role of HO-1 in AML cells. By quenching the ROS levels induced by these drugs we were able to prevent HO-1 up-regulation. This suggests that protection from apoptosis conferred to the AML cell is mediated through the generation of ROS. Interestingly, another report has shown enhanced ROS generation in response to ara-C in U937 cells [103].

Kim et al (2006) confirmed HO-1 involvement in cytoprotection against cisplatin exposure in the auditory cell line, H4I-OC1. Protection was provided by carbon monoxide and bilirubin which inhibited the generation of ROS [106].

Furthermore, a study from Nuhn et al (2008), has shown that HO-1 expression is usually increased in human pancreatic cancer cells and is increased further by the addition of cytotoxic agents [107]. Both ROS and HO-1 expression have also been shown to be up regulated in response to cisplatin treatment in A549 cells, a lung cancer cell line. Interestingly, when a HO-1 inhibitor, ZnPP was added, ROS expression was elevated even further [108]. Our study, however, is the first to show that chemotherapy-induced ROS levels may be linked to the expression of HO-1 in AML [101].

Silencing of HO-1 expression in both wt THP1 and U937 cells showed a strong decrease in the percentage of viable cells after ara-C or DNR treatment. Therefore we suggest inhibiting HO-1 could potentially enhance AML cell response to ara-C and DNR treatment *in vivo*. Kim et al (2008) also transfected A549 cells with HO-1 siRNA, a decrease in cell viability in response to cisplatin exposure was noted [108]. Whilst Fang et al (2004) have shown the presence of a HO-1 inhibitor *in vivo* can enhance tumour responsiveness to chemotherapeutic agents [109].

There are a number of different proposed mechanisms for the cytoprotective effect of ROS-induced HO-1 in AML cells. The first is by decreasing the levels of the pro-oxidant, haem [109, 110] the second by increasing the concentration of bilirubin, an antioxidant [109]; and the final mechanism is by increasing the levels of carbon monoxide, an anti-apoptotic molecule [66, 111, 112]. Together, these mechanisms may act either alone or in combination to protect cancer cells from apoptosis. Interestingly AML cells seem to have the ability to manipulate this pathway in order to provide themselves with a growth advantage over normal non-cancerous cells.

We have recently reported that basal levels of HO-1 are low compared to normal non-cancerous cells and that NF-κB regulation reduces basal HO-1 expression [49]. Moreover, Nrf2 activation is able to override this regulation [49]. In fact, a number of transcription factors can regulate HO-1 expression such as Nrf2, AP-1 and NF-κB [78-

80].   During this chapter we have concentrated on the role of HO-1 expression in regulating chemotherapy induced apoptosis but we are not in a position to exclude the involvement of other transcriptional elements.
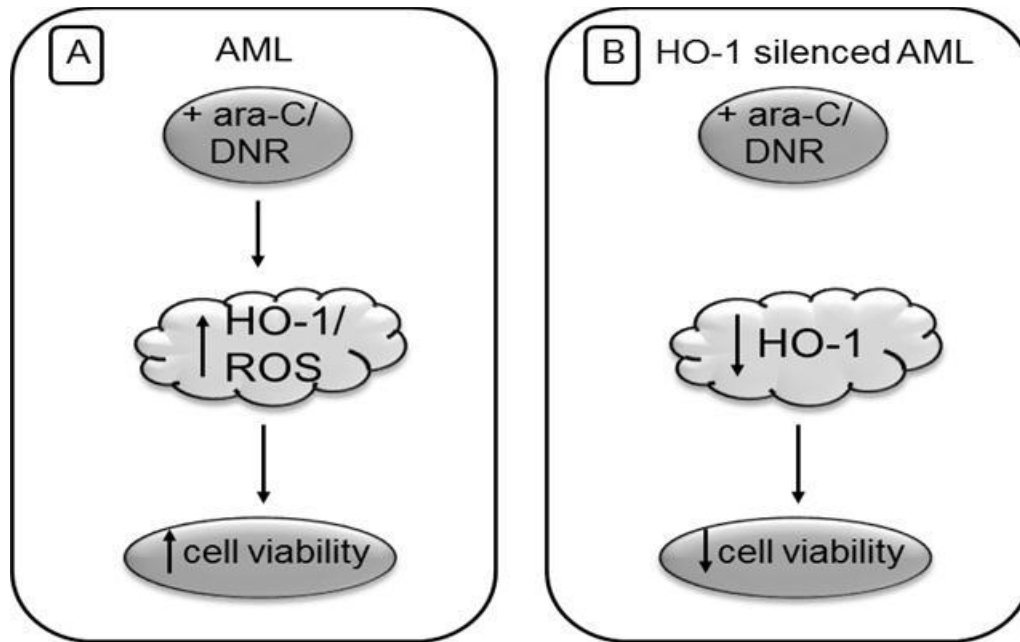
Figure 3.10: Overview of cell viability in relation to HO-1 expression. Diagrammatic representation of HO-1s expression in relation to cell viability. (A) ara-C and DNR or a combination thereof induce HO-1 mRNA and protein expression resulting increased cell viability and reduced cell apoptosis. (B) When HO-1 expression is silenced, cell viability is reduced thus increase cell apoptosis was seen [99].

To conclude, we report silencing HO-1 expression increases the in-vitro sensitivity of AML cells to ara-C and DNR, the two front-line chemotherapy agents currently widely used to treat the disease. Previously we have shown that HO-1 plays an important role in protecting AML cells from apoptotic stimuli and here we propose that this is likely to be clinically relevant. Therefore HO-1 warrants further investigation as a potential target for future therapeutic strategies.

Chapter 4: Exploring cytarabine resistance in AML cells

4:1 Introduction

Currently within the clinic setting ara-C and DNR are both used to treat AML in younger and fitter patients. After extensive literature review regarding AML ara-C resistance and the follow on from our groups focus we decided to investigate ara-C resistance in more detail. Over the last 10 years a number of research groups have developed various leukaemia resistant cells lines to front-line chemotherapeutic agents.

For example Oerlemans et al (2008) produced a THP1 bortezomib resistant cell line which over-expressed the proteasome subunit β5 (PSMB5) [113]. Bortezomib is licenced for use in MM treatment [5] however a number of groups have recently been investigating its use within AML treatment [114]. Whilst Funato et al (2000) developed an ara-C resistant K562 (K562/AC) cell line by increasing ara-C concentration and exposure over a 3 month period. KF-19, an human Myeloid Leukaemia cell line was established by Fukuda et al (1996), they also went on to develop three resistant sub lines which were resistant to ara-C, adriamycin and vincristine; KF-19AraC, KF-19ADR and KF-19VCR [115].

Funato et al (2000) then went onto explore K562/AC sensitivity towards other chemotherapeutics agents; vincristine, adriamycin and etoposide. The K562/AC cells still maintained a similar degree of sensitivity towards each of above agents compared to that of their parental K562 cells. However a decrease in Deoxycytidine Kinase (dCK) expression was noted. Thus the authors proposed a link between decreased dCK expression and ara-C resistance in leukaemia cells. During ara-C metabolism, ara-C is phosphorylated by dCK, before being incorporated into the cell's DNA. Therefore reduced dCK could result in cellular resistance to ara-C because ara-C initial phosphorylation is inhibited [44].

A number of ara-C resistance mechanisms within AML primary and cell lines have been proposed recently, briefly explained in Table 4.1.

Table 4.1: Resistance Theories

Cell line	Resistance mechanism	Reference
THP1 bortezomib resistant cell line	PSMB5 over-expression	[113]
K562/AC	dCK down regulation thus initial ara-C phosphorylation inhibited	[44]
K562/AC	IGF-1 up-regulation in response to ara-C	[43]
Ara-C resistant cell lines	DFS linked to hENTI expression. Absence of hENTI results in shorter DFS	[42]

Abe et al (2006) utilised Funatos' (2000) ara-C resistant K562/AC cells to explore insulin-like growth factor 1 (IGF-1) expression and its involvement in ara-C resistance. IGF-1 was subsequently up-regulated in the ara-C resistant cells, K562/AC. When ara-C sensitive K562 cells were incubated with both ara-C and the IGF-1 ligand, protection against ara-C induced apoptosis was seen. However the addition of IGF-1 receptor neutralizing antibody, significantly inhibited cell growth and increased apoptosis. Potentially indicating that IGF-1 is involved in cellular acquired ara-C resistance. The group goes on to further explore IGF-1 expression in 27 patient AML samples and found IGF-1 expression to be higher in patients in the refractory stage after therapy containing ara-C than those at diagnosis. Therefore, suggesting inhibiting IGF-1 could prove be a potential therapeutic approach to overcoming ara-C resistance [43].

Another proposed ara-C resistance mechanism involves Human equilibrative nucleoside transporter 1 (hENT1). Galmarini et al (2002) found that 93% of AML patients showed expression for hENT1 at diagnosis. The small minority of patients who did not express the transporter, had shorter Disease Free Survival (DFS) than those who did, therefore suggesting the absence of the transporter, hENTI could be involved in ara-C resistance [42]. hENT1 is one of the main transporters of ara-C in AML blast cells [116].

Ara-C resistant cell lines have also been shown to have increased Cytoplasmic 5'-nucleotidase (5NT) expression and enzyme activity compared to their sensitive controls. Galmarini et al (2002) have gone on to connect high 5NT expression in AML patients

with ara-C resistance [42]. However, the group have previously reported high 5NT expression is related to AML patient outcome [41]. But, they later went on to report CR (complete remission) is related to ara-C dose in patients expressing low levels of 5NT at diagnosis [42].

However we appear to be the first group to investigate the cytoprotective gene, HO-1s involvement and regulation in ara-C acquired resistance in AML cell lines.

4.2 Aims

In this chapter we aim to create an ara-C resistant THP1 cell line (THP1ara-C(1)) to explore the cytoprotective pathways involved within apoptosis. We want to investigate functional changes which have occurred due to ara-C acquired resistance and establish whether the acquired ara-C resistance is a transient or stable response.

4.3 Methods

Cell culture containing wt THP1 cells was refreshed with ara-C (1 µM) weekly. Ara-C resistance was achieved by stepwise (an increase of 0.2 µM per week) weekly media refreshments (Figure 4.1) these cells were referred to as THP1ara-C(1) [113] [44]. The dose used to refresh the cells was selected on the therapeutic concentration (1 µM) found in AML patients treated with ara-C [105]. Resistance to ara-C was assessed via apoptotic assay using both CellTitre-Glo® Luminescent Cell Viability Assay and Trypan Blue Exclusion Assay. To explore whether ara-C resistance was a transient or stable response, ara-C media refreshment was withdrawn for 4 weeks and ara-C resistance was explored again as previously described, these cells are referred to as THP1ara-C(1) TW.
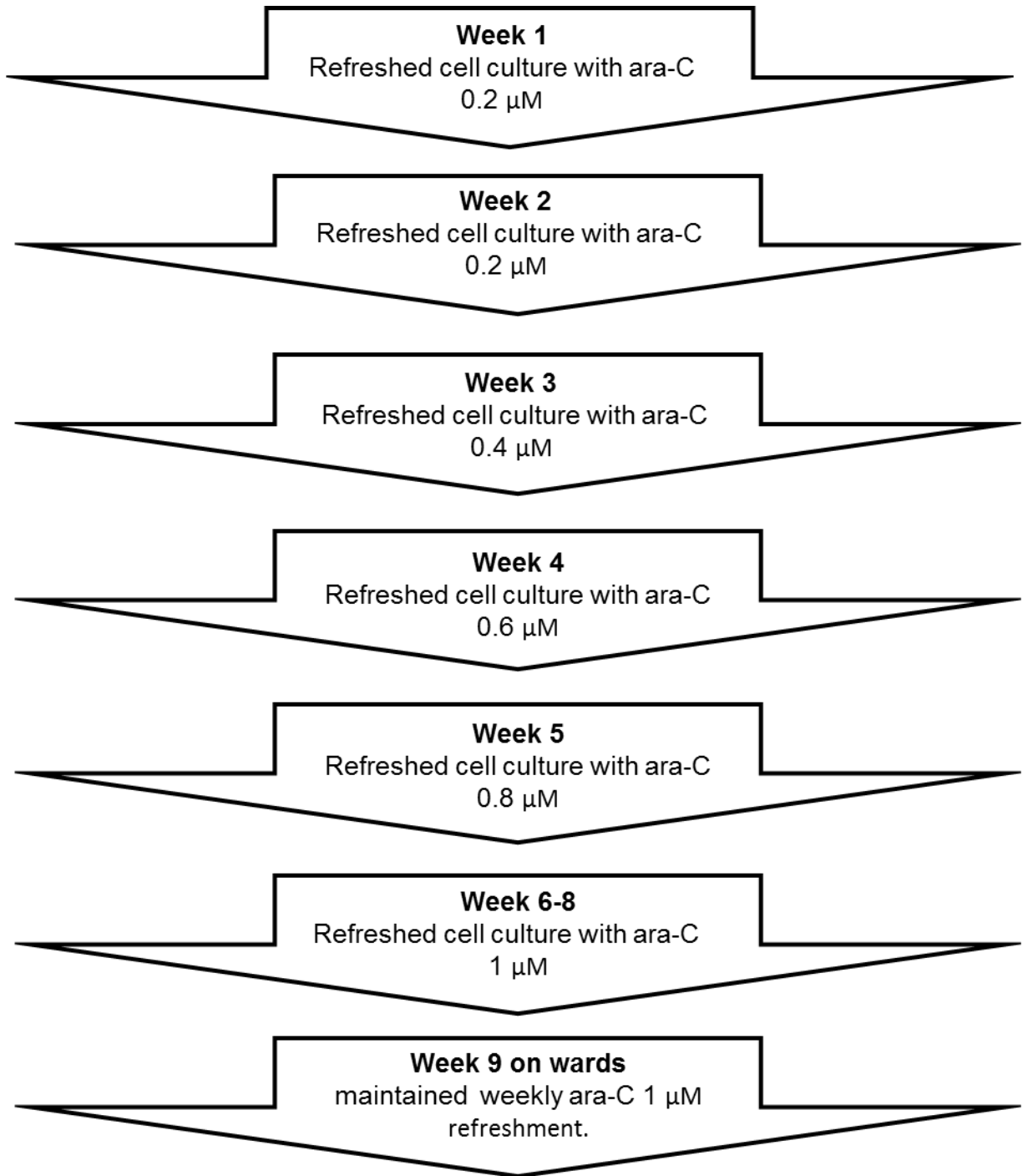
Figure 4.1: ara-C resistance development in THP1ara-C(1) over a 9 week period. wt THP1 cells were exposed to increasing doses of ara-C.

4.4 Results

4.4.1 Confirming resistance to ara-C. To confirm that THP1ara-C(1) cells had acquired a resistance to ara-C, both THP1ara-C(1) and wt THP1 cells were treated with increasing doses of ara-C (0.2- 200 μM) over 48 h. Figure 4.2 A shows increased cell death within the wt THP1 population compared to that of the THP1ara-C(1) cells in a dose dependant manner. Similarly Figure 4.2 B illustrates increased cell death within the wt THP1 population in response to 10 μM ara-C in a time-dependent manner and confirms that wt THP1 are significantly more susceptible to ara-C induced cell death than the THP1ara-C(1) population.

Next we wanted to explore whether the THP1ara-C(1) cells showed any resistance towards other chemotherapeutic agents also used in the treatment of AML. Figure 4.3 A-C demonstrates cell death in response to varying concentrations of bortezomib, etoposide and DNR after 24 h of drug exposure. Both wt THP1 and THP1ara-C(1) cells appear to be equally sensitive to etoposide (IC_{50} values for wt THP1 35 μM and THP1ara-C(1) 70μM) and DNR (IC_{50} values for wt THP1 0.5 μM and THP1ara-C(1) 0.55 μM) and show a significant decrease in cell viability with increasing chemotherapeutic concentration. Interestingly however, the THP1ara-C(1) cells show a small but significant reduction in sensitivity towards bortezomib (IC_{50} values for wt THP1 9 nM and THP1ara-C(1) 60 nM) compared to the wt THP1 cells (Figure 4.3 A).

Table 4.1 states wt THP1 and THP1ara-C(1) IC_{50} values for ara-C, bortezomib, DNR and etoposide. 48 h post treatment with ara-C, wt THP1 IC_{50} value is 6 μM whilst THP1ara-C(1) IC_{50} is increased to 100 μM. IC_{50} values for bortezomib, DNR and etoposide are after 24 h exposure.

Table 4.1: IC_{50} for chemotherapeutics used in AML treatment.

	IC_{50}	
chemotherapeutic agent	**wt THP1**	**THP1ara-C(1)**
ara-C	6 μM	100 μM
Bortezomib	9 nM	60 nM
DNR	0.5 μM	0.55 μM
etoposide	35 μM	70 μM
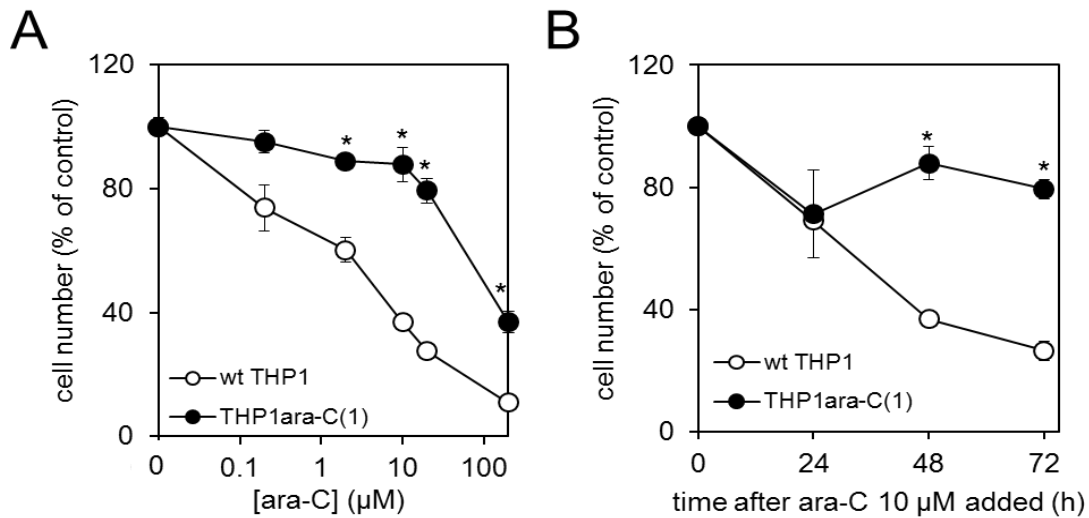
Figure 4.2: THP1ara-C(1) show decreased sensitivity towards ara-C compared to wt THP1. (A) Both wt THP1 and THP1ara-C(1) cells were incubated with varying doses of ara-C (0.2, 2, 10, 20, 200 µM) for 48 h before cell viability was analysed by CellTitre-Glo® Luminescent Cell Viability Assay (n = 3). (B) wt THP1 and THP1ara-C(1) cells treated with ara-C (10 µM) for 24, 48 and 72 h before cell viability was analysed by CellTitre-Glo® Luminescent Cell Viability Assay (n = 3). Cell number is expressed as a percentage of the control. Circles represent the mean ±SEM. Statistical significance was calculated by Student's t-test; *p≤0.05.
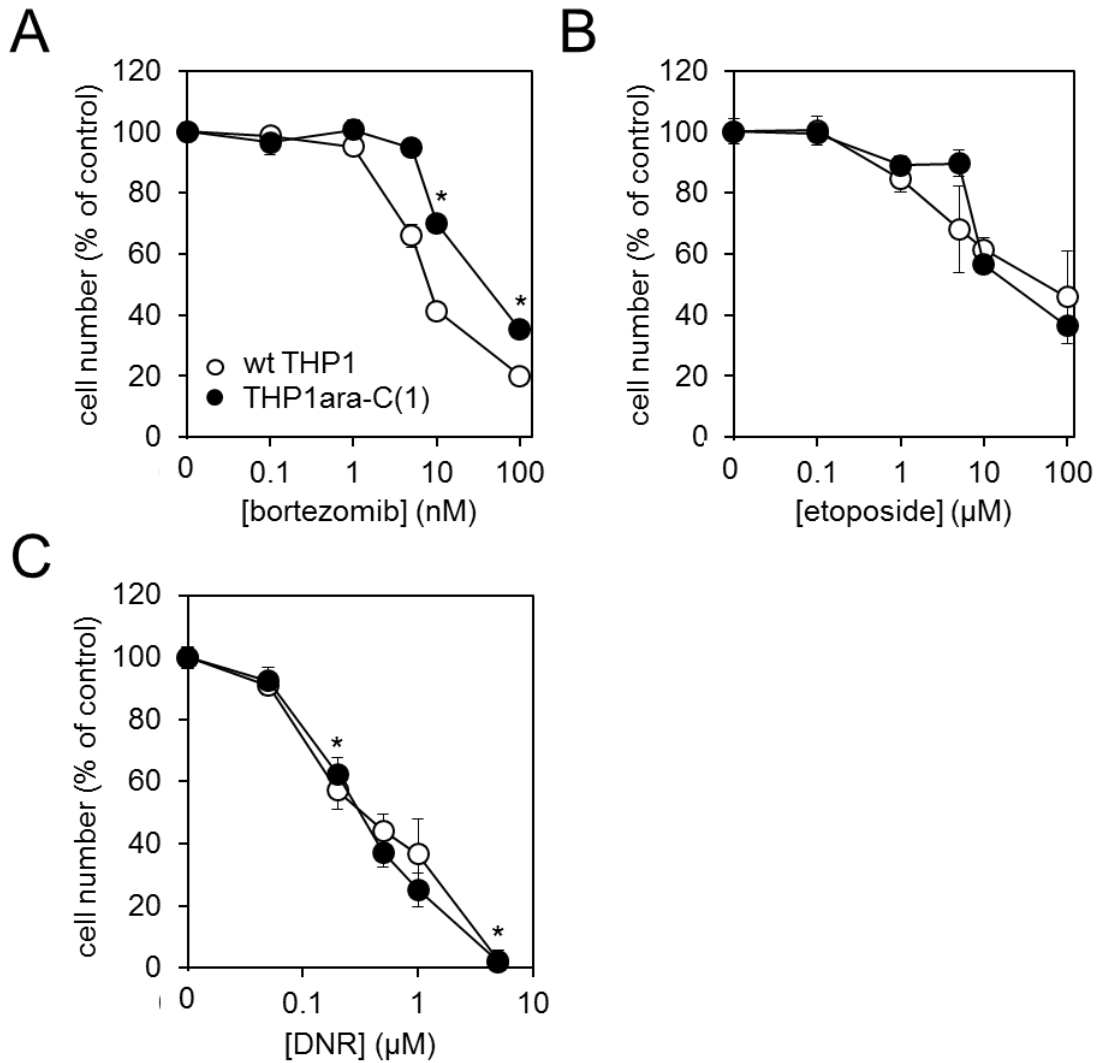
Figure 4.3: THP1ara-C(1) cell maintain their sensitivity towards other chemotherapeutics used to treat AML. (A) wt THP1 and THP1ara-C(1) cell were treated with varying dose of bortezomib (0, 0.1, 1, 5, 10, 100 nM) (B) etoposide (0, 0.1, 1, 5, 10, 100 μM) or (C) DNR (0, 0.05, 0.2, 0.5, 1, 5 μM) for 24 h before cell viability was analysed by CellTitre-Glo® Luminescent Cell Viability Assay. Cell number is expressed as percentage of control. Circles represent the mean ±SEM (n = 3). Statistical significance was calculated by Student's t-test; *p≤0.05.

4.4.2 Ara-C resistance alters cell doubling rate. To investigate any potential difference in cell proliferation rate between both wt THP1 and THP1ara-C(1) cells we used both Trypan Blue exclusion assay and a clonogenic methylcellulose assay (Figure 4.4). Figure 4.4 A shows the cell proliferation of both wt THP1 and THP1ara-C(1) cells as analysed by Trypan Blue exclusion assay. The cell doubling rate of THP1ara-C(1) is significantly increased compared to wt THP1 control cells. The doubling times for wt THP1 and THP1ara-C(1) are 2.2 days (SEM ± 0.10) and 2.7 days (SEM ± 0.07), respectively. Figure 4.4 B illustrates the number of THP1ara-C(1) colonies produced over 14 days is significantly reduced compared to that of the wt type THP1 cells. Figure 4.4 C examines the granularity of both cell types as assayed by flow cytometry. Both forward and side scatter have increased for THP1ara-C(1) indicating the cells have increased in volume and granularity.

Next we wanted to examine how continual ara-C exposure affected the cells ability to cycle. We investigated cell cycling in wt THP1, THP1ara-C(1) and THP1ara-C(1) Treatment Withdrawn (THP1ara-C(1) TW) cells (Figure 4.5). THP1ara-C(1) TW cells are ara-C resistant THP1ara-C(1) cells where weekly ara-C refreshment has been withdrawn. In the ara-C resistant THP1ara-C(1), a decrease in the number of cells undergoing G1 and G2 phase and an increase in the number of cells in S phase was reported in response to continual ara-C exposure compared to the control wt THP1 cells. Upon ara-C withdrawal (THP1ara-C(1) TW), both G1 and S phase appear to return to that of the control wt THP1 cells. Potentially suggesting the removal of continual ara-C exposure is rescuing the cells from S phase and allowing them to progress into G2 phase. Therefore ara-C is affecting the cells ability to progress from S phase to G2 phase.
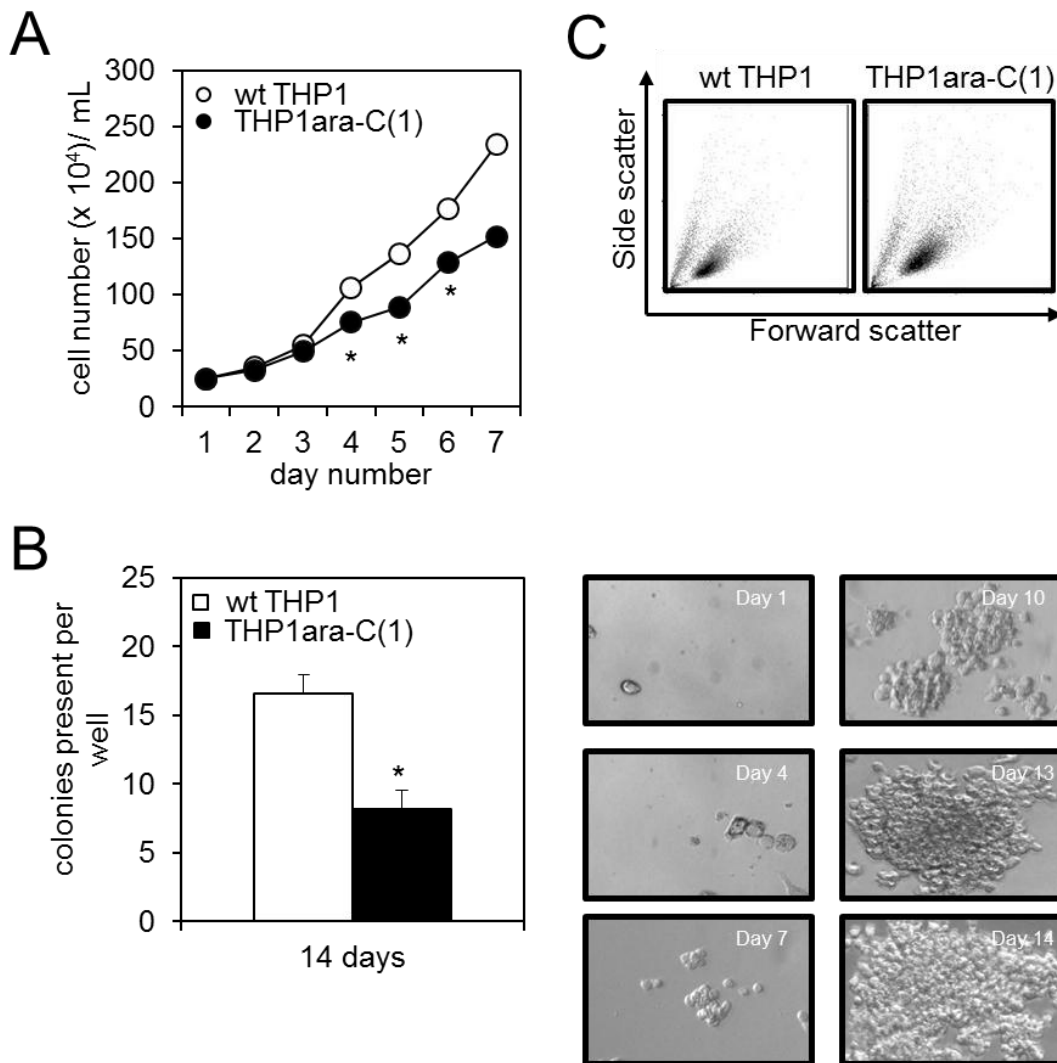
Figure 4.4: THP1ara-C(1) Cell proliferation is reduced compared to that of the wild type THP1 AML cells. (A) 25x10^4 wt THP1 and THP1ara-C(1) were cultured for 7 days and the number of viable cells was determined daily by Trypan Blue exclusion assay. Each circle represents the mean ±SEM (n = 3). (B) 2x10^4 wt THP1 or THP1ara-C(1) were plated in methylcellulose medium and incubated for 14 days. Number of colonies present was imaged on days 1, 4, 7, 10, 13, and 14 (right panel) and visualised counted on day 14. Each bar represents the mean ±SEM (n = 3). (C) Granularity (forward and side scatter) of wt THP1 and THP1ara-C(1) cells was measured using flow cytometry. Statistical significance was calculated by Student's t-test; *p≤ 0.05.
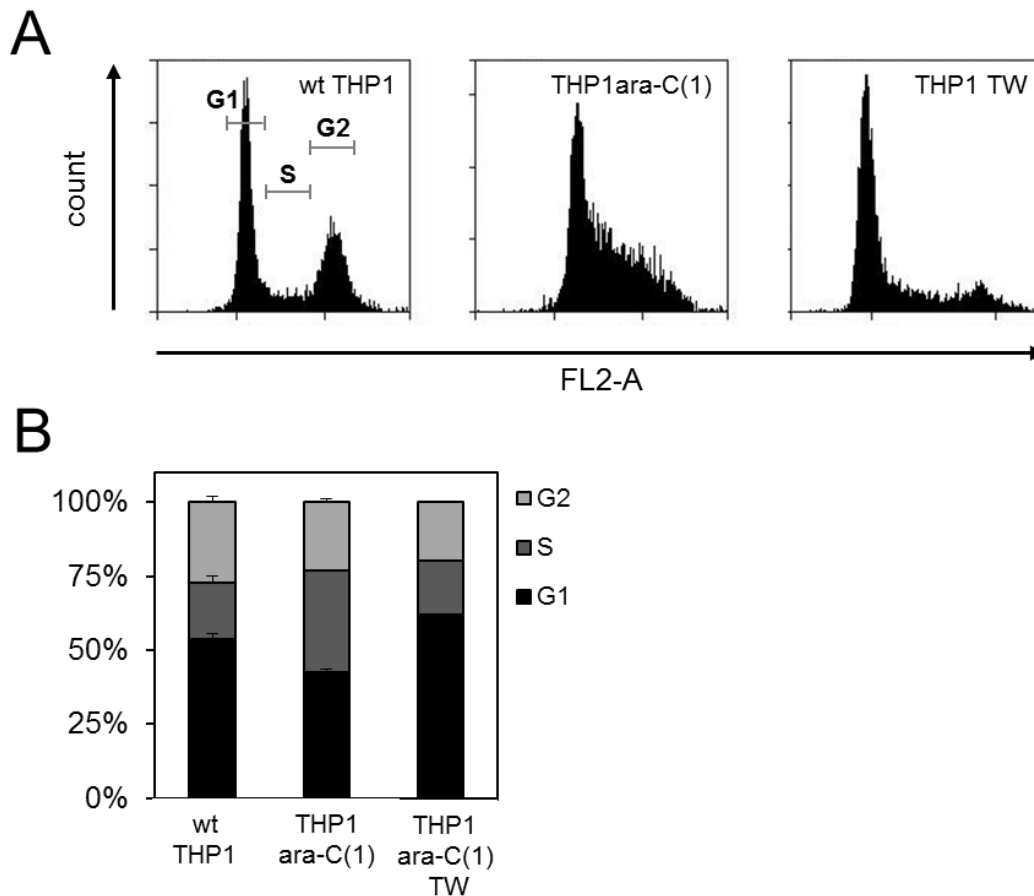
Figure 4.5: S phase is increased in THP1ara-C(1) cells. 2×10^6 wt THP1, THP1ara-C(1) and THP1ara-C(1) TW cells were harvested and fixed using 100% ethanol. Cells were stained with PI before analysis using flow cytometry. (A) Visual diagrammatic representation of cell cycling within each cell line. (B) Bar chart indicating percentage of cells in each phase of the cell cycle. Each bar represents the mean ±SEM (n = 3). Statistical significance was calculated by Student's t-test; *p≤ 0.05.

4.4.3 Exploring the effect of combination chemotherapy on ara-C resistant and non-resistant cells. During AML treatment, a number of chemotherapeutics agents can be used in combination; for example, ara-C and DNR are often combined in a '7+3' treatment regime [19, 20] Bortezomib is licenced for use in Multiple Myeloma treatment however we have previously explored bortezomib effect on AML cells and found them to be less sensitive towards bortezomib than their control non-cancerous cells [47]. Thus we decided to explore the effect of different combinations of these drugs on cell death in both the wt THP1 and THP1ara-C(1) cells.

wt THP1 and THP1ara-C(1) cells were treated with either ara-C (10 μM), DNR (0.2 μM), bortezomib (10 nM), or combinations thereof, for either 24 or 48 h (Figure 4.6 A-B). A significant increase in cell death was seen in both wt THP1 and THP1ara-C(1) cells in response to DNR, bortezomib and all drug combination treatments at 24 h. Again, THP1ara-C(1) showed reduced sensitivity towards bortezomib whilst they maintained sensitivity towards DNR. However, by 48 h post bortezomib treatment the number of viable THP1ara-C(1) cells is greatly reduced compared to that at 24 h, thus suggesting the THP1ara-C(1) cells experience a delayed response to bortezomib. As expected wt THP1 cells show a significant increase in cell death in response to ara-C alone 48 h post treatment, whilst both wt THP1 and THP1ara-C(1) show a significant increase in cell death in response to DNR, bortezomib and all combinations thereof.

4.4.4. Ara-C resistance alters basal expression of cytoprotective genes. We wanted to explore the basal expression of genes involved in cytoprotection thus we used qRT-PCR. As we have previously shown in chapter 3, upon ara-C and DNR treatment, HO-1 expression is increased in wt THP-1, HL60, U937 cell lines and AML patient samples. Thus we predicted HO-1 expression would also be increased in the ara-C resistant THP1 cells and thus wanted to examine other cytoprotective gene expression in response to prolonged ara-C exposure. Figure 4.7 A illustrates the expression of HO-1, NQO1 and Nrf2 mRNA, all of which are significantly down-regulated in THP1ara-C(1) cells. Figure 4.7 B examines protein expression of these cytoprotective genes and reiterates the results observed at mRNA level. Due to the nature of maintaining ara-C resistance in THP1ara-C(1) cells we wanted to further investigate the natural variation in cytoprotective gene expression over a 7 day time course. qRT- PCR was again utilised to investigate HO-1, NQO1, Nrf2 and GCLM mRNA expression (Figure 4.8 A). NQO1

and Nrf2 mRNA expression is down-regulated, relative to the control wt THP1 cells, throughout the 7 day time course. However, HO-1 is up regulated on Day 6 in the ara-C resistant THP1ara-C(1) cells. The increase in HO-1 regulation could be explained by the following scenario, the cells are usually refreshed with media and ara-C on Day 6, therefore prior to feeding we would expect elevated HO-1 levels due to increased cellular and oxidative stress induced by lack of media nutrients and the presence of ara-C. Nrf2 expression also appears slightly elevated on Day 6 therefore mirroring HO-1s, which is unsurprisingly as Nrf2 is known to induce the expression of HO-1.
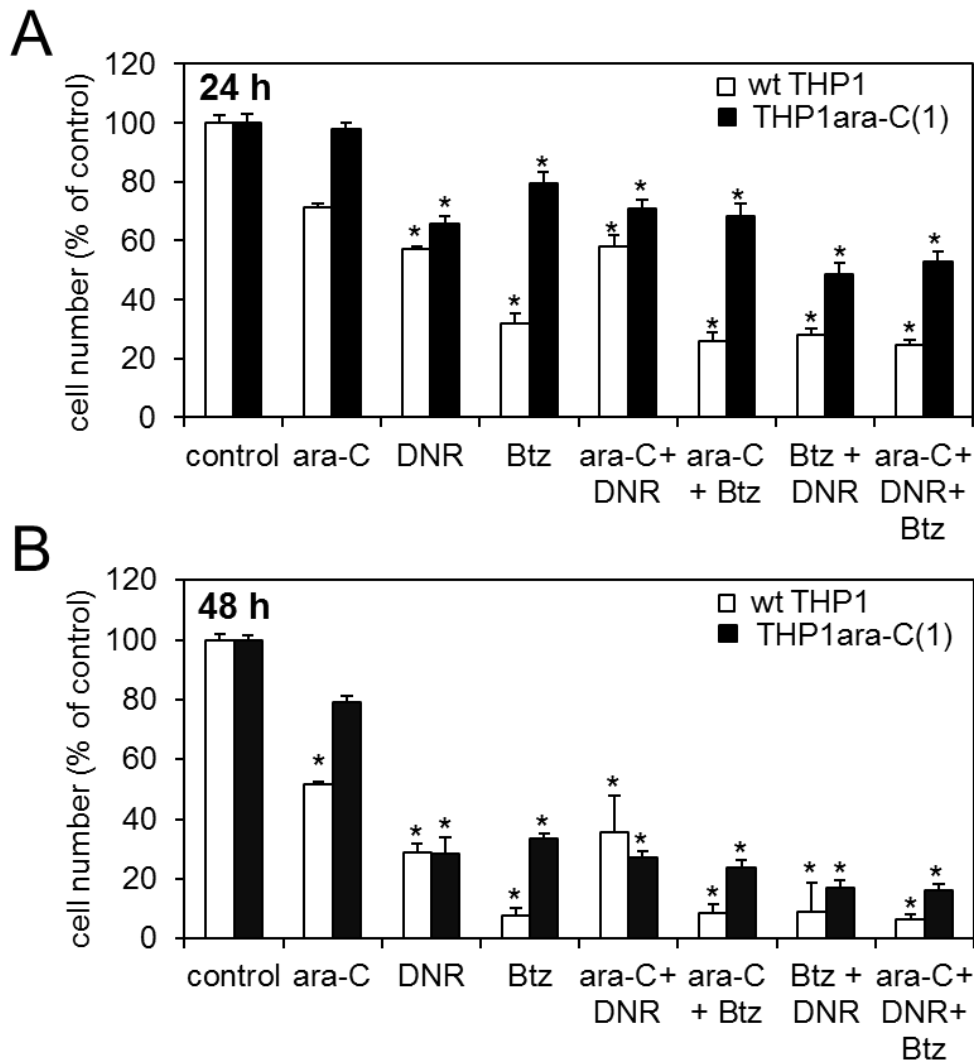
Figure 4.6: THP1ara-C(1) show delayed response to bortezomib but remain sensitive towards DNR and combinations of chemotherapeutic agents. (A) wt THP1 and THP1ara-C(1) cells were treated with ara-C (10 µM), bortezomib (10 nM), DNR (0.2 µM), or combinations thereof, for 24 h or (B) 48 h before cell viability was analysed using CellTitre-Glo® Luminescent Cell Viability Assay. Cell number is expressed as percentage of control. Bars represent the mean ±SEM (n = 3). Statistical significance was calculated by Student's t-test; *p≤0.05. Abbreviations Btz = Bortezomib.
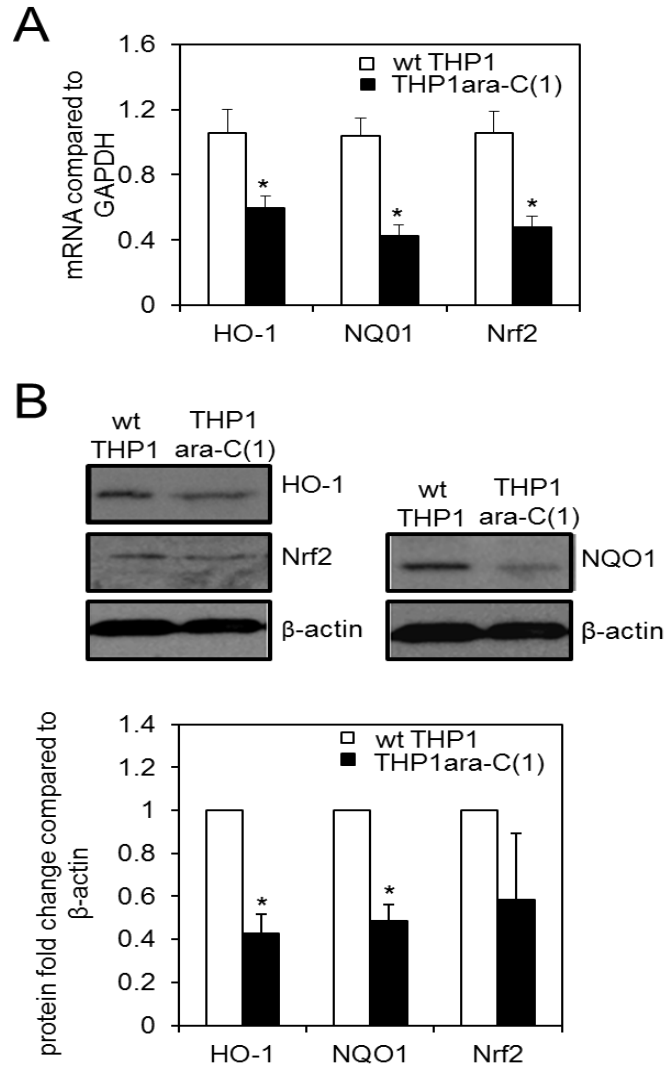
Figure 4.7: Basal expression of key cytoprotective genes is down-regulated in THP1ara-C(1) AML cells. (A) RNA was extracted from wt THP1 and THP1ara-C(1) for qRT-PCR analysis of basal HO-1, NQO1 and Nrf2 mRNA expression. Bars represent the mean ±SEM (n = 3). (B) Whole cell protein extracts were collected from wt THP1 and THP1ara-C(1) for Western immunoblotting analysis of basal HO-1, NQO1 and Nrf2 protein expression. Membranes were probed with β-actin to ensure equal protein loading across all samples (top panel). Quantity One 1-D Analysis software (BioRad) was utilised to quantify protein fold changes between cell types normalised to β-actin (bottom panel). Blots representative of three separate experiments. Bars represents mean ±SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.
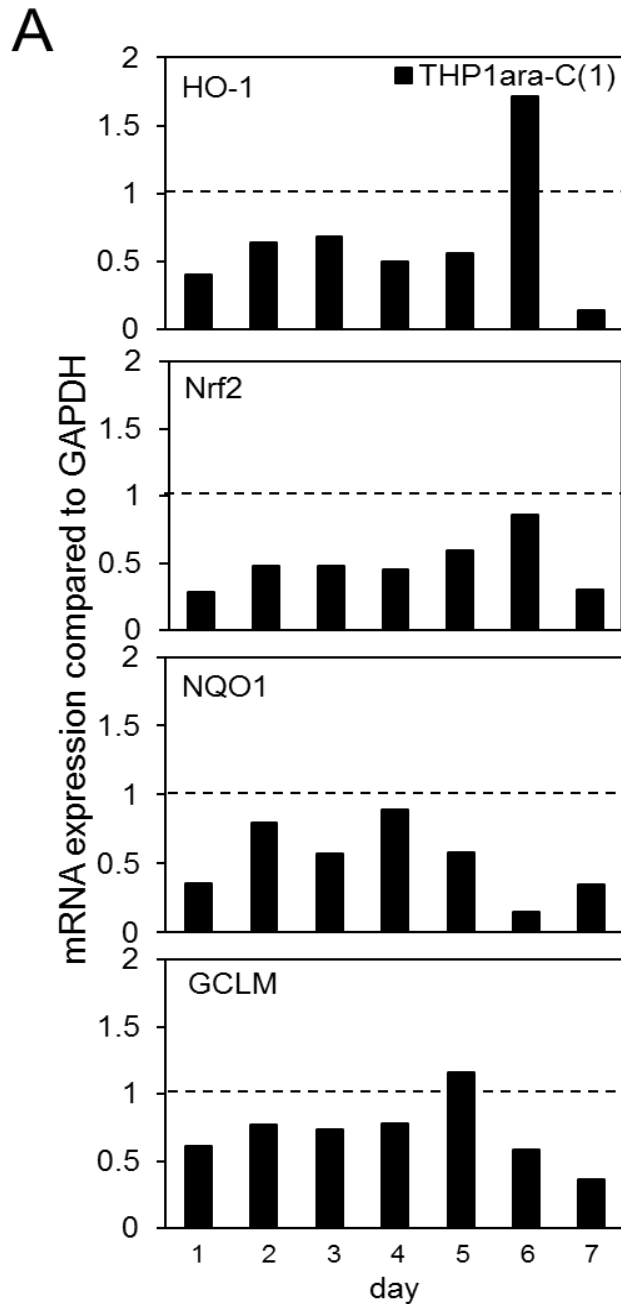
Figure 4.8: Examining natural variation of key cytoprotective genes over a 7 day time-course in THP1ara-C(1) cells. (A) RNA was extracted from wt THP1 and THP1ara-C(1) for qRT-PCR analysis of basal HO-1, NQO1, Nrf2 and GCLM mRNA expression over a 7 day time course. Data for each time-point is normalised to wt THP1 expression, represented by a dashed line. (Technical replicates = 2).

4.4.5 ROS production is elevated in ara-C resistant cells. Due to the altered expression of cytoprotective anti-oxidant genes in THP1ara-C(1) cells we further explored levels of ROS in these cells. Figure 4.9 A shows a significant increase in basal ROS production in THP1ara-C(1) cells. Next, we wanted to investigate ROS generation over a 7 day time course. Figure 4.9 B shows that ROS production cycles over the 7 day culture period and is dependent on levels of ara-C remaining in the culture medium. We decided to investigate how the enhanced ROS generation in the THP1ara-C(1) cells affected basal glutathione production. Figure 4.9 C shows a significant increase in glutathione production in the THP1ara-C(1) cells compared to control wt THP1s.

4.4.6 Ara-C resistant cells show increased ROS generation in response to further ara-C exposure. We wanted to explore the potential for ROS generation in THP1ara-C(1) and wt THP1 cells in response to either ara-C, DNR, bortezomib, or combinations thereof, after 24 h (Figure 4.10 A). ROS production is increased however not significantly, when either wt THP1 or THP1ara-C(1) cells are exposed to ara-C, DNR or a combination of both agents for 24 h. ROS generation is decreased, however not significantly, when either wt THP1 or THP1ara-C cells are exposed to bortezomib on its own or in combination with another chemotherapeutic agent.
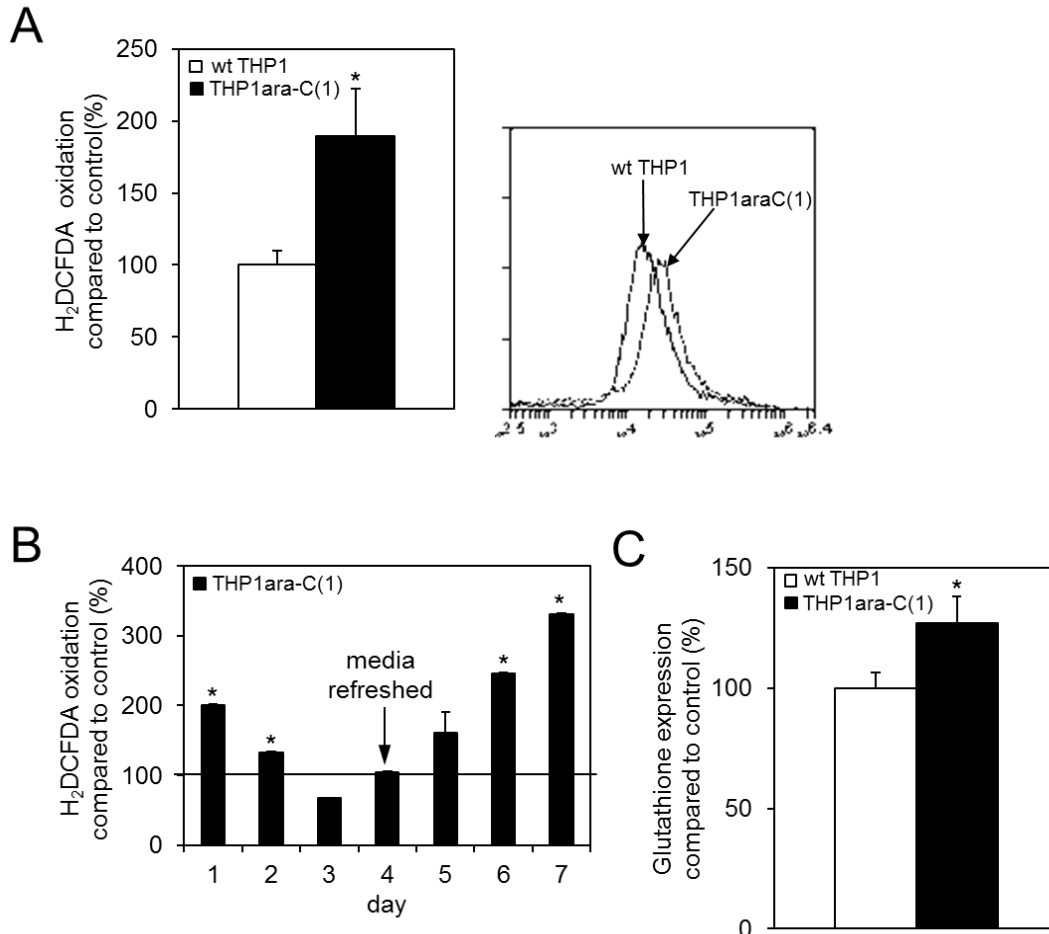
Figure 4.9: Basal ROS levels are up-regulated in THP1ara-C(1). (A) wt THP1 and THP1ara-C(1) cells were incubated with the fluorescent probe H_2DCFDA (1 μM) for 30 min before ROS production was measured using flow cytometry and is expressed as a percentage of wt THP1 levels (n = 7). (B) This was repeated daily over a 7 day time course with wt THP1 levels represented by a black line (n = 3). (C) Glutathione production was investigated in wt THP1 and THP1ara-C(1) cells using Promega GSH-Glo™ Glutathione assay. Bars represents mean ± SEM (n = 7). Statistical significance was calculated by Students t-test; * p≤0.05.
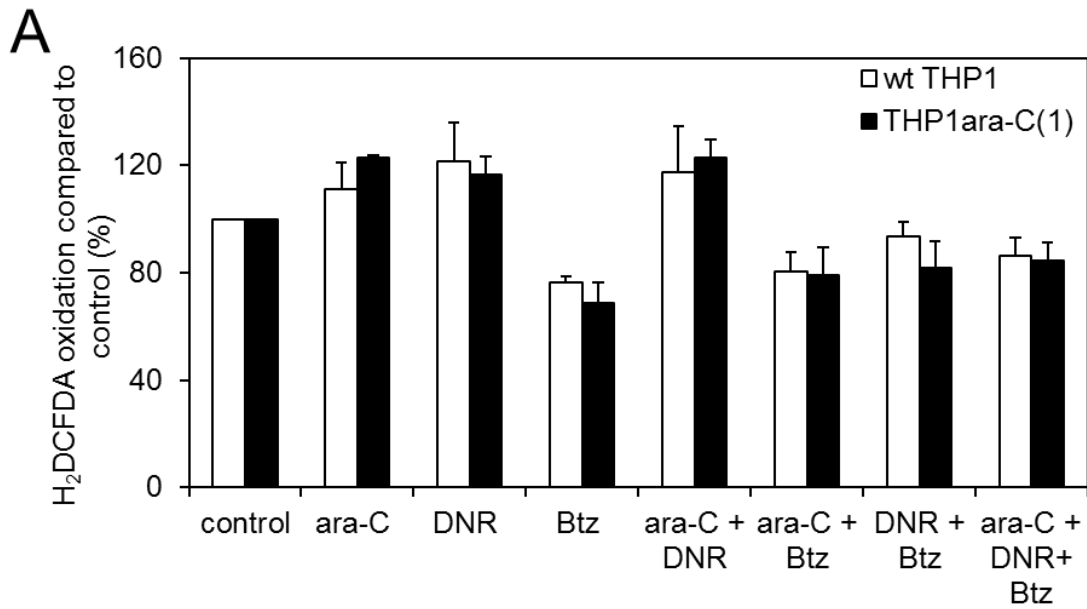
Figure 4.10: ROS production in wt THP1 and THP1ara-C(1) in response to chemotherapeutic agents. (A) Both wt THP1 and THP1ara-C(1) cells were treated with ara-C (5 µM), DNR (0.2 µM), bortezomib (10 nM) or combinations thereof for 24 h. Cells were collected and incubated with the fluorescent probe H$_2$DCFDA (1 µM) for 30 min before ROS production was measured using flow cytometry. Bars represents mean ±SEM (n = 3). Abbreviation Btz = bortezomib.

4.4.7 The effect of withdrawing ara-C from ara-C resistant THP1 cells. In order to find out whether ara-C resistance achieved in the THP1ara-C(1) cell line was a stable or transient response, weekly ara-C refreshment was withdrawn for 4 weeks prior to experimentation to produce a THP1ara-C(1) treatment withdrawn (THP1ara-C(1) TW) cell line. Cell doubling time was investigated and found to return to that of the wt THP1 AML cells over a period of 7 days (Figure 4.11 A). Next, we wanted to examine whether THP1ara-C(1) TW resistance towards ara-C was maintained. Wt THP1 and THP1ara-C(1) TW were treated with increasing doses of ara-C over a 72 h time course. Interestingly, resistance towards ara-C was maintained for at least 8 weeks after treatment withdrawal, suggesting a permanent changed has occurred to THP1ara-C(1) TW cells signalling pathways resulting in a transient resistance towards ara-C has been developed (Figure 4.11 B-C).

4.4.8 THP1ara-C(1) TW maintain reduced sensitivity towards combination chemotherapeutic treatment. (Figure 4.12 A-B) As we have previously shown that ara-C resistance confers a level of resistance to bortezomib we wanted to explore the resistance of THP1ara-C(1) TW to different combinations of ara-C (10 μM), bortezomib (10 nM), DNR (0.5 μM), for either 24 h or 48 h. At both 24 h and 48 h THP1ara-C(1) TW behaved in much the same way as THP1ara-C(1) (Figure 4.6 A-B).

4.4.9 ROS production is reduced in THP1ara-C(1) TW. ROS production and Glutathione expression was explored in wt THP1, THP1ara-C(1) and THP1ara-C(1)TW cells to examine the effect of withdrawing ara-C treatment. A significant reduction in ROS production and glutathione expression was seen in THP1ara-C(1) TW compared to both wt THP1 and THP1ara-C(1) cells (Figure 4.13 A-B). ROS generation was examined further, after 24 h treatment with various combinations of ara-C (5 μM), DNR (0.2 μM) or bortezomib (10 nM). (B) ROS production was increased in wt THP1 and THP1ara-C(1) TW when treated with either ara-C, DNR or a combination thereof. However when treated with Bortezomib or combinations containing Bortezomib, ROS generation was decreased in both wt THP1 and THP1ara-C(1) TW cells.

4.4.10 Nrf2 expression is increased after ara-C refreshment is withdrawn. Figure 4.14 A shows Nrf2 mRNA expression is significantly increased after weekly ara-C

refreshment has been withdrawn. Whilst both HO-1 and Nrf2 mRNA expression remains similar to that of the control wt THP1 cells.
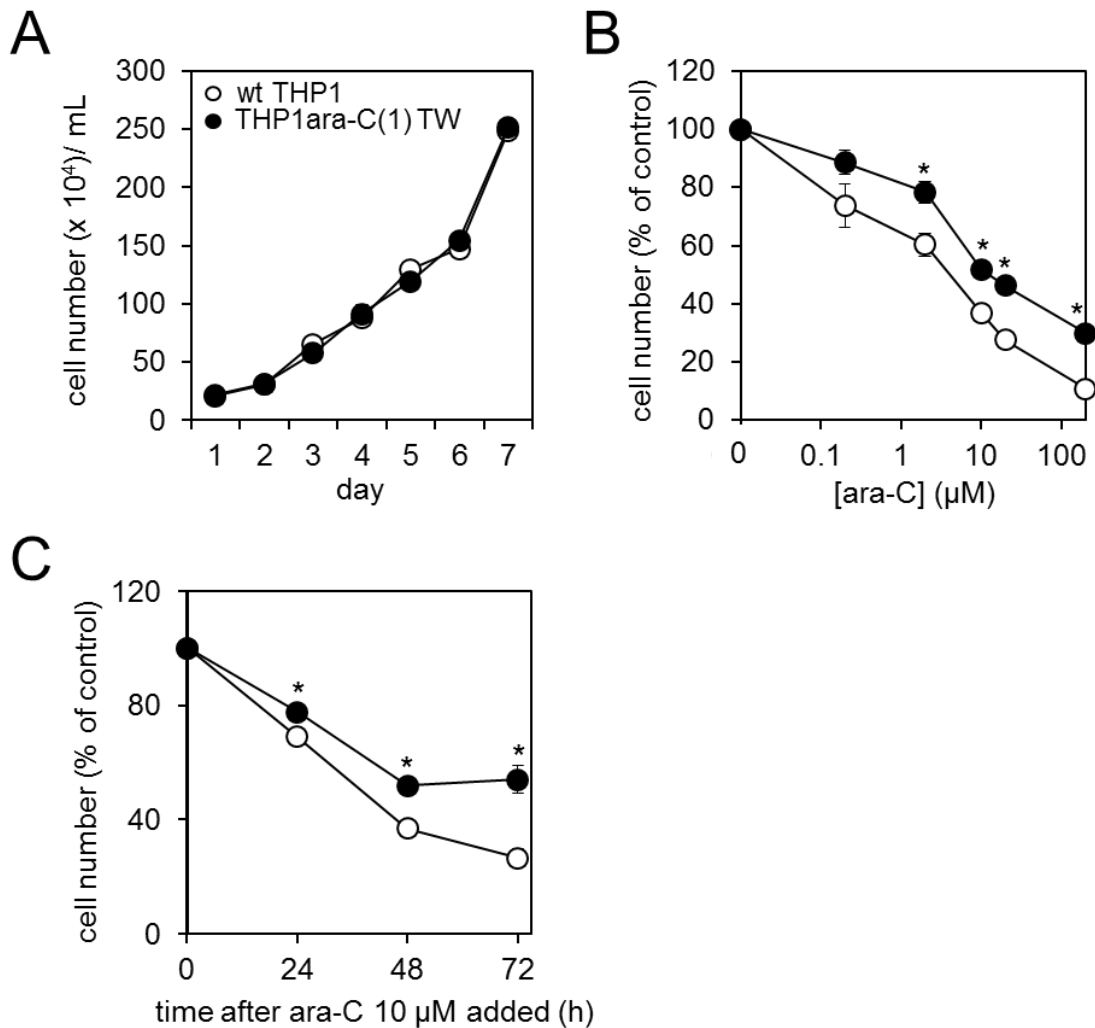
Figure 4.11: THP1ara-C(1) TW proliferation rate is returned to that of wt THP1s. (A) 25 x 10⁴ wt THP1 and THP1ara-C(1) TW were cultured for 7 days and the number of viable cells was determined daily by Trypan Blue exclusion assay (n = 3). (B) Both wt THP1 and THP1ara-C(1) TW cells were incubated with varying doses of ara-C (0.2, 2, 10, 20, 200 μM) for 48 h before cell viability was analysed using CellTitre-Glo® Luminescent Cell Viability Assay (n = 3). (C) wt THP1 and THP1ara-C(1) TW cells treated with ara-C (10 μM) for 24, 48 and 72 h before cell viability was analysed using CellTitre-Glo® Luminescent Cell Viability Assay (n = 3). Cell number is expressed as a percentage of the control. Circles represent the mean ±SEM. Statistical significance was calculated by Student's t-test; *p≤0.05.
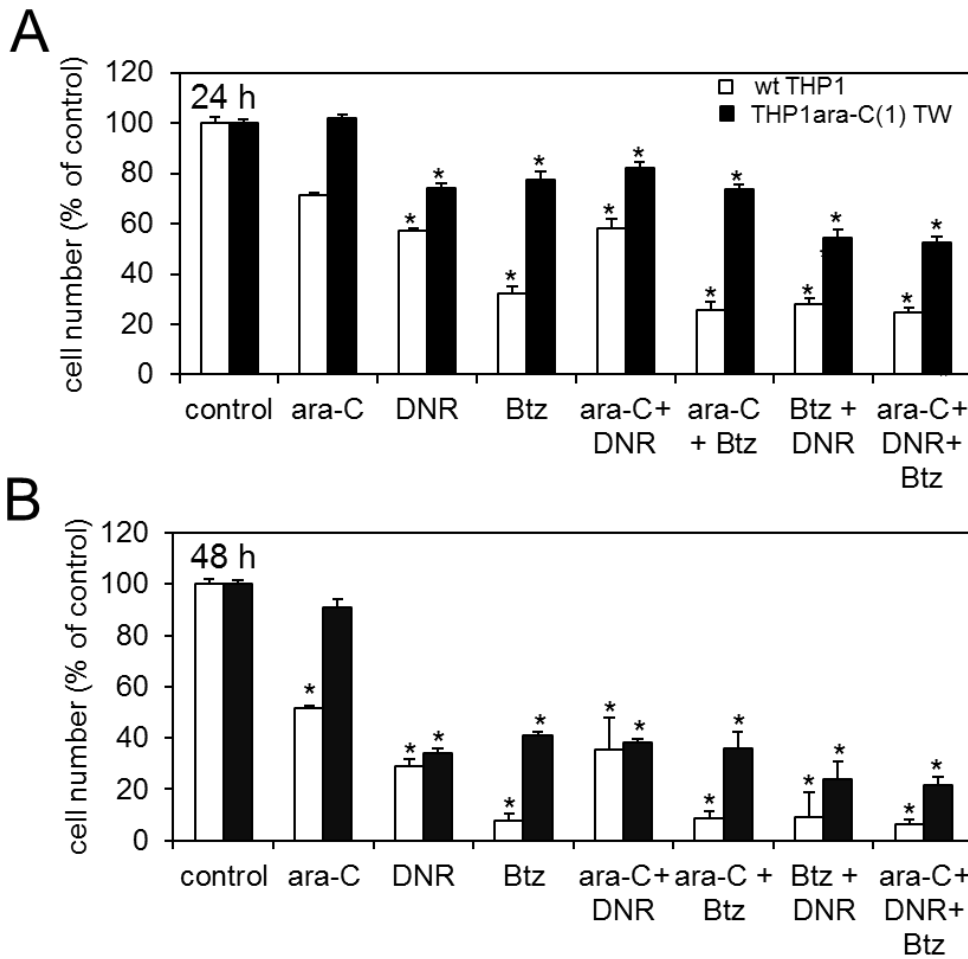
Figure 4.12: THP1ara-C(1) TW maintain delayed sensitivity towards combination chemotherapeutics therapy. (A) wt THP1 and THP1ara-C(1) TW cells were treated with ara-C (10 µM), bortezomib (10 nM), DNR (0.2 µM), or combinations thereof, for 24 h or (B) 48 h before cell viability was analysed using CellTitre-Glo® Luminescent Cell Viability Assay. Cell number is expressed as percentage of control. Bars represent the mean ±SEM (n = 3). Statistical significance was calculated by Student's t-test; *p≤0.05. Abbreviations Btz = Bortezomib.
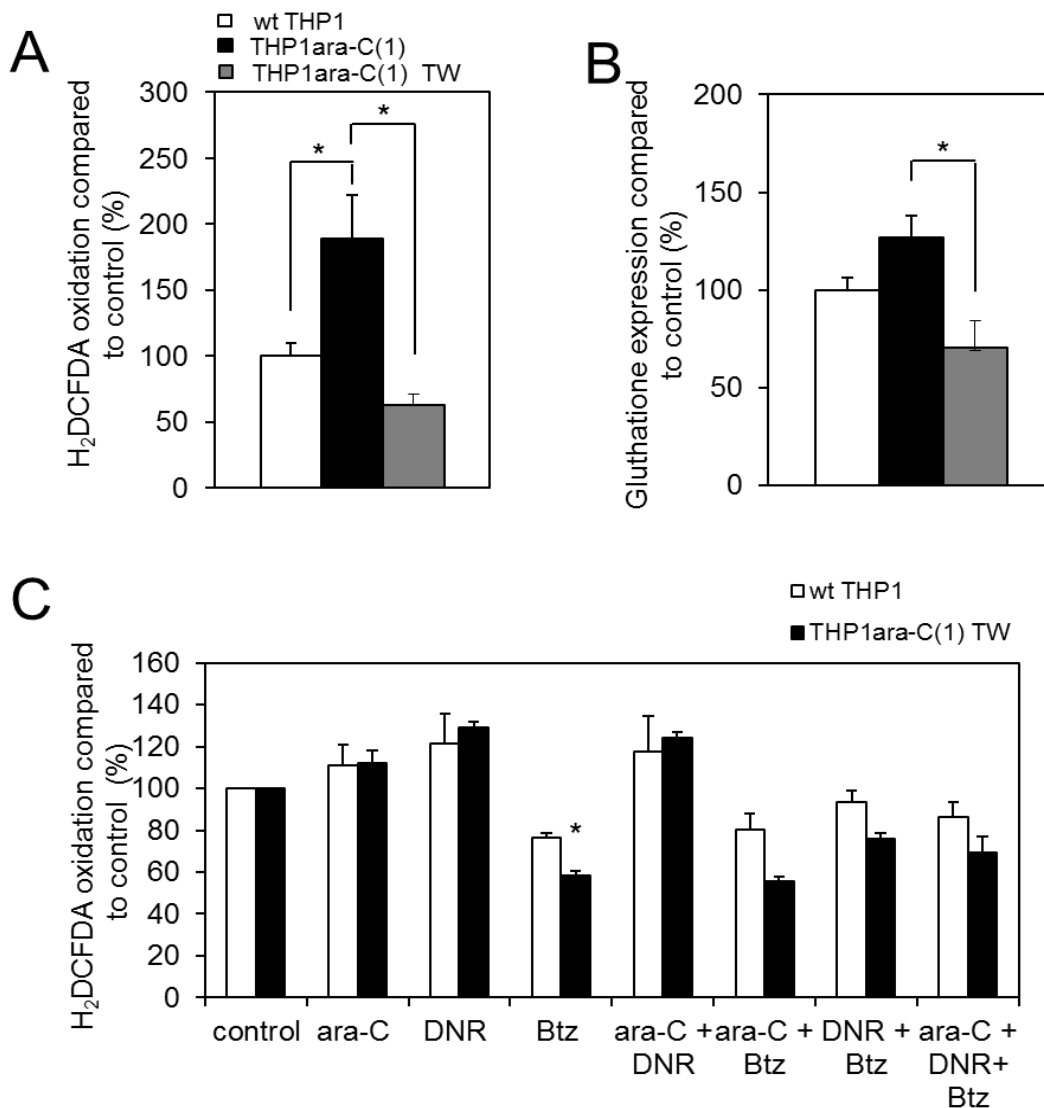
Figure 4.13: THP1ara-C(1) TW ROS production is reduced compared to both wt THP1 and THP1ara-C(1) cells. (A) Basal ROS production was investigated in wt THP1, THP1ara-C(1) and THP1 ara-C(1) TW cells following incubation H_2DCFDA (1 µM) for 30 min, using flow cytometry (n = 6). (B) Glutathione production was examined using Promega GSH-Glo™ Glutathione assay (n = 3). (C) wt THP1 and THP1ara-C(1) TW were treated with ara-C (10 µM), or bortezomib (10 nM) DNR (0.2µM) for 24 h before samples were collected and ROS production was measured as described above (n = 3). Bars represents mean ±SEM. Statistical significance was calculated by (A, B) ANOVO (C) Students t-test; * p≤0.05.
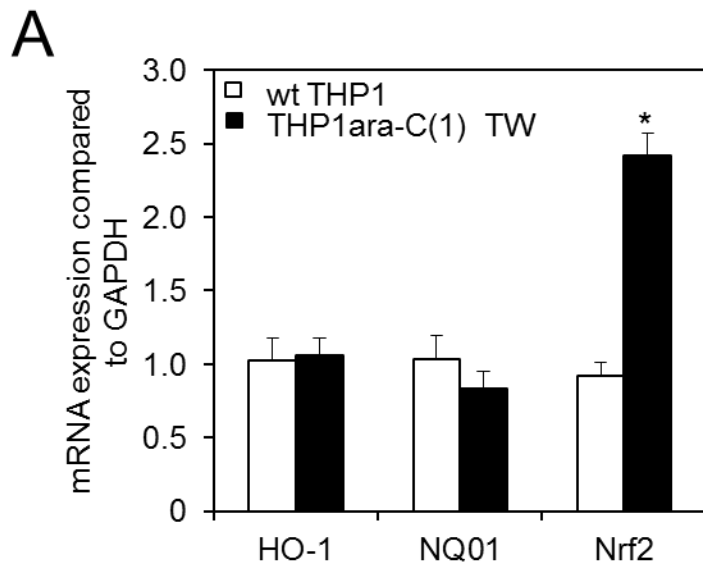
Figure 4.14: Regulation of Nrf2 is elevated in THP1ara-C(1) TW. (A) RNA was extracted from wt THP1 and THP1ara-C(1) TW for qRT-PCR analysis of basal HO-1, NQO1 and Nrf2 mRNA expression. (n = 3). Bars represent the mean ±SEM. Statistical significance was calculated by Students t-test; * $p \leq 0.05$.

4.5 Discussion

During this chapter we have explored ara-C resistance in wt THP1 an AML derived cell line. Ara-C resistance was developed within these cells, over a period of 3 months, by stepwise increments in ara-C exposure (Figure 4.1) producing THP1ara-C(1) cells. Over the last decade a number of groups within the field have used similar methods to develop AML cell lines resistant to ara-C, DNR and bortezomib [44, 91, 113, 115]. Initially we investigated the extent of the acquired ara-C resistance in our THP1ara-C(1) cells compared to their wt THP1 control. During the 3 month course of ara-C exposure the THP1ara-C(1) cells developed a gradual decreased sensitivity towards moderate ara-C concentrations. However, they have still maintained sensitivity towards greatly increased ara-C concentrations, for example, 10 to 20 times greater than their usual weekly refreshment concentration (1 μM).

Previously we have developed a lenalidomide and bortezomib resistant MM cell lines; RPM18226 and MM1S. They were produced by increasing the concentration and exposure of the desired chemotherapeutic agent over a 3 month period, thus producing RPMI8226 (Bz20 and Bz50) and lenalidomide (Len10 and Len20). Interestingly, basal HO-1 mRNA expression was increased in both lenalidomide and Bortezomib resistant RPMI8226 and MMIS cells [46]. However this does not appear to be the case in the ara-C resistant THP1 ara-C(1) cells.

Despite their resistance to ara-C we have shown that THP1ara-C(1) cells maintained a similar level of sensitivity towards other chemotherapeutic agents; DNR, etoposide and to a lesser extent bortezomib, compared to their control, wt THP1. This is, perhaps, unsurprising as both the anthracycline antibiotic, DNR and etoposide utilise different mechanisms of action to initiate apoptosis [5]. Previous work by Funato et al (2000) has shown similar results in the ara-C resistant K562-derived cell line (K562/AC). Funato et al (2000) exposed K562/AC cells to other chemotherapeutics; vincristine, adriamycin, and etoposide used in the treatment of AML and found that the cells still maintained sensitivity towards these chemotherapeutic agents similar to that of the parental K562 cells [44]. The K562 cells lacked Multidrug Resistant (MDR-1) and Multidrug Resistant Protein expression (MRP) which are both well known to be involved in drug resistance. Whilst dCK expression was reduced in the K562/AC cells which is consistent with other groups work involving ara-C resistance in leukaemia cell lines [44]. Fukuda et al (1996)

developed a new human myeloid leukaemia cell line, KF-19 and subsequent ara-C resistant; KF-19AraC. KF-19AraC showed decreased dCK expression along with cross resistance towards vincristine [115]. Suggesting decreased dCK expression and ara-C resistance are related. Funato et al went on to explore dCK effect within parental K562 cells, dCK expression was inhibited via antisense oligonucleotides before ara-C treatment which resulted in decreased sensitivity towards ara-C [44]. dCK phosphorylates ara-C into its active metabolite which is incorporated into the cells DNA. [33] A reduction in dCK would therefore result in reduced ara-CTP generation and DNA incorporation therefore leading to potential ara-C resistance [44]. In the future we would like to explore the connection between ara-C resistant in THP1ara-C(1) cells and dCK, MDR-1 and MRP expression and whether cross resistance to the vinca alkaloid; vincristine exists.

Oerlemans et al (2008) developed a bortezomib resistant THP1 cell line and noted an over expression of a mutant Proteasome subunit β5 (PSMB5) within the resistant cell line, thus linking bortezomib resistance to mutant PSMB5 over expression. The authors also confirmed resistance was not associated with increased expression of the MDR efflux pump [113].

Using a variety of detection techniques we observed decreased proliferation rate in THP1ara-C(1) resistant cells (doubling time = 2.7 days; SEM ± 0.07) compared to that of the wt THP1 cells (doubling time = 2.2 days; SEM ± 0.10). This suggests that, despite their resistance to ara-C in terms of cell death, continual ara-C exposure is affecting the THP1ara-C(1)cells, cell cycle and cell doubling time. However upon weekly ara-C removal, the cells doubling time is restored to that of the wt THP1 cells, potentially suggesting the presence of continual ara-C exposure is suppressing THP1ara-C(1) ability to grow. Interestingly Funato et al (2000) found there wasn't a difference in population doubling time between their ara-C resistant K562 and parental K562 cells [44].

We have previously shown increased HO-1 mRNA and protein expression in response to ara-C, DNR or a combination of both agents in AML patient samples and cell lines [101]. However this is not the case in the THP1ara-C(1) cells. There appears to be a significant decrease in both HO-1 mRNA and protein expression in response to

prolonged ara-C exposure along with NQO1, another cytoprotective gene. Interestingly Nrf-2 expression is also down regulated within these cells, thus potentially explaining HO-1's and NQO1's regulation. In chapter 6 we go on to explore Bach1's regulation in greater detail. Bach1 is known to regulation a number of cytoprotective genes through the ARE complex.

Kim et al (2008) reported a significant increase in HO-1 expression in A549 cells; a lung cancer cell line treated with cisplatin (10 μM). However, HO-1 basal expression is already significantly up regulated in A549 cells compared to other lung cancer cell lines. The authors go on to suggest A549 cells are more resistant to cisplatin than other lung cancer cell lines after investigating cell viability in response to a varying cisplatin concentrations, where A549 cells showed greater cell viability at each concentration than the other cell lines. Potentially suggesting the A549s' increased cisplatin resistance could be due to increased HO-1 expression. Therefore, when A549 cells were treated with both a; HO-1 inhibitor, ZnPP and cisplatin, a significant decrease in cell viability, was seen. Suggesting HO-1 is involved in cisplatin resistance. The authors then go onto the explore ROS generation in response to cisplatin or ZnPP or a combination thereof. ROS generation is increased in response to either of the above combinations and more so when both agents are used in combination. [108].This is similar to what we see in both wt THP1 and THP1ara-C(1) cells which are treated with either ara-C, DNR or in combination.

Both ara-C and DNR exposure have been shown to increase ROS generation in AML cell lines [101]. Overall ROS generation is also significantly increased in THP1ara-C(1) cells. However on further daily examination, ROS generation remains increased up until the day before ara-C weekly refreshment and thereafter. The decrease in ROS generation in comparison to the wt THP1 control could be due to ara-C half-life resulting in reduced ara-C present in the media or increased stress in the wt THP1 control cells due to their increased cell doubling time. In response to increased ROS generation, glutathione generation was also up regulated in the THP1ara-C(1) cells.

ROS generation in response to 24 h exposure to either ara-C or DNR or combinations thereof, was increased. However, when either wt THP1or THP1ara-C(1) cells were exposed to bortezomib or in combination with either ara-C or DNR, ROS production was

decreased, even though at least 80% of the THP1ara-C(1) population were viable and less so for wt THP1 cells. Potentially suggesting the pathways which bortezomib utilises to induce apoptosis are not reliant on ROS generation.

Increased ROS generation in response to chemotherapeutics within AML cells has been shown by a number of groups [101]. The antineoplastic, Niclosamide used to treat tapeworm infections [5] results in increased ROS generation in the AML cell line HL60, 2 h post exposure [117].
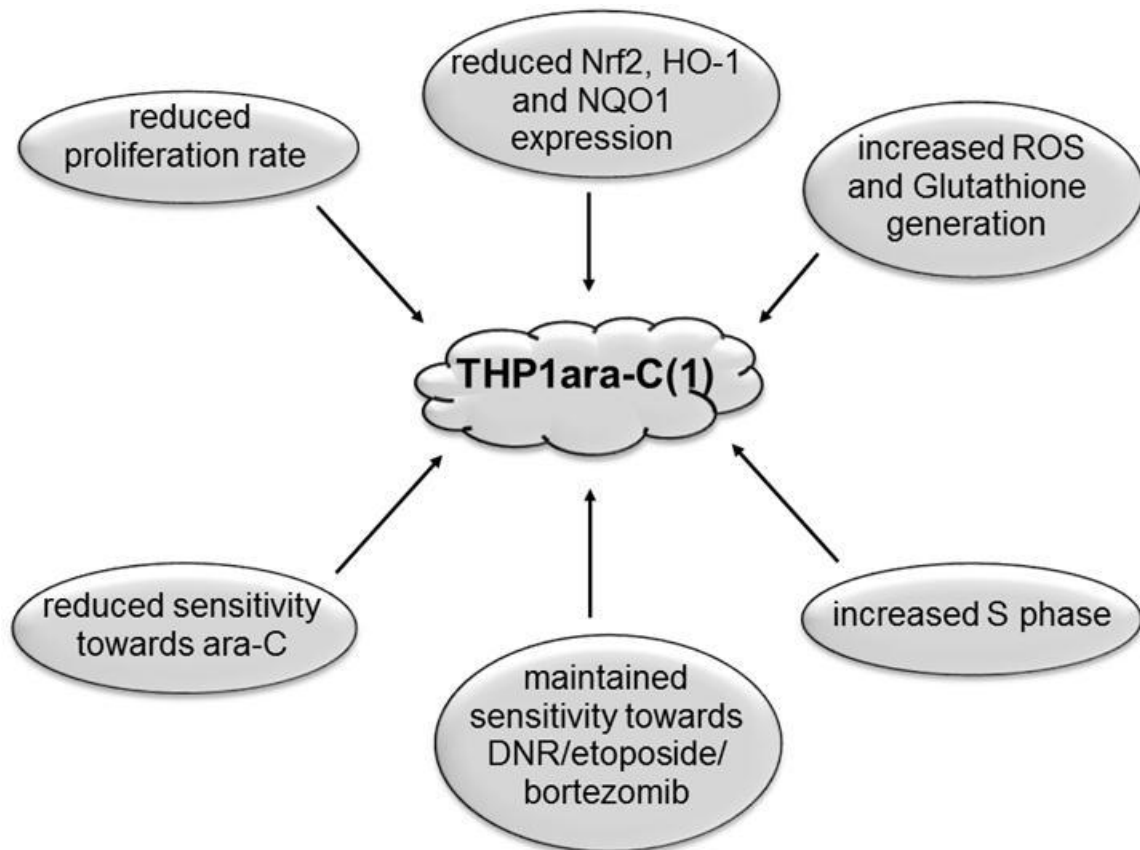
Figure 4.15: Characteristics differences between wild type THP1 cells and THP1ara-C(1) cells.

To conclude (Figure 4.16) the following cytoprotective genes; HO-1, NQO1, GCLM are significantly down regulated in THP1ara-C(1) cells along with the transcription factor Nrf2. However the regulation of Bach1 is significantly increased (explored in greater detail in Chapter 6). ROS generation is increased in response to ara-C exposure

however upon ara-C withdrawal ROS generation is reduced to below that of the control. Possibly suggesting the AML cells are utilising ROS generation to their advantage when exposed to continual ara-C.
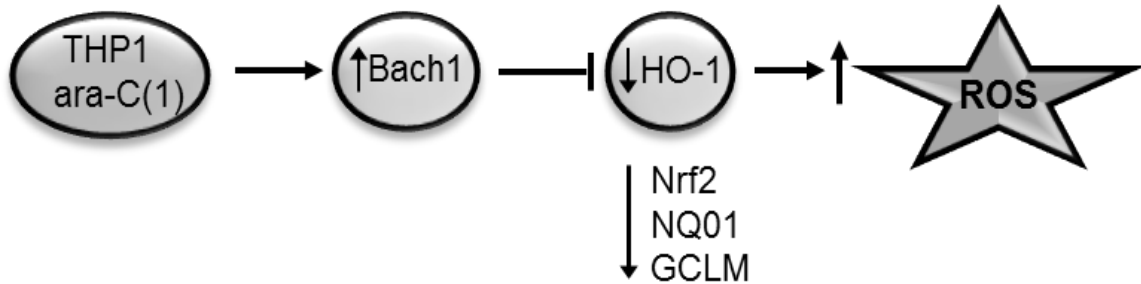
Figure 4.16: Proposed mechanism of action regarding cytoprotection and ROS generation.

Even though, there does appear to be a number of functional changes which have occurred due to acquired ara-C resistance. Some of these changes appear to be reversed upon withdrawal of continuous ara-C refreshment for examples cell doubling time and ROS generation.
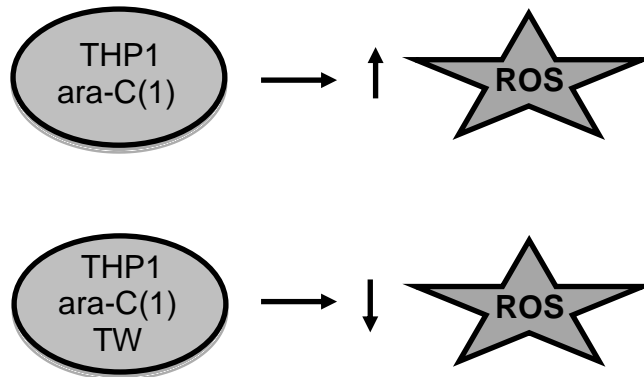
Figure 4.17: Upon ara-C withdrawal ROS generation is decreased.

However, upon ara-C withdrawal, THP1ara-C(1) TW still maintain a level of resistance towards ara-C and their sensitivity towards bortezomib and DNR which is comparable to the control wt THP1 cells. Thus potentially indicating the acquired ara-C resistance is a stable response and irreversible. It would be interesting to explore this response in vitro or with actual AML patient samples as opposed to cells lines and find out whether acquired ara-C resistance through continuous ara-C and DNR treatment cycles could be reversed upon chemotherapeutics withdrawal once the patients is in remission.

Chapter 5: Ara-C resistance in relation to differential microRNA expression

5.1 Introduction

We wanted to examine the regulatory role of miRNAs in the ara-C resistant AML cell line; THP1ara-C(1). Initially the SBI Cancer and Stem Cell miRNA Array was performed to identify any key miRNAs which were either up or down regulated in response to ara-c resistance. A number of miRNAs were highlighted and taken forward for further examination.

miRNAs are short non-coding evolutionary conserved RNAs, between 18 and 25 nucleotides in length. Their involvement in cell survival, proliferation and apoptosis has been implicated [8, 81, 88]. Recently a number of groups have investigated miRNA regulation within AML pathogenesis.

Cimmino et al (2005) have shown both miR-15a and miR-16-1 target and negatively regulate the protein B Cell Lymphoma (Bcl-2) in Chronic Lymphocytic Leukaemia (CLL). Bcl-2 repression results in induced apoptosis [118].

miR-21 has been shown to target both Phosphatase and Tensin Homolog Protein (PTEN) [91] and Programmed Cell Death Protein 4 (PDCD4) [93]. Li et al (2010) have suggested miR-21 is involved in ara-C sensitivity in the HL60 cell line, they found apoptosis was enhanced and ara-C sensitivity increased in response to anti-miR-21 oligonucleotide and ara-C treatment.

The quantification of miR-92a expression between plasma and leukaemia cells could potentially be used as a leukaemia monitoring parameter in ALL and AML patients [119]. miR-146a, miR-25, miR-26a, miR-25b and miR-196a have been found to be significantly associated with overall AML patient survival. Of which, miR-25 has been significantly associated with a good prognosis, whilst the other four miRNAs have linked to an negative outcome. In both AML and ALL patients which have a higher expression of miR-146a, a significantly shorter survival has been reported compared to that of patients with a lower miR-146a expression. After further analysis of additional patient samples the authors concluded miR-146a expression was significantly inversely associated with overall survival in AML patients thus it could be potentially be used as a prognostic marker [120].

5.2 Aims

During this chapter we aim to explore miRNA differential regulation in response to ara-C resistance in THP1ara-C(1) cells and explore miRNAs known to be involved in either proliferation or resistance and establish whether they are involved in THP1ara-C(1) reduced cell growth or acquired resistance.

5.3 Results

5.3.1. Key miRNA involved in Cancer or Stem Cells were highlighted by SBIs' QuantiMir Cancer or Stem Cell Arrays. MiRNA expression of miRNAs involved in either cancer or stem cell biology were investigated using the SBI's QuantiMir Arrays (Figure 5.1 A). MiRNAs highlighted by either the SBIs' QuantiMir Cancer or Stem Cell Array which fulfilled the following inclusion citeria were taken forward for further investigation (Figure 5.2); expression was either above 2 fold or below 0.5 fold of the control wt THP1 cells expression. miRNA was lsyed from synchronised control wt THP1 cells and ara-C resistant THP1ara-C(1) cells following the protocol stated in 2.8.5. then, differentail miRNA expression was analysed. Finially, miRNA expression profiling identified 15 potential miRNAs which fulfilled the inclusion criteria. Figure 5.2 A investigates the differential expression of highlighted miRNAs from the arrays previously mentioned.

miR-15a, miR-21, miR-92a, miR-149, miR-186, miR-200a and miR-222 miRNA expression was increased, whilst miR-101, miR-142-3p, miR-146a, miR1-83 and miR-196a miRNA expression was decreased in the ara-C resistant cells. Table 5.1 briefly highlights key miRNAs and their target genes which are examined in greater detail throughout his chapter.

Table 5.1: miRNA and their target gene.

miRNA	Targets	Reference
miR-15a	Bcl-2	[118]
miR-21	PTEN	[91]
	PDCD4	[93]
miR-145	BIM	[121]
miR-146a	TRAF6	[122]
	IRAK1	[123]
miR-196a	Bach1	[124]
	Annexin A1	[125]
	ERG	[126]
miR-200a	Keap1	[127]
miR-222	PTEN	[128]
	PUMA	[95]

A
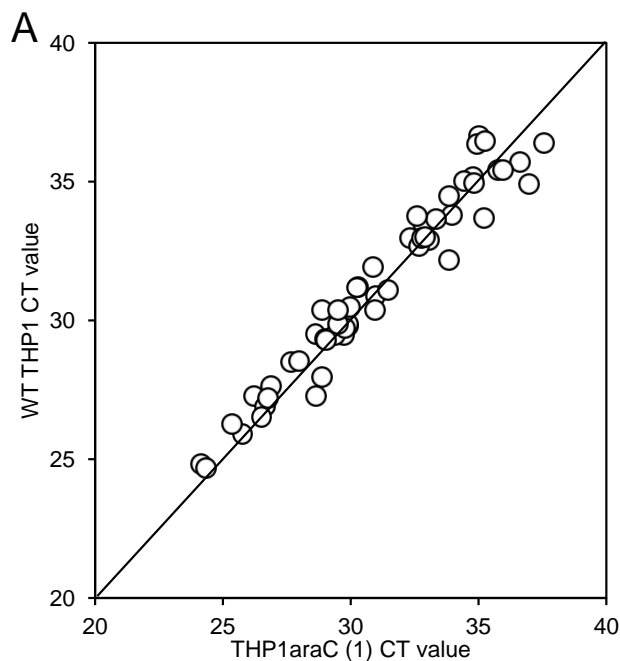
	CT values	
	THP1 araC(1)	THP1
miR-92a	26.23	27.28
miR-93	29.89	29.80
miR-9	28.62	29.52
miR-101	36.99	34.90
miR-103	29.90	29.89
miR-106a	30.97	30.90
miR-106b	33.06	32.92
miR-107	33.96	33.82
miR-125a-5p	29.74	29.49
miR-125b	32.81	33.50
miR-126	33.34	33.67
miR-132	34.78	35.18
miR-134	35.77	35.41
miR-140	36.63	35.72
miR-142-3p	30.31	31.23
miR-145	37.56	36.40
miR-146a	35.03	36.67
miR-149	32.58	33.78
miR-155	28.98	29.33
miR-15a	30.88	31.93
miR-15b	26.89	27.66
miR-16	26.65	26.91
miR-17*	32.31	32.96
miR-17	29.96	30.48
miR-181a	34.42	35.04
miR-181b	32.67	32.70
miR-181c	29.39	29.49
miR-183	35.23	33.70
miR-185	32.77	32.97
miR-186	34.96	36.35
miR-18a	31.44	31.10
miR-191	25.77	25.91
miR-195	26.78	27.22
miR-196a	33.85	32.20
miR-30b	26.51	26.52
miR-19a+b	29.02	29.30
miR-200a	30.25	31.19
miR-206	34.82	34.96
miR-21	28.87	30.38
miR-210	30.95	30.37
miR-372	35.24	36.46
miR-221	28.89	27.96
miR-222	28.63	27.30
miR-223	24.16	24.85
miR-23a	25.36	26.27
miR-24	29.78	29.73
miR-25	27.65	28.52
miR-26a	29.50	29.87
miR-27a+b	32.91	33.02
miR-30c	24.34	24.70
miR-29a+b+c	33.84	34.49
miR-30a*	29.50	30.37
miR-30a	27.97	28.54
miR-296	35.97	35.42

Figure 5.1: SBI QuanitiMir Cancer and SBI QuanitiMir Stem cell Array. (A) miRNA was extracted from both wt THP1 and THP1ara-C(1) cells before SBI's Cancer and Stem Cell Array was performed using qRT-PCR (n = 1). Table (right panel) states CT values for each miRNA included in the scatter plot.
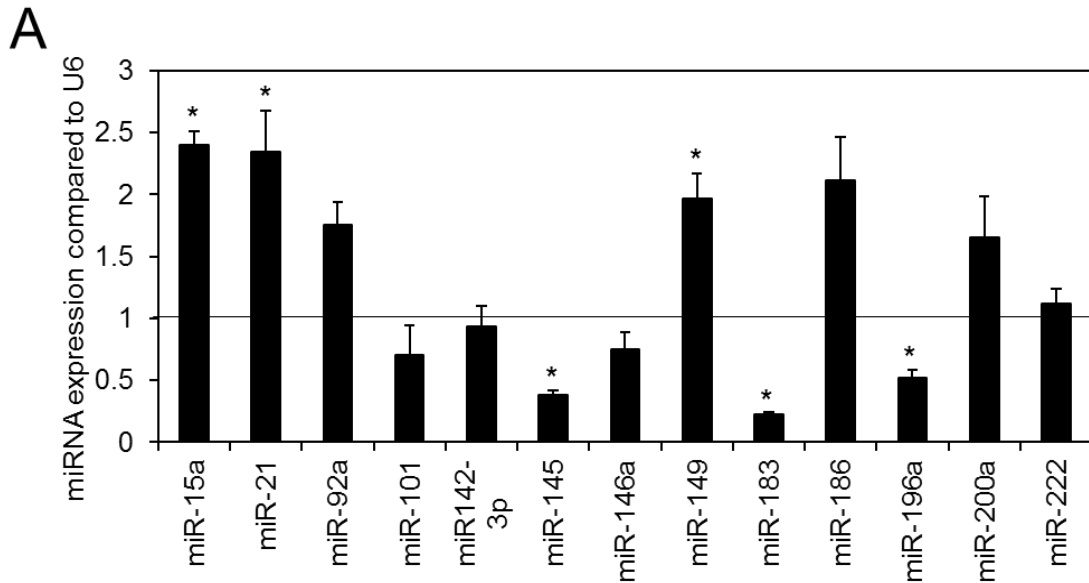
Figure 5.2: miRNAs highlighted by the previous SBI QuantiMir Arrays taken forward for further investigation. (A) miRNA was extracted from wt THP1 and THP1ara-C(1) for qRT-PCR analaysis. THP1ara-C(1) fold change was standarised against control wt THP1 (represented by black line) expression which was normalised to one. Bars represents mean ±SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.

5.3.2 miR-15a expression is up regulated in the ara-C resistant, THP1ara-C(1) cells.
miR-15a targets the pro-survival protein Bcl-2 in CLL [118]. Even though miR-15a expression is significantly up-regulated (Figure 5.3 A) in THP1ara-C(1) cells. Its target gene, Bcl-2 (Figure 5.3 B) expression is indifferent between both wt THP1 and THP1ara-C(1) cells. These results appear inconclusive of whether miR-15a does target Bcl-2 in the ara-C resistant THP1ara-C(1) cells. We also need to consider both miR-15a and Bcl-2 could potentially have other targets which affect their expression [129].

5.3.3 miR-21 is unaffected by ara-C treatment. miR-21 has been shown to target PTEN in K562 cells [91]. Figure 5.4 A shows miR-21 is significantly up regulated in THP1ara-C(1) cells, (B) whilst its target gene PTEN is significantly down regulated. Potentially confirming there is a miRNA and mRNA target relationship between miR-21 and PTEN in THP1ara-C(1) cells. Both wt THP1 and THP1ara-C(1) cells were treated with additional ara-C for 24 h before miR-21 and PTEN mRNA expression in response to ara-C treatment was investigated using qRT-PCR. (C) miR-21 miRNA expression is significantly reduced further in wt THP1 cells in the presence of ara-C treatment, however its expression is indifferent between the control THP1ara-C(1) cells and additional ara-C treated THP1ara-C(1) cells. (D) In response to decreased miR-21 miRNA expression in wt THP1, there is a slight increase in PTEN mRNA expression. However their does not appear to be a difference in PTEN mRNA between THP1ara-C(1) treated and non-treated populations. These results appear inconclusive of whether miR-21 is affected by the presence of ara-C treatment.
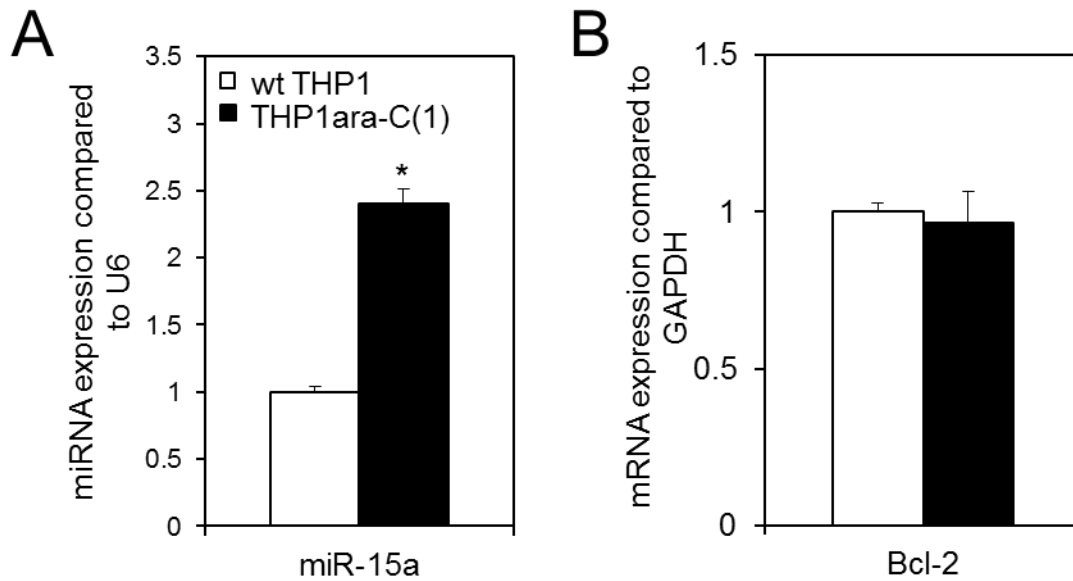
Figure 5.3: miR15a expression is up regulated in the ara-C resistant THP1ara-C(1) cells. miRNA and mRNA was collected from wt THP1 and THP1ara-C(1) cells, (A) miR-15a miRNA basal expression and (B) Bcl-2 mRNA basal expression was analysed using qRT-PCR. Bars represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.
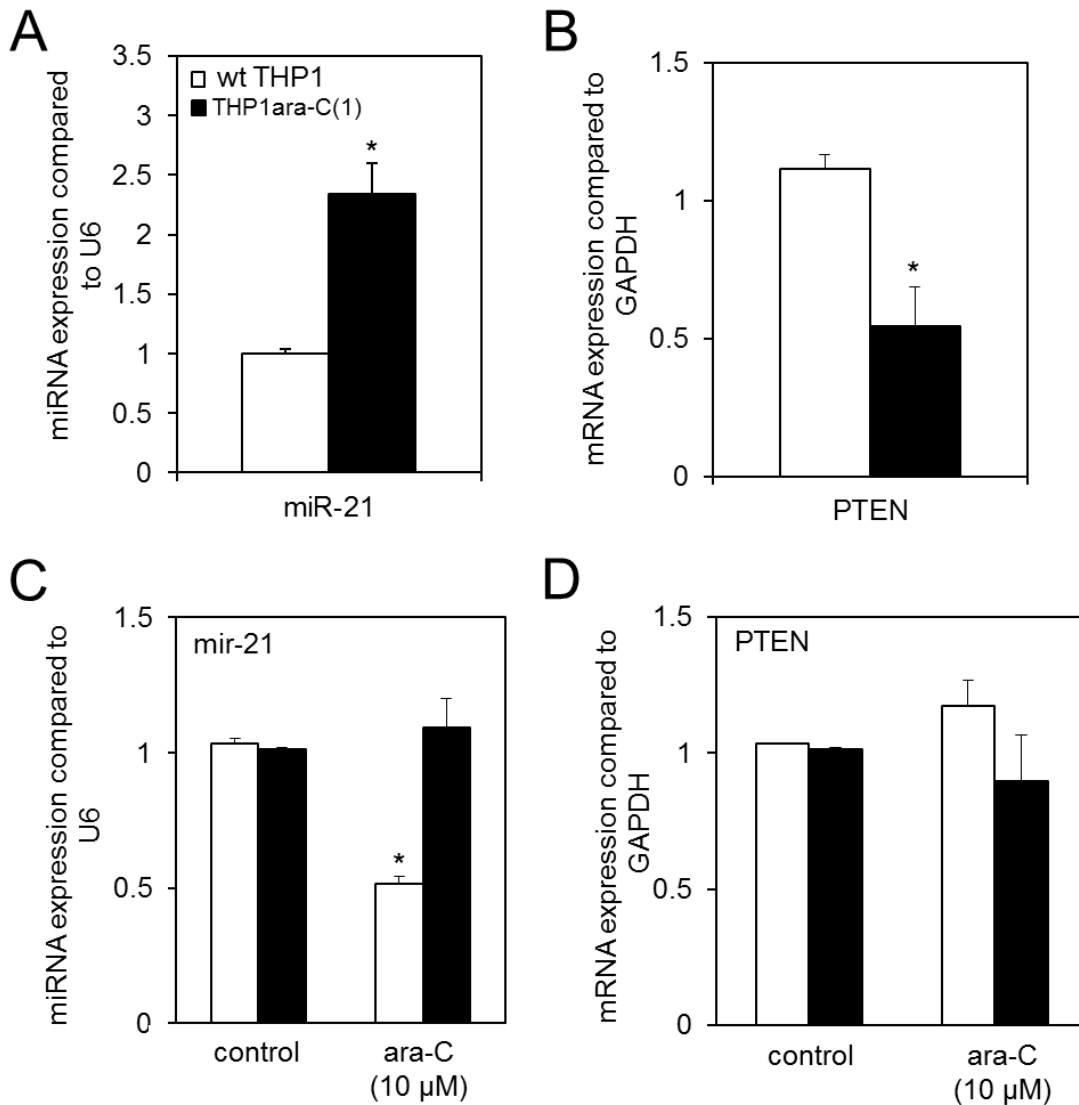
Figure 5.4: miR-21 targets PTEN. miRNA and mRNA was collected from wt THP1 and THP1ara-C(1) cells before (A) miR-21 miRNA (B) PTEN mRNA expression were examined using qRT-PCR. wt THP1 and THP1ara-C(1) cells were treated with ara-C (10 µM) for 24 h before miRNA and mRNA was collected for qRT-PCR investigation, (C) miR-21 miRNA and (D) PTEN mRNA expression. Bars represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.

5.3.4 miR-34a expression is up regulated in THP1ara-C(1) cells whist their cell proliferation rate is repressed. miR-34a has been shown to target SIRT1 [96] whilst p53 has been shown to target miR-34a [130]. miR-34a is significantly up regulated by 14 fold in the ara-C resistant THP1ara-C(1) cells compared to their control wt THP1s cells (Figure 5.5 A). In response, its target gene SIRT1 mRNA is significantly down-regulated confirming the miRNA and its target gene relationship in the ara-C resistant THP1ara-C(1) cells. p53 mRNA expression is also down-regulated in the THP1ara-C(1) cells.

We have previously shown during chapter 4 that the time taken for THP1ara-C(1) cells to double is significantly longer than that of wt THP1 control cells (Figure 4.3 A). We have also shown that upon withdrawal of ara-C refreshment, cell doubling rate returns back to that of the wt THP1 control cells (Figure 4.11 A). Thus suggesting miRNAs affected by continual ara-C exposure could be involved in cell doubling. We therefore wanted to explore miR-34a expression in THP1ara-C(1) TW cells. Ara-C refreshment was withdrawn for at least 4 weeks (Figure 5.5 C) prior to the experiment**, m**iR-34a expression is significantly reduced in THP1ara-C(1) TW cells possibly suggesting its involvement in cell doubling rate.

5.3.5 miR-145 rescues AML cells from apoptosis. miR-145 expression is significantly down regulated in THP1ara-C(1) compared to control wt THP1 cells (Figure 5.6 A). miR-145 indirectly targets the pro apoptotic gene Bim [121, 131], its mRNA expression is unchanged by the presence of reduced miR-145 expression (Figure 5.6 B) in the THP1ara-C(1) cells. Bim, a member of the Bcl-2 family, is in involved in mitochondrial apoptosis [132]. The anti-apoptotic proteins Bcl-2 and Bcl-xl and pro apoptotic protein Bax mRNA expression are also unchanged between both wt THP1 and THP1ara-C(1) cells (Figure 5.6 C).

Next we were intrested in establishing the functional repsonse of miR-145 in relation to ara-C treatment. miR-145 was overexpressed using a mimic which was transfected via nulecofection into both wt THP1 and THP1ara-C(1) cells. Cells were treated 8 h post transfection with ara-C (10 µM) before cell viabailty was examined 48 h post ara-C treatment. No difference between cell viabilty was observed after ara-C treatment with increased miR-145 expression in wt THP1 cells (Figure 5.7 A). However (Figure 5.7 B) when miR-145 expression was over expressed in THP1ara-C(1) cells, a signiifcant

increase in cell viabilty post ara-C treatment was seen. Thus potentially suggesting increased miR-145 expression is involved in cell survival and chemoresistance responses.
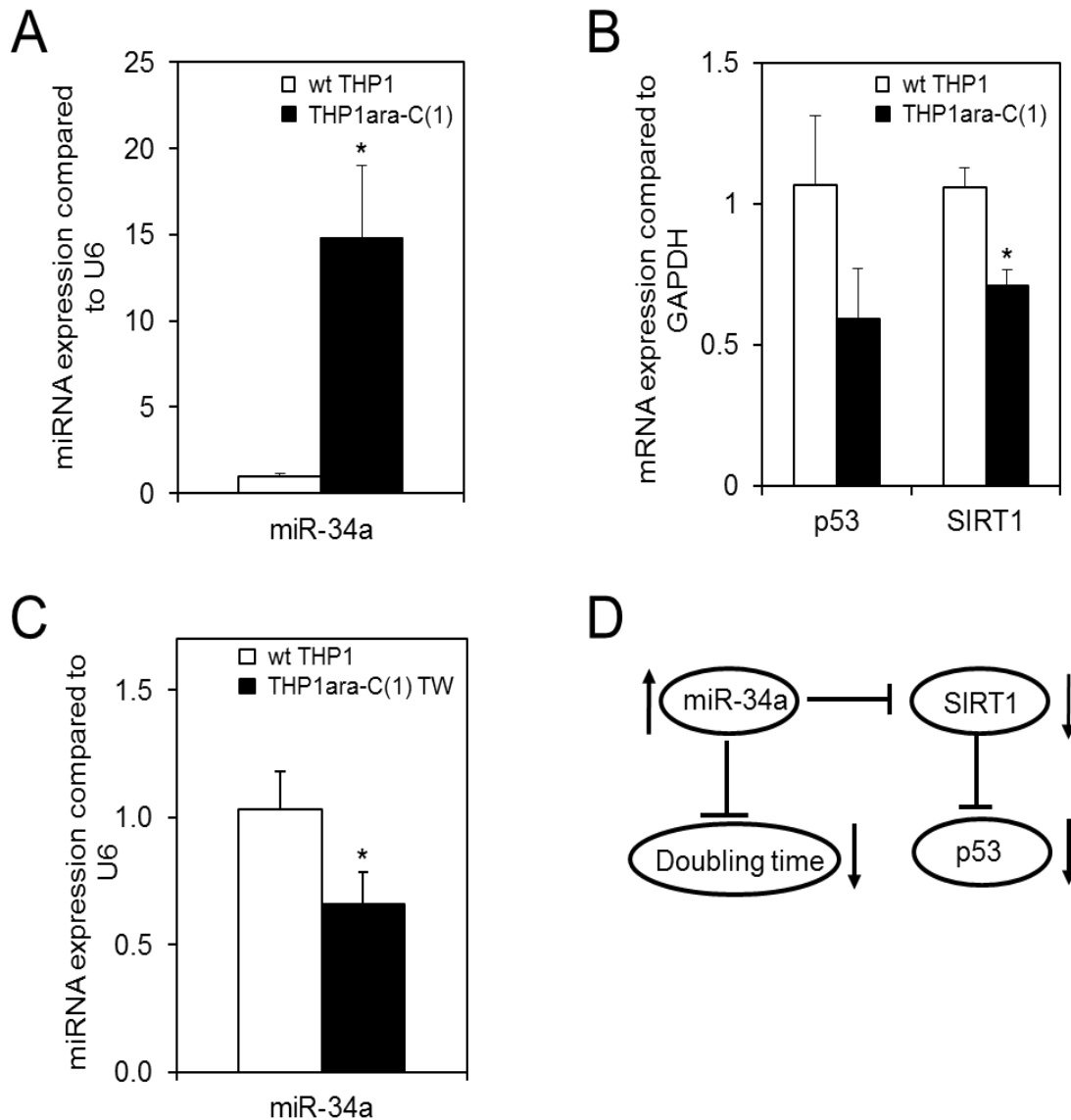
Figure 5.5: A correlative relationship is shown between induced miR-34a expression and repressed cell doubling. miRNA and mRNA was collected from wt THP1 and THP1ara-C(1) cells (A) miR-34a basal expression and (B) p53 and SIRT1 mRNA expression was analysed using qRT-PCR. (C) miRNA was collected from wt THP1 and THP1ara-C(1) TW cells, basal miR-34a expression was analysed using qRT-PCR. (D) Diagrammatic representation of how cell doubling time is potentially regulated by miR-34a. Bars represent mean ± SEM. (n = 3) Statistical significance was calculated by Students t-test; * p≤0.05.
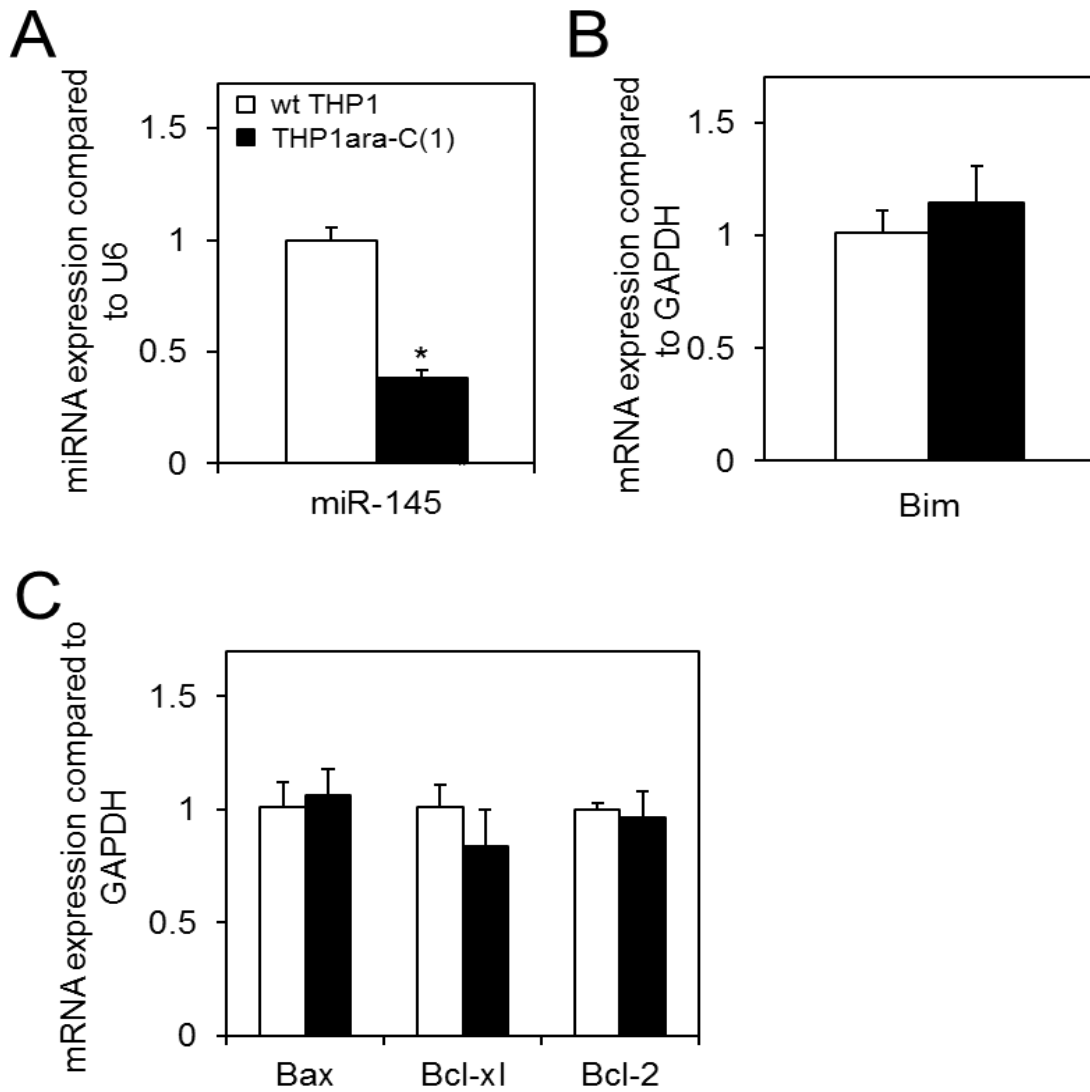
Figure 5.6: miR-145 targets BIM, a pro-apoptotic gene. miRNA and mRNA was collected from wt THP1 and THP1ara-C(1) cells before qRT-PCR analysis (A) miR-145 miRNA expression, (B) Bim mRNA expression and (C) Bax, Bcl-xl and Bcl-2 mRNA expression. Bars represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.
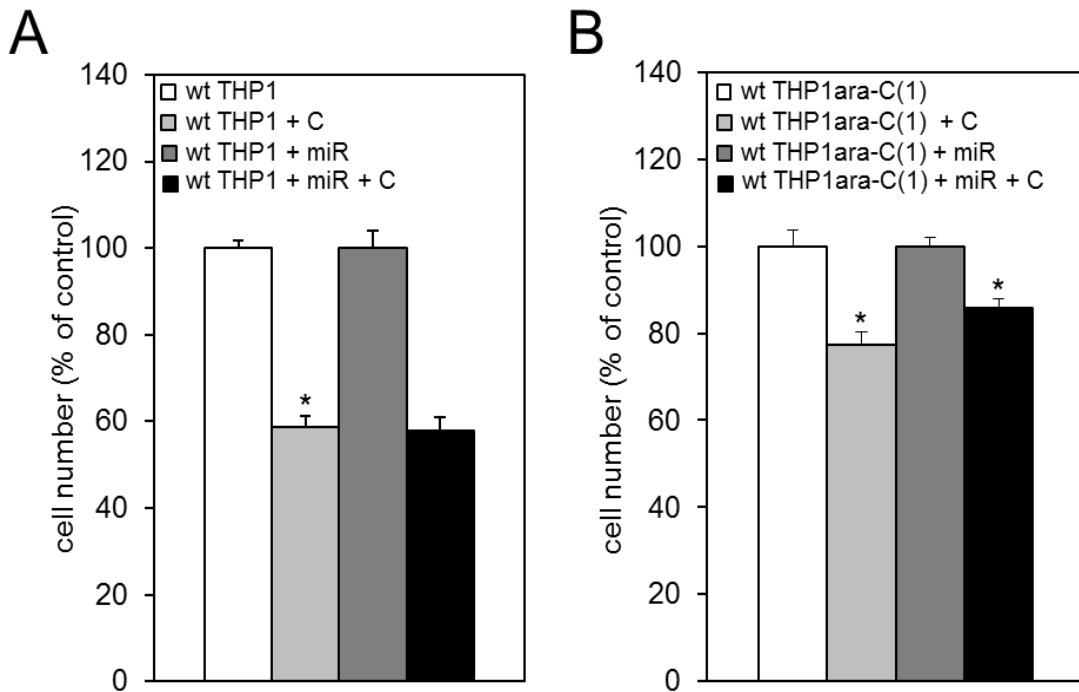
Figure 5.7: miR-145 rescues AML cells from apoptosis. 2×10^6 wt THP1 and THP1ara-C(1) cells were transfected via nucleofection (Amaxa) with either miR-145 mimic (25 µM) or unscrambled miR-RNA (25 µM) control before (A) wt THP1 and (B) THP1ara-C(1) cells were treated with ara-C (10 µM) 8 h post transfection. 48 h post ara-C treatment cell viability was analysed using CellTitre-Glo® Luminescent Cell Viability Assay. Bars represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05. Aberrations, miR = miR-196a mimic, C = ara-C (10 µM).

5.3.6 IRAK1 and TRAF6 are all down regulated in THP1ara-C(1) cells. miR-146a has been show to target both IRAK1 and TRAF6 [122] [123], however they are all down regulated in the THP1ara-C(1) cells of which IRAK1 is significant (Figure 5.8 A-B). Potentially suggesting the relationship between miR-146a and its target genes is specific to certain disease models.

miR-222 is known to target both PTEN and PUMA expression [128] [95]. There is a slight increase in miR-222 expression (Figure 5.8 C) in the ara-C resistant THP1ara-C(1) cells, in response, both (D) PTEN and PUMA mRNA is down regulated. However these experiments do not prove a miRNA- mRNA targeting relationship instead a trend.
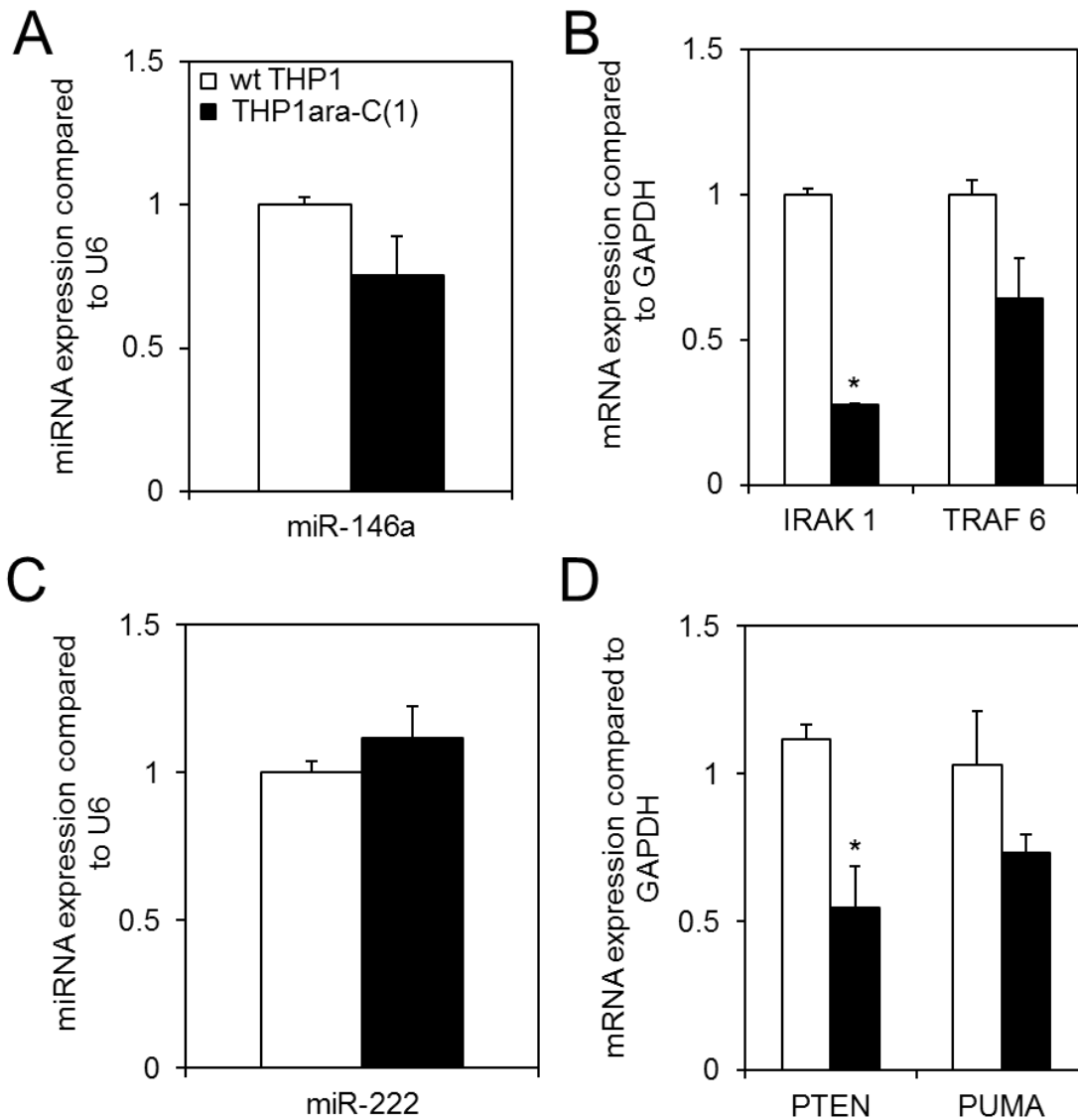
Figure 5.8: miR-146a targets IRAK1 and Traf6 whilst miR-222 targets both PTEN and PUMA. miRNA and mRNA was collected from wt THP1 and THP1ara-C(1) cells,(A) miR-146a miRNA expression, (B) IRAK1 and Traf6 mRNA expression, (C) miR-222 miRNA expression and (D) PTEN and PUMA expression was analysed using qRT-PCR, Bars represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.

5.4 Discussion

A number of miRNAs involved in both cancer and stem cell biology were highlighted by SBIs' QuantiMir Cancer and Stem cell Array and thus taken forward for further examination. The selection of miRNA taken forward was based on the inclusion criteria previously mentioned in 5.3.1. Highlighted miRNAs were differentially expressed between the ara-C resistant and non-resistant THP1 cells. The aim of this chapter was to explore miRNA regulation in response to acquired ara-C resistance.

The following highlighted miRNAs miR-15a, miR-21, miR-92a, miR-149, miR-186, miR-200a and miR-222 were all up-regulated in the ara-C resistant; THP1ara-C(1) cells whilst miR-101, miR-142-3p, miR-145, miR-146a, miR-183 and miR-196a were all down regulated. miR-196a regulation in regards to ara-C resistance will be further discussed in Chapter 6.

Dai et al (2011) noted an increase in miR-101 expression in docetaxol resistant head and neck squamous cell carcinoma cell lines; UMSCC-1 and SQ20B. Interestingly, a decrease in miR-101 expression was seen in the THP1ara-C(1) cells. Potentially suggesting miR-101 is involved in chemotherapeutic acquired resistance [133].

We have previously shown the THP1ara-C(1) have developed a reduced rate of proliferation compared to their wt THP1 control cells (Chapter 4; Figure 4.3 A-C). Over-expressed miR-15a and miR-16-1 in both K562 and HL60 cells has been shown to significantly inhibit cell proliferation. Gao et al (2011) observed miR-15a and miR-16-1 indirectly targets the oncogene, Wilms' tumour gene (wt1). They suggest miR-15a and miR-16-1 act as tumour suppressors by down regulating wt1 and therefore regulating cell proliferation [129]. wt1 is also known to regulate Bcl-2 expression [134]. We have shown an increase in miR-15a expression in the THP1ara-C(1) cells compared to their wt THP1 control cells. Thus, increased miR-15a expression could potentially be responsible for inhibited cell proliferation rate. We would like to explore the effect of a miR-15a mimic on cell proliferation within the THP1ara-C(1) cells.

miR-15a also targets the anti-apoptotic gene Bcl-2 in CLL. When Bcl-2 is repressed by miR-15a, apoptosis is induced in the human megakaryoblastic leukaemia cell line, MEG-01 [118]. However, miR-15a is significantly up regulated in the ara-C resistant, THP1ara-

C(1) cells, whilst Bcl-2 expression remains indifferent between both ara-C resistant and non-resistant cells. In light of the above findings, I would expect the anti-apoptotic gene Bcl-2, expression to be increased in response to continuous ara-C exposure in THP1ara-C(1) cells. However this does not appear to be the case. Possibly suggesting miR-15a does not target Bcl-2 in AML cells, Bcl-2 has a different function or other miRNAs are also acting on Bcl-2 expression within THP1ara-C(1) cells for example miR-125b or miR-155 [135]. We would like to explore miR-16-1 role and expression profile within ara-C resistance.

The relationship between miR-21 and ara-C resistance has been explored by a number of research groups in different cell lines. miR-21 is known to target PTEN [91] and PDCD4 [92, 93]. Li, et al (2010) observed in HL60 cells, the presence of an antimiR-21 oligonucleotide significantly sensitised the cells to ara-C treatment by inducing apoptosis, increased PDCD4 regulation was also seen, potentially explaining the cells response [93]. A similar response was reiterated in K562 cells by Hu et al (2010). miR-21 was yet again inhibited by an antisense oligonucleotide, cell apoptosis was enhanced whilst PDCD4 regulation was increased [92]. miR-21 up regulation was also seen in K562 DNR resistant cells, however when PTEN expression was silenced, cell survival was significantly increased. The same response was seen when miR-21 was over expressed [91].

We have also seen a significant increase in miR-21 expression and a subsequent decrease in its target gene, PTEN mRNA expression in the ara-C resistant THP1ara-C(1) cells. Potentially reinforcing miR-21 involvement in ara-C acquired resistance. When wt THP1 and THP1ara-C(1) cells were treated with ara-C, 24 h post ara-C treatment a significant decrease in miR-21 expression and increased PTEN mRNA expression was seen in the wt THP1 cells, however miR-21 expression remained indifferent between the control and ara-C treated THP1ara-C(1) cells. We have previously shown in Chapter 4; Figure 1 A decreased cell viability in response to ara-C (10 µM) in wt THP1 control cells whilst cell viability remained the same in the THP1ara-C(1) cells. Therefore, we have shown miR-21 expression is decreased in response to ara-C treatment in non-resistant AML cells. Further investigation is required regarding miR-21 and PTENs relationship in response to ara-C exposure and induced apoptosis in both ara-C resistant and non-resistant THP1 cells. We would also like to explore PDCD4

regulation and find out its involvement in ara-C induced apoptosis within the THP1ara-C(1) cells.
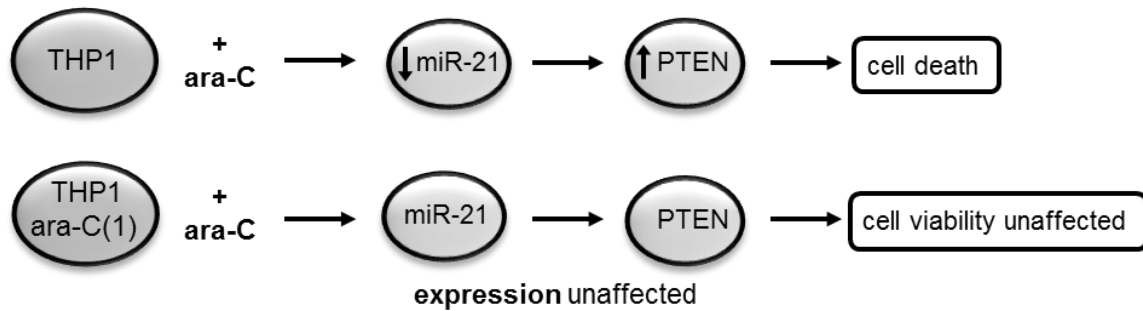
Figure 5.9: Exploring miR-21 and PTEN relationship in response to ara-C treatment.

A number of studies have examined the role of miR-34a in cancer over the last four years. miR-34a expression has been shown to be reduced in both cancer cell lines and patient samples compared to control non-cancerous cells by a number of authors. For example Li. Y et al (2009) reported miR-34a expression is reduced in human glioblastoma tissue [136] whilst Li. L et al (2012) have shown reduced expression in 5 breast cancer cell lines and 14 out of 17 patient samples [137]. When both breast cancer cells and glioblastoma cells were transfected with miR-34a cell growth was inhibited [136, 137]. miR-34a was significantly up regulated in the ara-C resistant THP1ara-C(1) cells compared to their wt THP1 control whilst cell growth rate was significantly reduced. However when weekly ara-C refreshment was withdrawn from the THP1ara-C(1) cells for a minimum of 4 weeks, miR-34a expression returned to below that of control wt THP1 cells, whilst their cellular growth rate was returned to that of the wt THP1 control cells. Suggesting the presence of continuous ara-C exposure could be involved in both miR-34a regulation and cell proliferation rate. A similar response was seen by Ichimura et al (2010) when they stimulated K562 cells with PMA [97]. miR-34a's target gene, SIRT1 [96] was down regulated in response to miR-34a increased expression. miR-34a could potentially regulate cell proliferation via SIRT1 and Bcl-2 regulation, due to down regulated SIRT1 and Bcl-2 in response to over expressed miR-34a via transient transfection [137]. However Bcl-2 was not down regulated in our THP1ara-C(1) cells, thus this theory is probably not likely for this particular disease model.

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Havelange et al (2011) have shown both miR-145 and miR-196a are positively correlated with pro-apoptotic genes such as BIM and PTEN based on their miRNA-mRNA profiling integration analysis [121]. When miR-145 is over expressed using an oligonucleotide, apoptosis was enhanced in K562 cells, an increase in BIM protein expression was also seen [121]. Due to the lack of predicted miRNA and mRNA interaction sites, BIM is regulated indirectly by miR-145 [121, 131]. In the ara-C resistant THP1ara-C(1) cells, miR-145 expression is down regulated basally when compared to the control wt THP1 cells whilst BIM expression is only marginally up-regulated, which could potentially be explained by the indirect relationship.

However, over-expressed miR-145 in the ara-C resistant THP1 cells; THP1ara-C(1) rescues them from additional ara-C exposure and induced cell death. Diagram 5.2 briefly demonstrates this response.
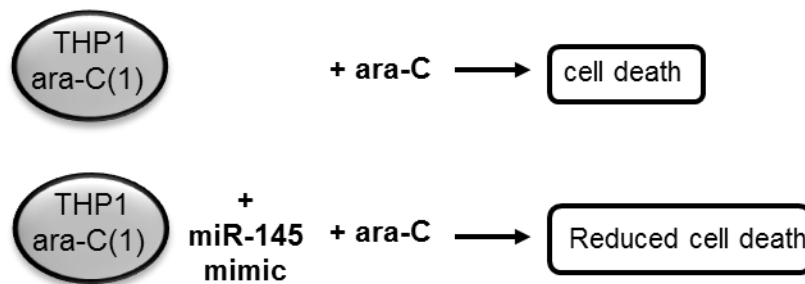
Figure 5.10: Increased miR-145 expression protects ara-C resistant AML cells from ara-C induced cell death.

miR-146a has been shown to target TRAF6 in SNK6 a NK/T Cell Lymphoma (NKTL) cells [122] and IRKA1 [123]. However miR-146a, TRAF6 and IRKA1 are all down regulated in the ara-C resistant TH1ara-C(1) cells.

We have shown a slight increase in miR-222 expression in the ara-C resistant THP1ara-C(1) cells. miR-222 has been shown to target both PTEN [128] and PUMA [95]. A decrease in both PTEN and PUMA mRNA expression was seen in the THP1ara-C(1) cells therefore re-iterating the miRNA and mRNA targeted relationship in AML cell lines.

To conclude we have explored the regulation of a number of miRNAs which were initially highlighted by SBIs Cancer and Stem Cell Arrays throughout this chapter. We have

shown over expressed miR-145 rescues ara-C resistant; THP1ara-C cells from high concentration of ara-C induced death. We have also explored the expression of miR-15a and miR-34a known to be involved in the regulation of cell proliferation.

Chapter 6: miR-196a and Bach-1 relationship in wild type THP1 and THP1ara-C(1) cells

6.1 Introduction

Previously we have highlighted and discussed both Cancer and Stem cell specific miRNAs which were differential expressed in ara-C resistant and non-resistant THP1 cells. miRNAs are short (between 18 and 25 nucleotides) evolutionary conserved RNAs [8, 88]. miR-196a was highlighted by the array and down regulated in the ara-C resistant THP1ara-C(1) cells compared to their wt THP1 control cells. miR-196a has been shown to target Annexin A1 [125], ETS transcription factor (ERG) [126], HOXA5 [138], HOX-C8 [139] and Bach1 [124]. We have previously shown the transcription factor Bach1 is up regulated in the ara-C resistant, THP1ara-C(1) cell line. Thus we feel the relationship between miR-196a and its target gene Bach1 in ara-C resistance warrants further investigation.

Recently a number of groups have explored miR-196a and its regulatory effect on its target genes. Luthra et al (2008) reported an inverse relationship between the expression of miR-196a and one of its targets, annexin A1 in twelve oesophageal, breast and endometrial cancer cell lines. The relationship was also observed in a further ten esophageal adenocarcinomas. The authors went on to investigate miR196a and annexin A1 expression in paired normal and tumour tissues from ten patients, they observed an increase in miR-196a expression and a subsequent decrease in annexin A1 expression in the tumour samples analysed. Over expression of miR-196a resulted in decreased annexin A1 expression in oesophageal, breast and endometrial cancer cell lines thus confirming miR-196a targets annexin A1 [125]. miR-196a has also shown to inhibit proliferation and enhance differentiation of mesenchymal stem cells which are derived from human adipose tissue, by binding to HOXC8 3-'UTR [140]. Both miR-196a and miR-196b have been identified as regulators of ERG, Coskun et al (2011) explored the expression of both miRNAs during hematopoietic differentiation. A significant increase in miR-196a and miR-196b expression was seen in AML patient samples compared to control healthy samples [126].

Bach1 has been shown to regulate HO-1 expression in AML cells. Miyazaki et al (2001) observed reduced Bach1 mRNA expression and subsequent increased HO-1 mRNA expression which resulted in enhanced cell survival [70]. Hou et al (2010) concluded miR-196a represses Bach1, by directly acting on its 3'-UTR therefore transnationally repressing its expression and furthermore leading to the up regulation of HO-1

expression in human hepatoma cells. miR-196a has also been shown to inhibit hepatitis C virus expression (HCV) in human hepatocytes [124].

miRNAs can target the regulation of both transcription factors and genes involved in cellular cytoprotection. Alongside miR-196a, miR-155 is also known to target and repress Bach1 expression in endothelial cells therefore allowing the induction of HO-1, its expression is induced via the NF-ΚBpathway by TNFα [141]. miR-101 and miR-132 are both predicted to target Nrf2 expression by microRNA.org [142]. miR-217 and miR-377 combined target HO-1 protein expression which leads to reduced HO-1 enzyme activity. When both miR-217 and miR-377 are knocked down HO-1 reduction is reversed in HEK 293 cells [143]. miR-128, miR-220b and miR-200c are all predicted to target HO-1 expression using mircoRNA.org [142]. miR-122 has been shown to target HO-1 in Hepatocellular carcinoma cell line, Huh-7-cells [144].

We have previously shown the expression of HO-1 (Chapter 3) is increased in response to chemotherapeutics treatment. However we next wanted to explore the regulation of other cytoprotective genes in response to ara-C acquired resistance. This was examined with the aid of a ara-C resistant cell line developed in house; THP1ara-C(1). Interestingly a number of cytoprotective genes; HO-1, NQO1 and GLCM and their transcription factor Nrf2, were all down regulated in response to acquired ara-C resistance. Hence why we wanted to explore Bach1 regulation in THP1ara-C(1) cells. miR-196a has been shown to regulated Bach1, thus its and Bach1 relationship will be further explored during the content of this chapter.

6.2 Aims

The aim of this chapter is to explore both miR-196a and Bach1s relationship and expression in relation to ara-C acquired resistance in THP1ara-C(1) cells. We also want to confirm whether the regulation of miR-196a affects ROS generation and establish whether this is involved in ara-C resistance. Finally explore whether differential expression in miR-196a reverses ara-C acquired resistance.

6.3 Results

6.3.1 Exploring the regulation of miRNAs involved in cellular cytoprotection. (A) SBI QuantiMir Cancer and Stem Cell Arrays combined results. miR-196a known to target Bach1, is down regulated in the ara-C resistant THP1ara-C(1) cell and is highlighted by a red dot. We were interested in investigating the expression of miRNAs which regulate BACH1, HO-1 or Nrf2 expression therefore we identified potential miRNAs using both microRNA.org [142] and relevant literature. (B) The expression of the identified miRNAs is displayed and represented as fold change. miR-377, miR-217, miR-155, miR-200b and miR-128 were all up-regulated in ara-C resistant THP1ara-C(1) cells whilst miR-132, miR-101, miR-122 and miR-196a were down regulated. Whilst miR-200c regulation was indifferent between the ara-C resistant and non-resistant THP1 cells.

Both miR-155 and miR-196a target Bach1 expression however their expression is subsequently different. Whilst HO-1 is targeted by miR-128, miR-200b, miR-217 and miR-377, their regulation is up, compared to the non-resistant control wt THP1 cells however miR-122 is down regulated compared to the other miRNAs and miR-200c regulation is indifferent between the ara-C resistant, THP1ara-C(1) and non-resistant wt THP1 cells. Nrf2 is targeted by both miR-101 and miR132 of which both are down regulated in the ara-C resistant, THP1ara-C(1) cells. A diagrammatic representation of the miRNA and their target genes mentioned above is present in
 Figure 6.1 C.

Table 6.1: miRNAs and their target genes.

miRNA	Target	Reference Source
miR-155	Bach1	[141]
miR-196a*		[124]
miR-122	HO-1	[144]
miR-128		microRNA.org [142]
miR-200b		microRNA.org [142]
miR-200c		microRNA.org [142]
miR-217		[143]
miR-377		[143]
miR-101	Nrf2	microRNA.org [142]
miR-132		microRNA.org [142]

A

B

	Fold change	
	wt THP1	THP1ara-C(1)
miR-132	1	0.25
miR-101	1	0.32*
miR-122	1	0.09*
miR-377	1	2.98
miR-217	1	8.71
miR-155	1	3.84
miR-200b	1	1.93*
miR-200c	1	1.03
miR-196a	*1*	*0.62**
miR-128	1	1.82*

C
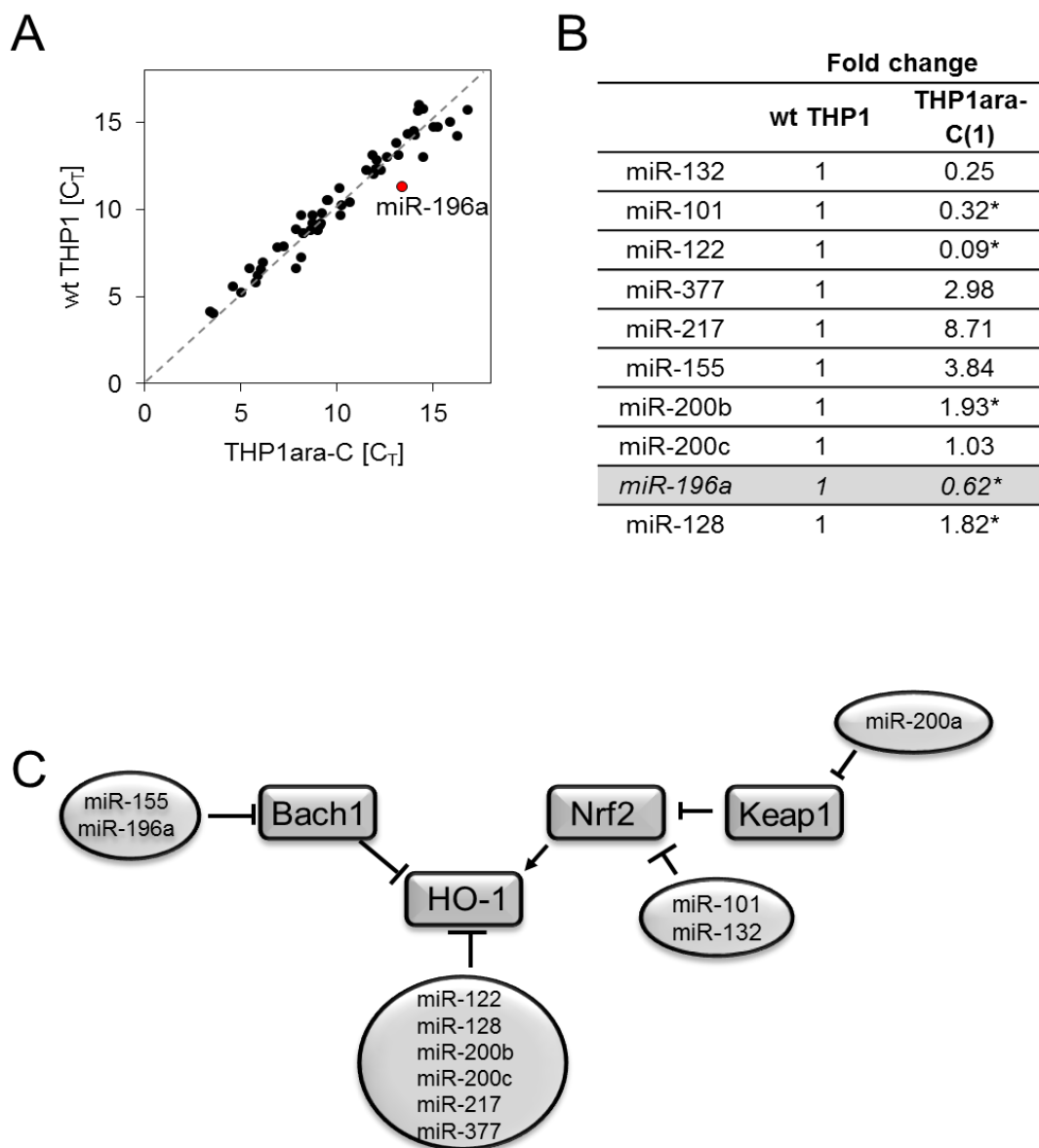
Figure 6.1: miRNA which target cytoprotective genes HO-1 and Nrf2 in ara-C resistant, THP1ara-C(1) cells. (A) SBI QuantiMir Cancer and SBI QuantiMir Stem cell Array was performed on both wt THP1 and THP1ara-C(1) cells, samples were analysed using qRT-PCR. Results from both arrays were combined and presented (n = 1). (B) miRNAs involved in cellular cytoprotection, table represents miRNA fold change (n = 3). (C) Diagrammatic representation of miRNAs and corresponding cytoprotective target genes. Circles represent mean ± SEM, Statistical significance was calculated by Students t-test; * p≤0.05.

6.3.2 Bach1 basal expression is up regulated in THP1ara-C(1) cells in response to mi-196a down regulation. miR-196a has been shown to target the transcription factor, Bach1 [124]. The SBI QuantiMir Cancer Array indicated miR-196a expression was down regulated in the THP1ara-C(1) cells compared to their control, wt THP1 cells. Thus we wanted to explore the regulation of Bach1 in response to down regulated miR-196a. Figure 6.2 shows (A) miR-196a miRNA expression and (B) Bach1 mRNA expression which was investigated using qRT-PCR. miR-196a expression is significantly down regulated in THP1ara-C(1) cells whilst the regulation of Bach1, its target gene is significantly up regulated in THP1ara-C(1) cells compared to that of the wt THP1 control. Bach1 protein expression was analysed using Western immunoblotting. Figure 6.3 A reiterates the above point, increased Bach1 protein expression was seen in THP1ara-C(1) cells (top panel). Difference in Bach1 expression from three independent Western blots was explored in greater detail using Quantity One 1-D Analysis Software which showed Bach1 expression was greater in THP1ara-C(1) cells.

6.3.3 miR-196a mediates Bach1 expression. Next we wanted to confirm the direct targeting relationship between the miRNA, miR-196a and its target gene, Bach1. Genomic DNA extraction, primer PCR design and subcloning of the pMIR-REPORT reporter construct, were performed by Dr Lyubov Zaitseva as described previously [52]. This was examined using the Dual Glo Luciferase Assay System Kit (Figure 6.4 B). Previously mentioned, Bach1 is significantly up regulated in the ara-C resistant THP1ara-C(1) cells (Figure 6.2 B). miR-196a mimic was transfected into HEK 293 cells which significantly reduced pMIR-REPORT 3'UTR-Bach1 luciferase activity. Figure 6.4 C explores different miR-196a mimic concentrations and its effect on pMIR-REPORT 3'UTR-Bach1 luciferase activity. A significant decrease in pMIR-REPORT 3'UTR-Bach1 luciferase activity was seen in response to varying miR-196a mimic concentration. To further explore and confirm the relationship between miR-196a and Bach1, (Figure 6.4 D) miR-196 mimic was transfected into THP1ara-C(1) cells via nucleofection, before Bach1 mRNA expression was examined using qRT-PCR. A significant decrease in Bach1 mRNA expression was seen in the THP1ara-C(1) cells further confirming miR-196a and Bach1s relationship.
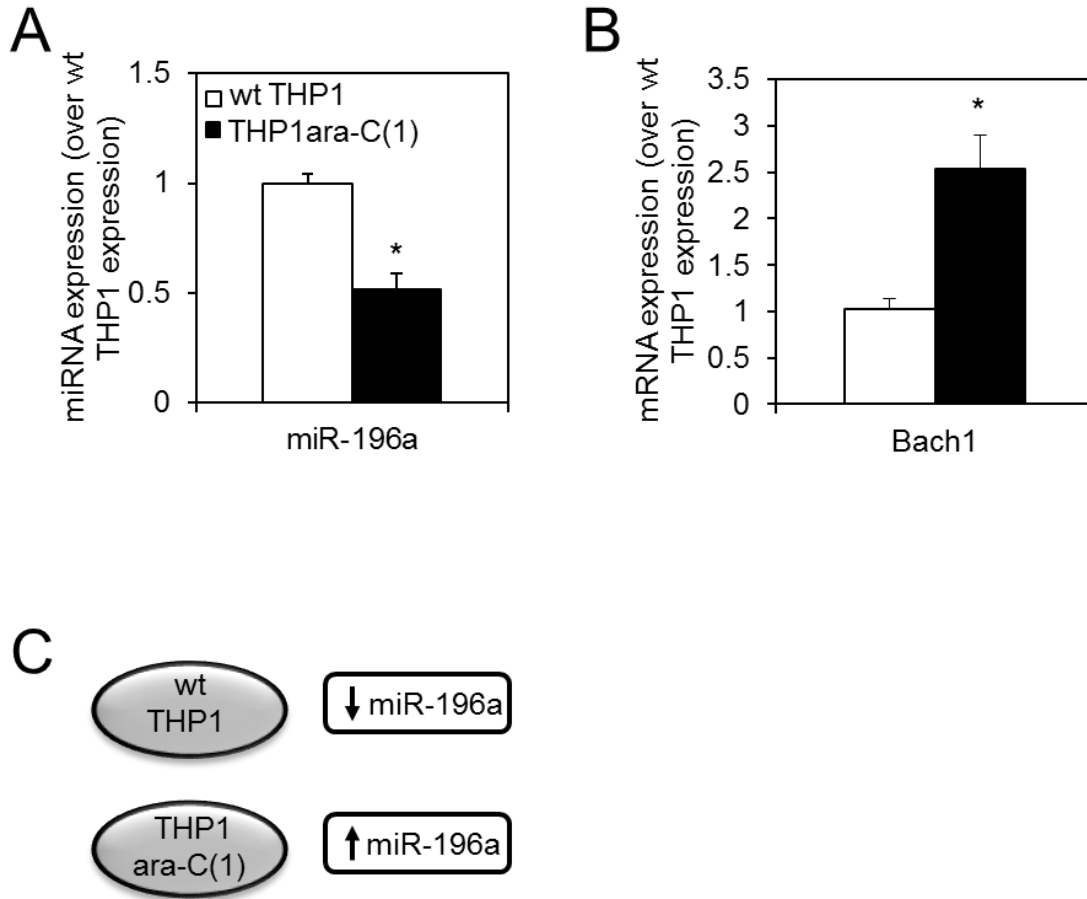
Figure 6.2: miR-196a expression is down regulated in THP1ara-C(1) cells. (A) miRNA and mRNA was collected from wt THP1 and THP1ara-C(1) cells before miR-196a basal expression and (B) Bach1 basal expression was analysed using qRT-PCR. (C) Diagrammatic representation of miR-196a expression in both wt THP1 and THP1ara-C(1) cells. Bars represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.
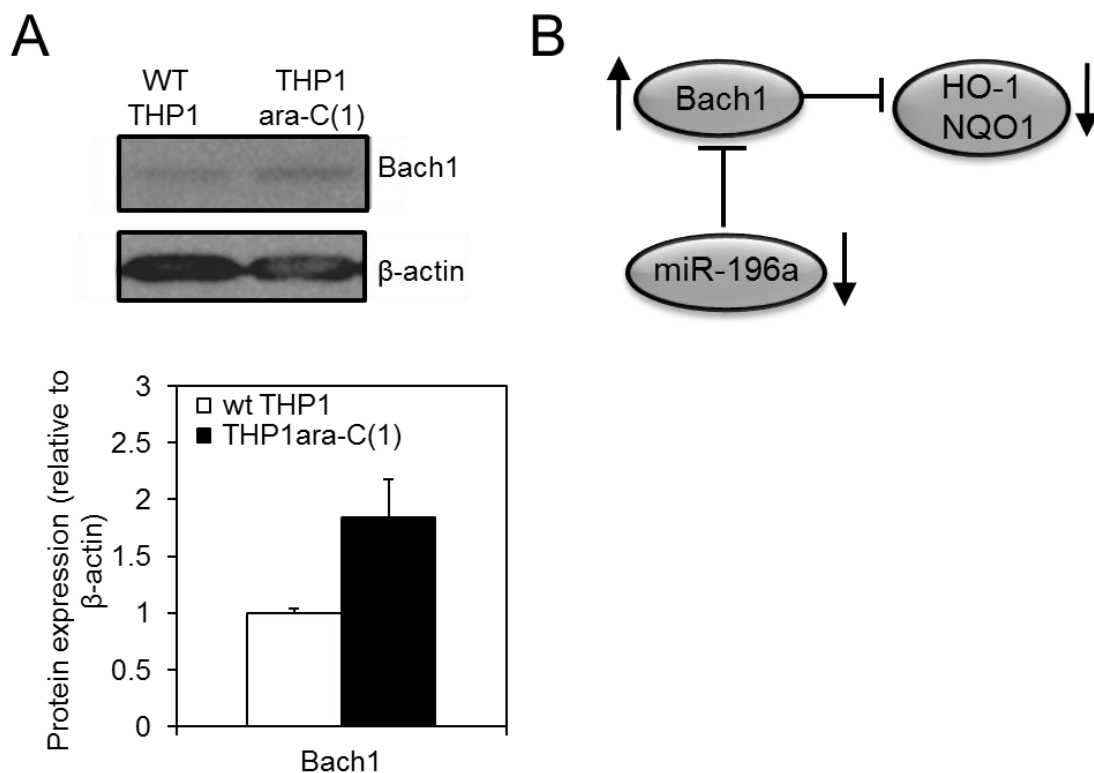
Figure 6.3: Bach1 expression is up regulated in ara-C resistant, THP1ara-C(1) cells. (A) Whole cell protein extracts were collected from wt THP1 and THP1ara-C(1) for Western immunoblotting analysis of basal Bach1 protein expression (top panel) Membranes were probed with β-actin to ensure equal protein lysate loading across samples. Quantity One 1-D Analysis software (BioRad) was utilised to quantify protein expression changes between cell types (bottom panel). Blot is representative of three separate experiments, however Quantity One 1-D Analysis is representative of all three experiments. (B) Diagrammatic representation of predicted signalling pathway involving miR-196a and Bach1. Bars represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.
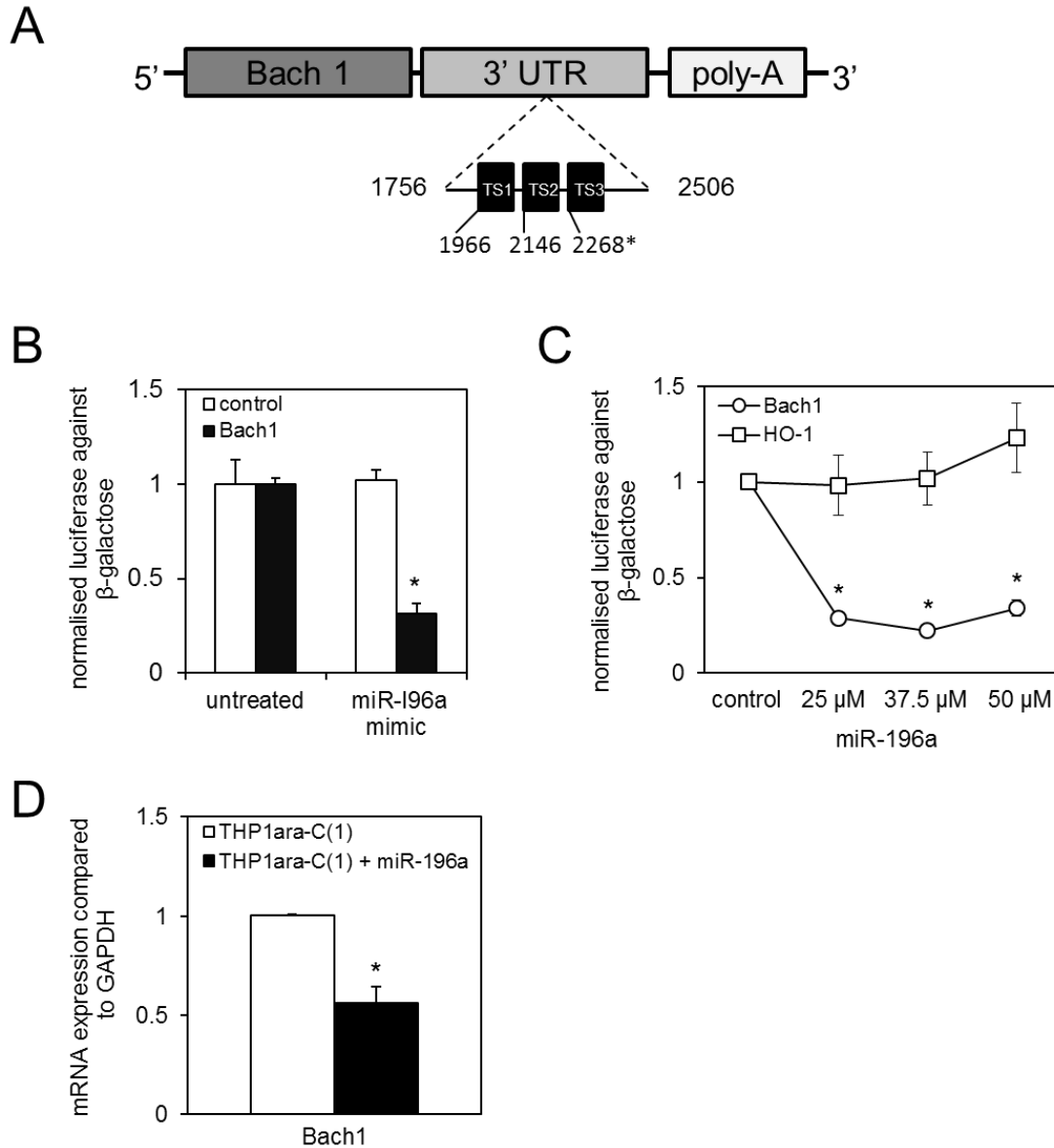
Figure 6.4: miR-196a mediates Bach1 regulation. (A) Bach1 and miR-196a 3'UTR diagrammatic representation. (B) HEK 293 cells were plated onto 6-well plates at 3.5-4 x10⁵/well and transfected using Lipofectamine™ 2000 with total 500 ng DNA (250 ng pMIR-REPORT™-Luciferase or pMIR-REPORT -3'UTR-Bach1 and 250 ng pMIR-REPORT™-β-gal Control Plasmid) with optional 25 µM miR-196a mimic and incubated before luciferase and β-galactosidase activities were measured 48 h after transfection using Dual-Glo Luciferase Assay System Kit (Applied Biosciences) on a WALLAC_Envision, 2103 Multi Label Reader (n = 3). (C) HEK 293 cells were transfected

as before with optional 25 µM, 37.5 µM or 50 µM miR-196a mimic before luciferase and β-galactosidase activities were measured as before (n = 1). (D) 2 x10^6 THP1ara-C(1) cells were transfected with either scrambled control siRNA (25 µM) or miR-196a siRNA (25 µM) via nucleofection technology and incubated for 24 h before Bach1 mRNA expression was analysed using qRT-PCR. Bars or Circles represent mean ± SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.

6.3.4 ROS generation is reduced in ara-C resistant THP1ara-C cells in response to reduced Bach1 expression. Previously shown in Chapter 4 Figure 4.10 A and again here, in Figure 6.5 A basal ROS generation is significantly increased in THP1ara-C(1) cells compared to that of their control wt THP1. Therefore, we next wanted to investigate whether miR-196a was involved in ROS generation in ara-C resistant; THP1ara-C(1) cells. (B) miR-196a mimic was transfected into THP1ara-C(1) cells using nucleofection before incubation with H_2DCFDA, cells were harvested and ROS generation analysed using flow cytometry. However, when miR-196a is over-expressed a decrease in ROS generation was observed, potentially indicating miR-196a is involved in ROS regulation. We next wanted to investigate cell viability using CellTitre-Glo® Luminescence Viability Assay in response to increased miR-196a expression and reduced ROS generation. (Figure 6.6 A) Cell viability was reduced over a 48 h time course in the presence of increased miR-196a expression. (B) Finally, we wanted to explore what effect increased miR-196a expression would have on the cells ability to cycle. Both wt THP1 and THP1ara-C(1) cells were transfected with either the miR-196a mimic or unscrambled control via nucleofection. A decrease in S phase was seen in both wt THP1 + M and THP1ara-C(1) + M cells in response to increased miR-196a expression compared to their relevant controls. However a slight increase in G1 and a decrease in G2 phase was seen in the THP1ara-C(1) + M compared to their control; THP1ara-C(1) cells.

6.3.5 In response to over-expressed miR-196a, Bach1 expression was down regulated whilst the cytoprotective gene, HO-1 expression was enhanced in the ara-C resistant THP1ara-C(1) cells. Both wt THP1 and THP1ara-C(1) cells were transfected with either miR-196a mimic or unscrambled control before Bach1 and HO-1 mRNA expression was analysed using qRT-PCR. Figure 6.7 A shows a decrease in Bach1 mRNA and an increase in HO-1 mRNA expression in response to the miR-196a mimic in both ara-C resistant THP1ara-C(1) cells and control wt THP1 cells. Increased Bach1 mRNA expression and decreased HO-1 mRNA expression was also seen between the wt THP1 control cells and the ara-C resistant THP1ara-C(1) control cells.
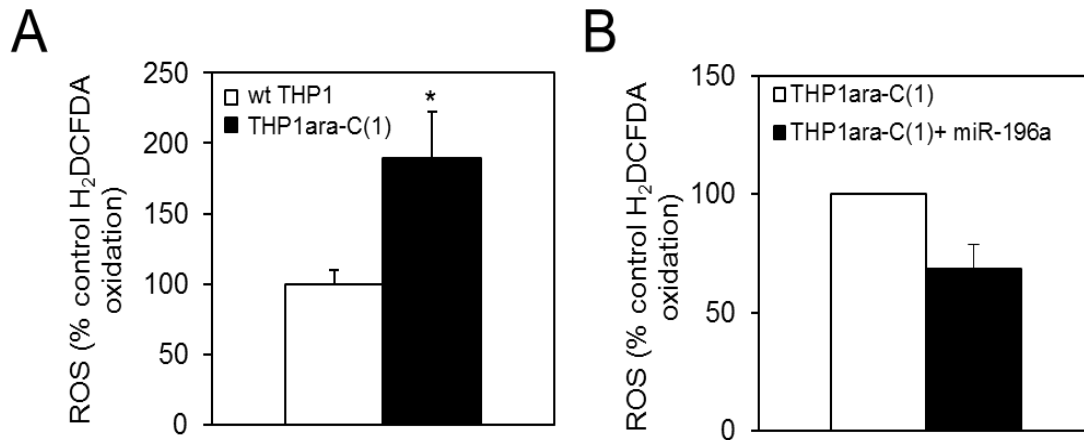
Figure 6.5: ROS regulation is reduced in response to increased miR-196a expression. (A) wt THP1 and THP1ara-C(1) AML cells were incubated with H_2DCFDA (1 µM) for 30 min. Cells were harvested and re-suspended in PBS. ROS production was measured using flow cytometry (n = 7). (B) 2 x10^6 THP1ara-C(1) cells were transfected with either unscrambled control siRNA (25 µM) or miR-196a siRNA (25 µM) via nucleofection technology and incubated for 24 h before being incubated with H_2DCFDA (1 µM) for 30 min. Cells were treated as previously outlined in A. Bars represent the mean ±SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.
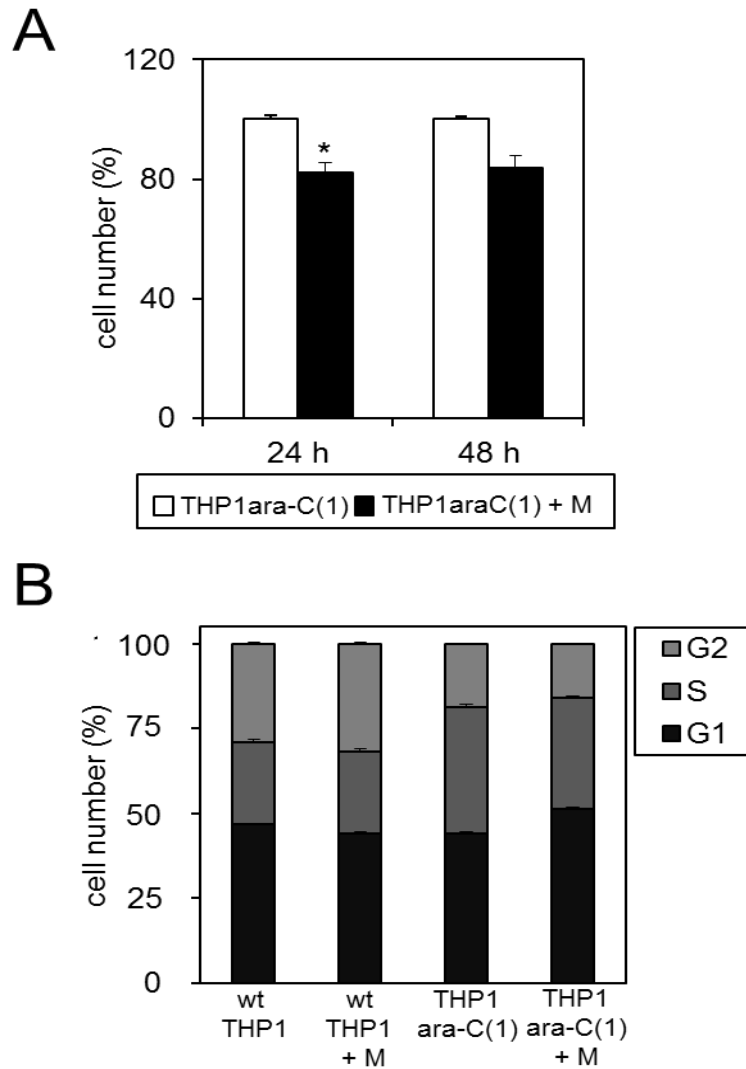
Figure 6.6: Increased miR-196a expression results in decreased cell viability. (A) cell viability was assessed 24 and 48 h post transfection with CellTitre-Glo® Luminescence Viability Assay (n = 3). (B) 2 x10^6 wt THP1 and THP1ara-C(1) cells were transfected with either unscrambled control siRNA (25 µM) or miR-196a siRNA (25 µM) via nucleofection technology and incubated for 48 h before cells were fixed and stained with PI before Cell cycling was determine using flow cytometry. Bars represent the mean ±SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05. (Abbreviations M = miR-196a mimic).
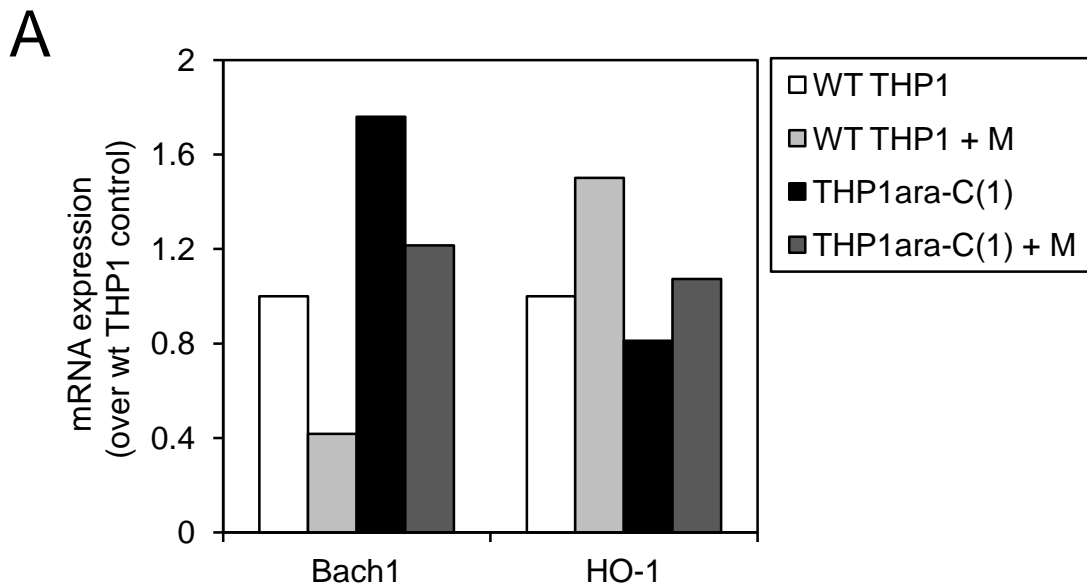
Figure 6.7: HO-1 expression is increased in the presence of increased miR-196a expression. (A) 2 x 10^6 wt THP1 and THP1ara-C(1) cells were transfected with either unscrambled control siRNA (25 µM) or miR-196a siRNA (25 µM) via nucleofection technology and incubated for 48 h before mRNA was collected from wt THP1, wt THP1 + M, THP1ara-C(1) and THP1ara-C(1) + M cells before Bach1 and HO-1 mRNA expression was analysed using qRT-PCR. Abbreviations M = miR-196a mimic.

6.3.6 ROS generation is increased further after additional ara-C exposure in THP1ara-C(1) cells. THP1ara-C(1) cells were treated with ara-C (10 µM) for 24 h and then incubated with H$_2$DCFDA for a further 30 min before ROS generation was determined using flow cytometry. Figure 6.8 A shows a significant increase in ROS generation in response to additional ara-C exposure. (B) THP1ara-C(1) cells were transfected with either unscrambled siRNA or miR-196a mimic 24 h before ara-C (10 µM) treatment. Cell viability was determined 24 and 48 h post ara-C treatment using CellTitre-Glo$^®$ Luminescence Viability Assay. Cell viability remained unchanged with the addition of ara-C at both 24 and 48 h which we have previously shown (Chapter 4: 4.2 A) However, a decrease in the percentage of viable cells was seen in the presence of increased miR-196a expression at both 24 and 48 h, of which 24 h was significant. Finally, we wanted to explore cell viability in response to both increased miR-196a expression and additional ara-C treatment. A significant decrease in cell viability was seen at both 24 and 48 h potentially suggesting increased miR-196a expression is reversing THP1ara-C(1) cells ara-C acquired resistance.

6.3.7 Even when weekly ara-C refreshment is withdrawn, Bach1 mRNA expression remains elevated in THP1ara-C(1) TW cells. We wanted to explore whether both miR-196a miRNA and Bach1 mRNA expression were altered upon withdrawal of ara-C weekly refreshment. Therefore we withdraw weekly ara-C refreshment for at least 4 weeks before examining miRNA and mRNA expression using qRT-PCR. Figure 6.9 A indicates miR-196a is down regulated, whilst its target gene, (B) Bach1 mRNA expression is significantly increased in THP1ara-C(1) TW cells. Suggesting up regulated Bach1 expression is involved in ara-C acquired resistance as opposed to just the presence of continual ara-C exposure.
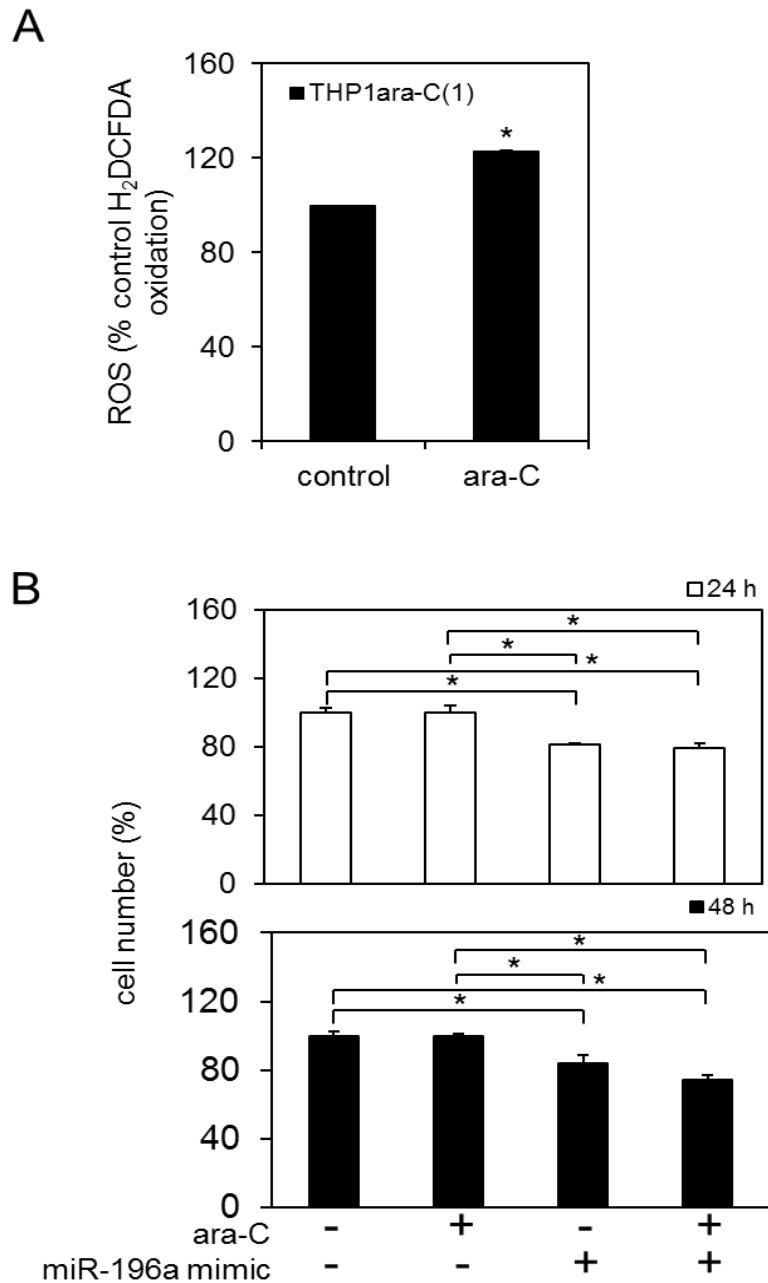
Figure 6.8: ROS generation is increased further when ara-C resistant cells; THP1ara-C(1) are treated with additional ara-C. (A) 1 x 10^6 THP1ara-C(1) cells treated with ara-C (10 μM) for 24 h before cells were collected and incubated with the fluorescent probe H$_2$DCFDA (1 μM) for 30 min before ROS production was measured using flow cytometry. (B) 2 x 10^6 THP1ara-C(1) cells were transfected with either unscrambled control siRNA (25 μM) or miR-196a siRNA (25 μM) via nucleofection technology and incubated for 24 h before ara-C treatment (10 μM). Cell viability was

assessed 24 and 48 h post ara-C treatment with CellTitre-Glo® Luminescence Viability Assay (n = 3). Bars represents mean ±SEM (n = 3). Statistical significance was calculated by (A) Students t-test; * p≤0.05 and (B) ANOVA; * p≤0.05.
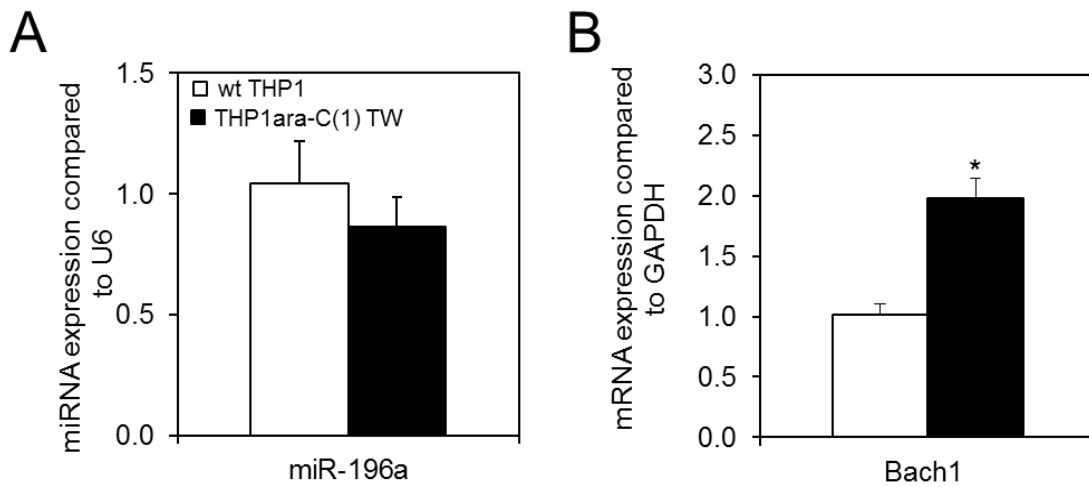
Figure 6.9: Bach1 mRNA expression in THP1ara-C(1) TW remains elevated. (A) miRNA and mRNA was collected from wt THP1 and THP1ara-C(1) TW cells miR-196a basal expression and (B) Bach1 basal expression was analysed using qRT-PCR. Bars represent the mean ±SEM (n = 3). Statistical significance was calculated by Students t-test; * p≤0.05.

6.4 Discussion

We have previously explored the expression profile of key cytoprotective genes involved in cellular cytoprotection. Therefore we wanted to examine the expression profile of miRNAs either known or predicted to target genes involved in cellular cytoprotection. We have previously shown in Chapter 4 Nrf2, HO-1 and NQO1 are all down regulated in the ara-C resistant THP1ara-C(1) cell line. Figure 6.10 highlights miRNAs known to target both transcription factors and genes whos expression is differential regulated in the THP1ara-C(1) cells compared to their control wt THP1 counterparts.
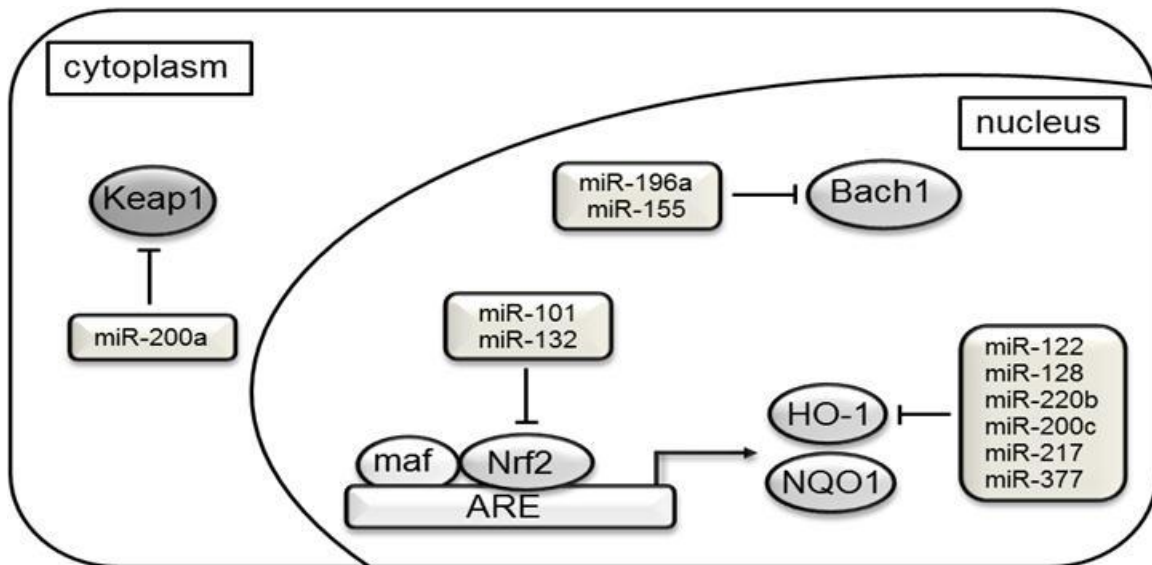
Figure 6.10: Overview of miRNA targets. A diagrammatic representation of which miRNAS have been proven or predicted to target Bach1, HO-1, Keap1 and Nrf2s expression.

HO-1 is down regulated in the ara-C resistant THP1ara-C(1) cells, however all of the miRNAs either known or predicted to target HO-1 expression, which we examined were up regulated apart from miR-122.

Both miR-101 and miR-132 are predicted to target Nrf2 by microRNA.org [142], however, their expression is down regulated alongside Nrf2s in the THP1ara-C(1) cells. Either suggesting both of these miRNAs do not target Nrf2 in AML cell lines or their effect on Nrf2 expression is over thrown by other miRNAs/ transcription factors. Further

investigation to confirm the miRNA and mRNA targeting relationship would be required to comment on the involvement of these two miRNAs.

Both miR-155 and miR-196a have previously been shown to regulate Bach1 expression. miR-155 miRNA and Bach mRNA were both up regulated in the ara-C resistant cells, THP1ara-C(1) cells whilst miR-196a expression was down regulated. miR-155 have been shown to target Bach1 in endothelial cells [141]. However there appears to be a difference in miR-196a and miR-155 miRNA expression in the THP1ara-C(1) cells. The difference in miRNA expression could be due to miR-155 posing a different function within AML cells and potentially therefore is not targeting Bach1. Or miR-196a and Bach1s targeting relationship is greater, therefore miR-155 effect on Bach1s regulation is being over ridden. However both miR-196a and miR-155 are known to repress Bach1 expression [124, 141]. We also need to consider miR-155 and miR-196a regulate a number of other genes as well as Bach1 and their expression is also directly or indirectly regulated by other transcription factors and genes.

Here we have shown miR-196a is down regulated in the ara-C resistant THP1ara-C(1) cell line compared to its wt THP1 control. Xia et al (2008) have also reported decreased miR-196a expression in SGC7901/VCR cells, a multi-drug resistant gastric cancer cell line [145]. Potentially linking miR-196a to cell acquired drug resistance. Hou et al (2010) have previously reported miR-196a targets Bach1 in human hepatoma cells [124]. We have also confirmed miR-196a and Bach1s relationship in HEK 293 cells using the Dual Glo Detection Luciferase Assay Kit. Bach1 expression is up regulated in the ara-C resistant THP1ara-C(1) cells, whilst the cytoprotective transcription factor and genes; Nrf2, HO-1 and NQO1 are all down regulated as previously shown. When miR-196a was over expressed in THP1ara-C(1) cells a significant decrease in Bach1 expression was seen, thus reiterating that Bach1 is one of miR-196a targets. In response to over expressed miR-196a miRNA, the cytoprotective gene HO-1 expression is increased in both wt THP1 and THP1ara-C(1) cells.

We experienced a number of technical problems during the optimisation of the Dual Glo Detection Luciferase Assay which are outlined in Diagram 6.13. Briefly, wt THP1 cells are notoriously known to be difficult to transfect, thus we eventually decided to use HEK 293 cells as a tool to confirm the miRNA and mRNA relationship instead, even though

they are a different type of cell line, after extensive literature review we established the norm in miRNA research is to present the relationship between the miRNA under investigation and its target mRNA in HEK 293 cells.

We have previously shown in Chapter 4 Figure 4.9 A, ROS expression is elevated in the ara-C resistant THP1ara-C(1) cells. However when miR-196a is over expressed ROS generation is in turn reduced. Potentially suggesting miR-196a is implicated in ROS generation and cellular cytoprotection. However, ROS generation is increased further in response to ara-C (10 µM) exposure in additional to their weekly ara-C refreshment in THP1ara-C(1) cells. Furthermore, when THP1ara-C(1) cells are treated with additional ara-C (10 µM) and miR-196a is over expressed via the addition of a miR-196a mimic and nucleofection, decreased cell viability was seen. Potentially suggesting reduced Bach1 expression and reduced ROS generation is reversing THP1ara-C(1), ara-C acquired resistance.

When miR-196a was over expressed in the ara-C resistant THP1ara-C(1) cells, the percentage of cells undergoing each phase of the cell cycling was altered. A reduction in S phase and an increase in G1 phase was seen in the THP1ara-C(1) cells.

However when continual ara-C treatment is withdrawn; decreased basal miR-196a expression and increased Bach1 mRNA expression is still seen in the THP1ara-C(1) TW cells.
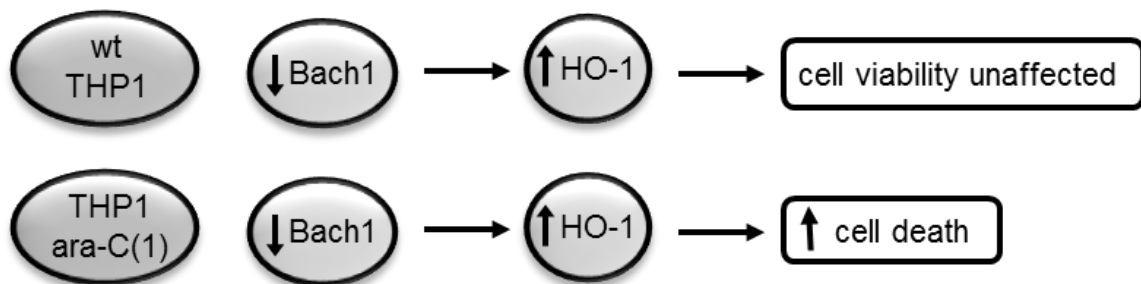
Figure 6.11: Reduced Bach1 expression leads to increased ara-C induced cell death.

To conclude reducing Bach1 expression in ara-C resistant THP1ara-C(1) cells reverses ara-C acquired resistant whilst reducing ROS generation. Therefore targeting and inducing miR-196a expression in acquired ara-C resistant AML cells could potentially

prove to be a viable method of treatment. However further investigating surrounding miR-196a and Bach1 relationship needs to be explored in vitro with ara-C resistant AML patients samples.
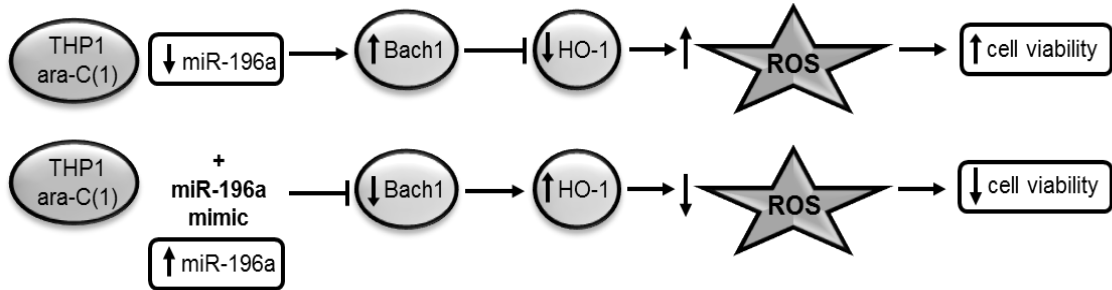
Figure 6.12: Overview of reversing ara-C acquired resistance in THP1ara-C(1) cells.

Method of Transfection
Chemical transfection (Lipofectamine™2000) was initially used to transect DNA plasmids followed by nucleofection (amaxa) to transfect miRNA siRNA.
Problem: 80% of the cells died during the process, therefore decided to transfect both DNA plasmids and miRNA siRNA at the same time.
Result: Nucleofection (amaxa technology)

DNA concentration
Aware of DNA toxicity towards cells therefore optimised via titration (500-1000ng total DNA).
Result: 800ng total plasmid DNA

Transfection duration
Optimised collection point from 24 to 48 h. Monitored effect of DNA on protein expression found 48 h was an effective time point for sample collection..
Results: 48 h incubation before collection

Cell line used
Immortalized AML cell lines are notoriously difficult to transfect with DNA plasmids. HEK 293 cells were used as a tool line due to their good transfection efficiency.
Result: HEK 293

Suitable control
Background luciferase activity in empty vector and -3'UTR constructs of different proteins can not be normalised between constructs.
Result: Used empty vector as control

Figure 6.13: Luciferase time line. Diagrammatic represenation of the problems encounterd during luciferase assay optimisation.

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Chapter 7: Discussion

7.1 Overview of results. We have shown an increase in both HO-1 expression and ROS generation in AML primary cells and AML derived cell lines in response to ara-C and DNR exposure. However when HO-1 was silenced, cell viability in response to chemotherapeutic exposure was increased indicating HO-1s involvement in acquired cellular resistance. Next we explored ara-C acquired resistance by developing a ara-C resistant form of an AML derived cell line THP1 (THP1ara-C(1), by continual exposure to ara-C. Differential regulation of the cytoprotective gene HO-1 was seen in response to ara-C acquired resistance whilst ROS generation was enhanced. However upon withdrawal of continual ara-C exposure, ROS generation is once again reduced but the cells still maintain resistance towards ara-C. Finally miRNAs which target cytoprotective gene expression were explored in both the wt THP1 and THP1ara-C(1) cells. miR-196a known to target Bach1 is down regulated in response to ara-C acquired resistance whist its target is up-regulated.

7.2 Does HO-1 rescue AML cells from induced cell death? We have previously shown in AML derived cell lines, NF-κB inhibition results in acquired cellular resistance towards TNF induced cell death. Furthermore an increase in HO-1 mRNA expression was seen in response to TNF stimulation when NF-κB was inhibited TNF stimulation induced ROS generation, which in turn induced Nrf2 mRNA expression followed by HO-1 mRNA induced expression and thus protection from cell death [57]. Increased basal NF-κB expression has been reported in AML cells compared to their non-cancerous counterparts [46]. We have also previously shown HO-1 acts as a silent anti-apoptotic factor. Its expression is revealed and induced only when NF-κB is inhibited. Whilst HO-1s basal expression is suppressed in AML cells compared to control CD34$^+$ non-malignant cells. In AML cells HO-1 mRNA suppression is regulated by NF-κB subunits p50 and p65. When both NF-κB and HO-1 mRNA expression was inhibited in AML cells a significant decrease in cell viability was reported compared to the non-cancerous control cells. This potentially suggests, inhibiting both HO-1 and NF-κB expression could be a viable therapeutic target for targeted AML treatment [49]. FLIP has been shown to indirectly induce HO-1 mRNA regulation in AML cells in response to TNF stimulation, however this response does not occur in non-cancerous control cells. When FLIP$_L$s expression is knocked down, an increase in HO-1 mRNA expression within a small population of these cells is reported displaying cytoprotection and increased resistance towards TNF induced cell death [76]. Finally we have reported induced Nrf2 mRNA

expression in response to bortezomib exposure which subsequently leads to induced HO-1 mRNA expression and cytoprotection in AML derived cell lines [47]. A similar induction in HO-1 expression was also seen in both primary AML cells and cell lines in response to ara-C, DNR or a combination thereof exposure [101].

The regulation of HO-1 in response to TNF stimulation differs between both AML and non-cancerous human monocytes. Suggesting the regulation of both transcription factors and genes involved in the cells cytoprotective responses are differential regulated during cancer progression. We have shown TNF regulation is responsible for the expression of the transcription factor Nrf2, via the generation of ROS in human monocytes. Nrf2 then subsequently induces the expression of both cytoprotective and detoxifying genes. However, the expression of HO-1 mRNA is reduced in response to TNF stimulation within these cells, consistent with other reported results [146]. This is the reverse of what was reported in AML derived cell lines stimulated by TNF.
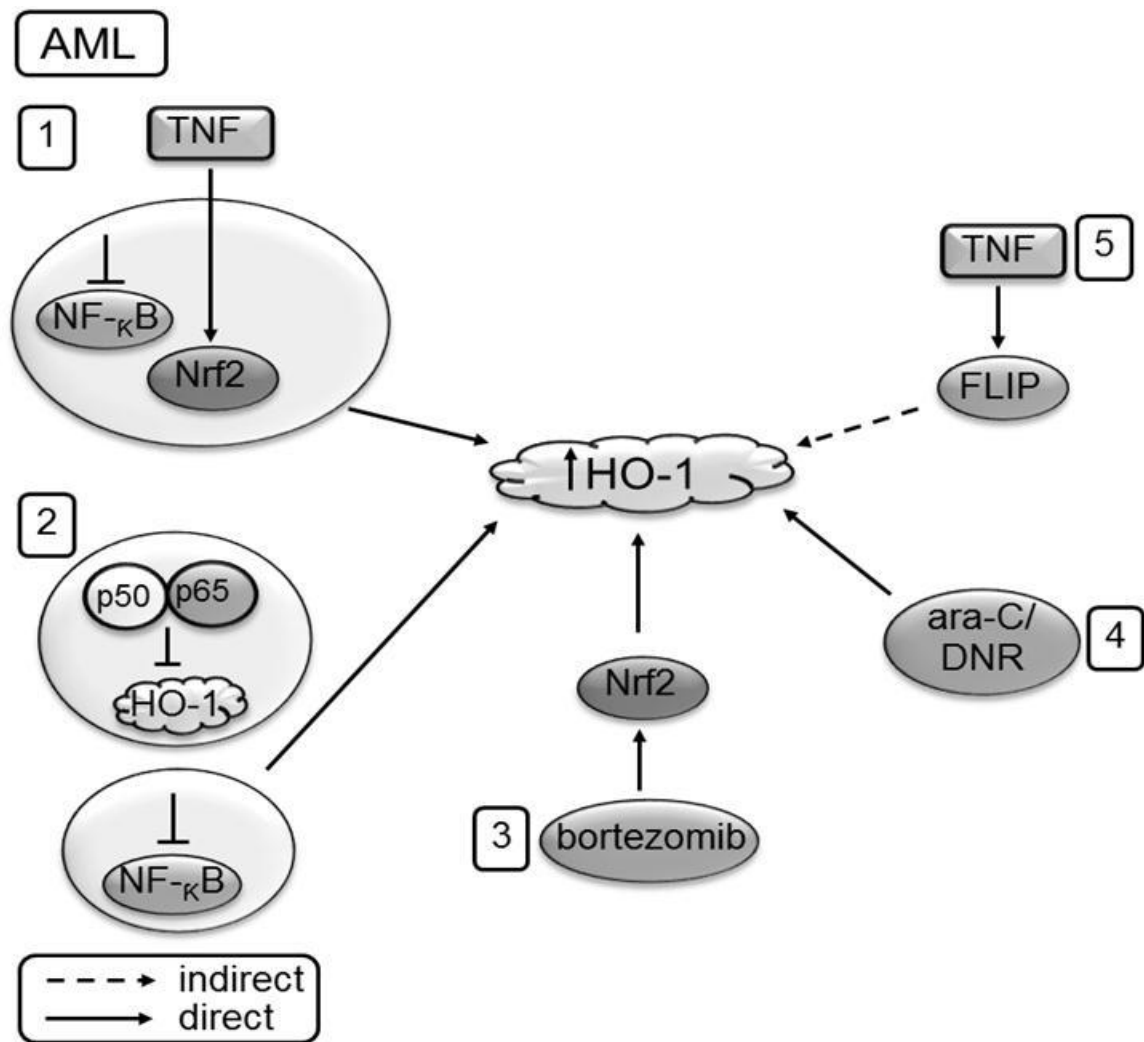
Figure 7.1 The regulation of HO-1 in response to chemotherapeutic exposure, TNF stimulation and NF-κB inhibition within AML cells. Diagrammatic representation of results reported by Professor MacEwan's laboratory regarding HO-1 induction in response to various stimuli in primary AML cells and AML derived cell lines. (1) TNF stimulation induces Nrf2 expression which induces HO-1 expression [57]. (2) NF-κB subunits p50 and p65 inhibit HO-1 expression however inhibition of NF-κB results in induced HO-1 expression [49]. (3) Bortezomib exposure up-regulates Nrf2 expression which in turn induces HO-1 expression [47]. (4) Ara-C and DNR exposure induces HO-1 expression [101]. (5) FLIP indirectly induces HO-1 expression in response to TNF stimulus [76] .
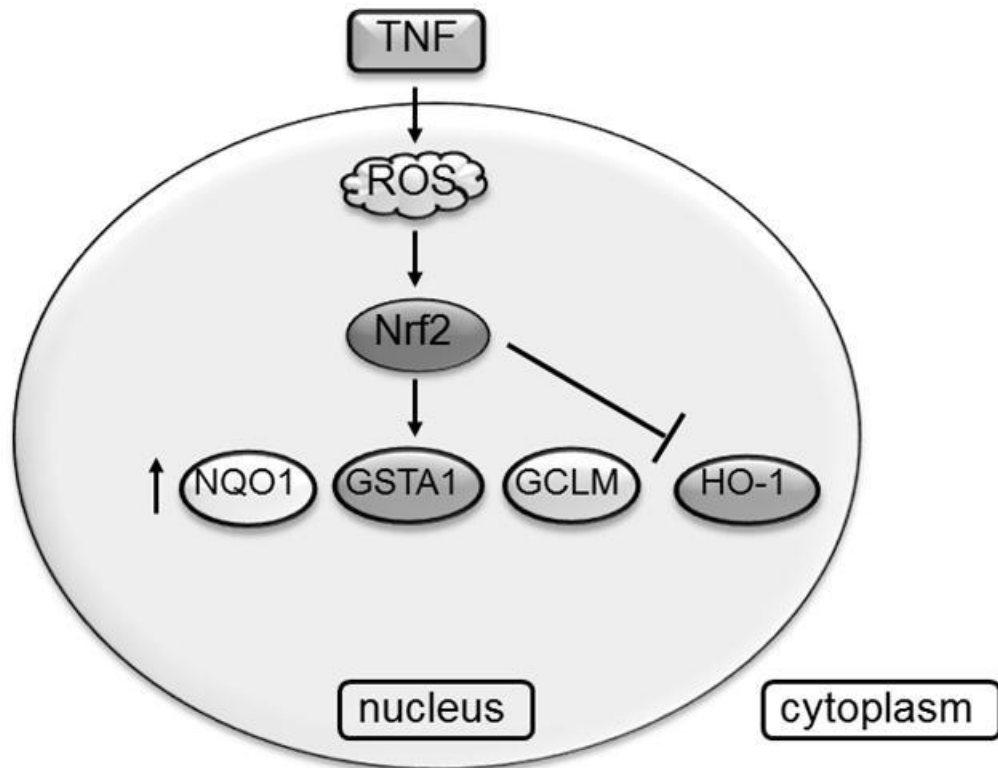
Figure 7.2: The transcription factor, Nrf2 induces cytoprotective and detoxification gene expression in response to TNF activation in human monocytes. ROS generation regulates the transcription factor, Nrf2 activation in response to TNF stimulation. Nrf2 subsequently induces NQO1, GSTA1 and GCLM mRNA expression. However HO-1 mRNA remains un-regulated. Diagram based on results reported by Rushworth et al (2011) [146] .

The concept behind HO-1 potentially rescuing AML cells from undergoing induced apoptosis either by TNF stimulation or exposure to chemotherapeutic agents has been extensively researched within the group. The AML cells ability to induce HO-1 expression and potentially lead to resistance upon chemotherapeutic exposure lead to the development of the ara-C resistant THP1 cell line (THP1ara-C(1)). Where we explored cytoprotective gene expression and proliferation rate, in response to continual ara-C exposure and acquired ara-C resistance during the course of this research project.

However basal Nrf2, HO-1 and NQO1 mRNA and protein expression was reduced in response to continual ara-C exposure. Finally, we explored miRNAs and their target genes expression involvement in cytoprotection within AML cells and ara-C resistance development.

7.3. Ara-C and DNR both induce HO-1 expression. Initially in chapter 3 we reported for the first time, HO-1 mRNA and protein expression is induced in response to exposure to ara-C, DNR or a combination thereof in both AML derived cell lines and AML patient samples. Furthermore, when the chemotherapeutics agents were used in combination, a further increase in HO-1 mRNA and protein induction was seen. This could be due to both ara-C and DNR utilising different modes of action. A number of groups have reported induced HO-1 expression in response to chemotherapeutic exposure within leukaemia cells. For example Barrera et al (2012) observed an increases in HO-1 mRNA expression in response to bortezomib exposure in the MM cell line RPMI8226 [46].

ROS generation in both wt THP1 and U937 cells was also increased in response to ara-C or DNR exposure. Interestingly ROS generation was enhanced with increasing chemotherapeutic exposure time. A number of other agents for example, the antieoplastic, niclosamide has been shown to also induce ROS generation [117]. Niclosamide has been observed inhibiting the NF-KBpathway whilst inducing ROS generation resulting in induced cellular apoptosis. Jin et al (2010) went on to explore the connection between both processes involved in apoptosis and revealed they were independent of each other. Interestingly when niclosamide was used in combination with other agents; ara-C, DNR or etoposide its affect was synergetic. These results show promise and enhance niclosamide potential as an chemotherapeutic agent suitable for trial consideration in AML treatment [117].

Interestingly when HO-1 expression was silenced in both wt THP1 and U937 cells, cell viability was reduced upon chemotherapeutic exposure. Potentially indicating HO-1 is involved in cellular cytoprotection and acquired resistance. In hindsight we should have explored the effect of HO-1 silencing on ROS generation to establish whether ROS is involved in acquired chemotherapeutic resistance.
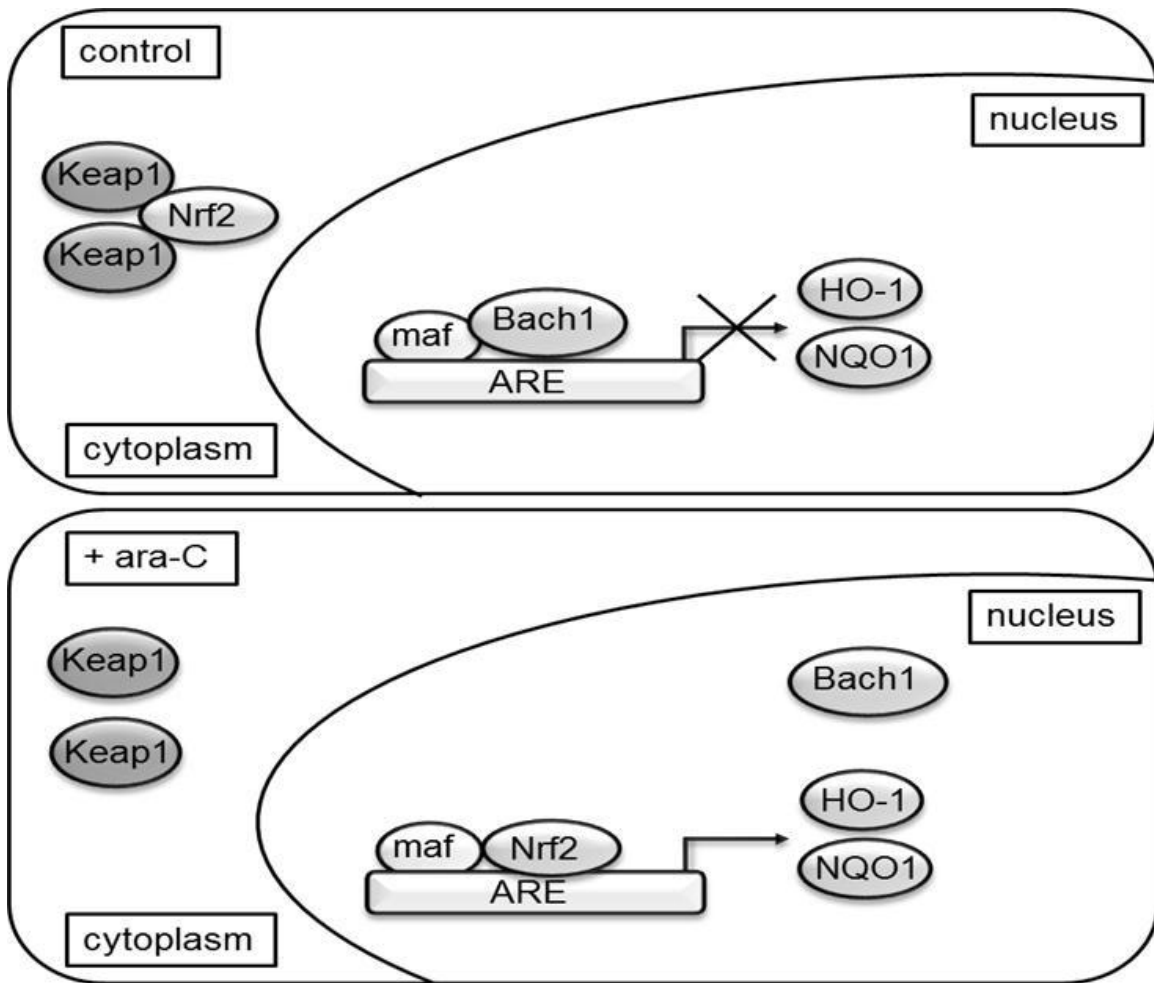
Figure 7.3: HO-1 expression is induced in response to ara-C exposure in AML cells. Diagrammatic prediction of cytoprotective gene regulation in response to ara-C stimulation. Control; In the cytoplasm, Nrf2 is bound to Keap1, whilst Bach1 is bound to MARE within the nucleus. Upon ara-C stimulation, Nrf2 is released from Keap1, its transported from the cytoplasm into the nucleus and binds to MARE. Subsequently up regulating the expression of the cytoprotective genes.

7.3.1 Development of ara-C acquired resistance and its impact on induced cell apoptosis. To be able to explore ara-C acquired resistance further we developed a ara-C resistance THP1 cell line (THP1ara-C(1)) by increasing ara-C exposure gradually over a 9 week period. THP1ara-C(1) cells were refreshed weekly with ara-C (1 µM). The developed cell line was used as a model to explore the relationship between ara-C acquired resistance and cytoprotective gene regulation in response to chemotherapeutic exposure within AML cells.

The THP1ara-C(1) cells showed decreased sensitivity towards therapeutic concentrations of ara-C compared to their wt THP1 control cells. However the ara-C resistant THP1ara-C(1) cells have maintained sensitivity towards other chemotherapeutic agents used in AML treatment; DNR and etoposide and to a lesser extent bortezomib. Similar findings were reported by Abe et al [42] when exploring the K562ara-C cell lines sensitivity towards etoposide [42-43], indicating ara-C utilises a different method of action compared to the other agents investigated. Decreased dCK mRNA expression was reported within the ara-C resistant K562ara-C cell line. dCK phosphorylates ara-C into ara-CTP which is subsequently incorporated into cellular DNA. Thus dCK action is imperative to ara-C therapeutic function. The decrease in dCK expression potentially explains K562ara-C acquired ara-C resistance [44].

As previously mentioned, HO-1 mRNA and protein expression was induced in response to ara-C, DNR or a combination thereof exposure in both AML derived cell lines and AML primary cell. However in the ara-C resistant THP1ara-C(1) cells; Nrf2, HO-1 and NQO1 expression was down regulated even though they were continual exposed to ara-C. Interestingly ROS generation remained elevated in response to ara-C exposure in both wt THP1 and THP1ara-C(1). Cell proliferation rate was affected and reduced in response to continual ara-C exposure and acquired resistance.

7.3.2 Continual ara-C exposure withdrawn affects both proliferation rate and miR-34a expression. Next we explored the effect of removing continual ara-C refreshment producing the ara-C resistant THP1ara-C(1) Treatment Withdrawn (THP1ara-C(1) TW) sub line. THP1ara-C(1) TW maintained sensitivity towards ara-C similar to that of the resistant THP1ara-C(1) cells. However both ROS and gluthatione generation was below that of the control wt THP1 cells. Interestingly Nrf2 mRNA expression was up regulated

in response to ara-C withdrawal but HO-1 and NQO1 mRNA was unchanged between them and their control wt THP1 counterparts. Furthermore, an increase in proliferation rate was seen and returned to that of the control wt THP1 cells.

Ichimura et al reported repressed proliferation in response to over expressed miR-34a in K562 cells [97]. A similar response was seen in our ara-C resistant THP1ara-C(1) cells, were miR-34a was over expressed whilst cell proliferation rate was reduced compared to the wt THP1 control cells. However, when weekly ara-C refreshment was withdrawn from the media both cell proliferation rate and miR-34a miRNA expression were returned to that of the wt THP1 control cells. Potentially indicating cell proliferation rate and miR-34a expression are responsive towards ara-C refreshment and thus reversible.

Abe et al (2006) assumed all cellular characteristics between both ara-C resistant and sensitive K562 cells remained identical unless differential expression was implicated in ara-C resistance [43]. Even though differential expression in cytoprotective genes was seen between our wt THP1 and both ara-C resistant THP1ara-C(1) and THP1ara-c(1) TW cells, miR-34a expression differed between the THP1ara-C(1) and THP1ara-C(1) TW cells. However, when continual ara-C refreshment was withdrawn, miR-34a expression was down regulated whilst cellular proliferation rate was also increased.

7.3.3 Differential miRNA expression between ara-C resistant and non-resistant THP1 cells. miR-222 has been shown to target PTEN and PUMA. We have observed no difference in miR-222 between the THP1ara-C(1) and wt THP1 cells. Interestingly Bai et al (2011) reported a decrease in miR-222 expression in their DNR resistant K562 leukaemia cells which is the reverse to what we have reported in ara-C resistant THP-1 cells [91]. Suggesting miR-222 is involved in DNR acquired resistance and not ara-C acquired resistance. However a decrease in both PTEN and PUMA mRNA expression was seen.

Bai et al (2012) observed down regulated miR-181a expression in the ara-C resistant HL60 cell line (HL60/Ara-C) which showed reduced sensitivity towards both ara-C at 1 and 10 μM [96]. Similar to what was seen in the ara-C resistant THP1ara-C(1) cells. miR-181a has been reported and confirmed to target Bcl-2 in HL60 cells. The authors noted increased basal Bcl-2 protein expression in the ara-C resistant HL60/Ara-C cells.

However when miR-181a was over expressed in the ara-C resistant HL60/Ara-C cells, sensitivity towards ara-C treatment was developed alongside decreased Bcl-2 expression. Moreover, when Bcl-2 expression was knocked down using siRNA a similar survival response was seen therefore confirming miR-181a role within ara-C acquired resistance. The authors go on to suggest the expression of miR-181a during ara-C treatment could potentially be targeted as a possible AML treatment [99].

However Bcl-2, Bcl-xl and Bax expression is indifferent between both the ara-C resistant, THP1ara-C(1) and control wt THP1 cells. Thus potentially indicating Bcl-2 is not involved in ara-C acquired resistance in THP1 cells. Interestingly miR-15a has also been shown to target Bcl-2 in MEG-01 cells [118], yet again this does not appear to be the case in the ara-C resistant THP1ara-C(1) cells.

Yang et al (2011) miR-28 has been shown to target Nrf2 in breast epithelial cells. Over expression of miR-28 results in a reduction in endogenous Nrf2 mRNA and Nrf2 protein expression which is independent of Keap1 involvement with Nrf2 and its Keap1 protein expression. The authors go on to suggest miR-28 could control breast cancer progression by utilising the Nrf2 pathway [147].

7.4 Exploring miR-196a and its target, Bach1s relationship. We have reported reduced miR-196a and increased Bach mRNA expression in the ara-C resistant THP1 ara-C(1) cell line compared to their wt THP1 control. As previously mentioned Bach1 inhibits the expression of cytoprotective genes such as HO-1 and NQO1. Thus potentially explaining why these cytoprotective genes and their transcription factor Nrf2 regulation is reduced in response to ara-C acquired resistance. However ROS generation still remains elevated in the presence of continual ara-C exposure. Furthermore, when miR-196a expression was increased via the addition of a miR-196a mimic, both Bach1 and ROS generation were reduced. Upon stimulation with additional ara-C a decrease in cell viability was seen therefore suggesting miR-196a is potentially involved in reversing the acquired ara-C resistance developed by these cells (THP1ara-C(1).

7.5 General conclusions

To conclude HO-1 is involved in cellular cytoprotective responses against the front-line chemotherapeutic agents; ara-C and DNR, in both primary AML samples and AML derived cell lines. We show here for the very first time a crucial role for HO-1 and its transcription factor Nrf2, involvement in drug-resistance mechanisms developed by AML cells. We have shown acquired ara-C resistance is a stable response and irreversible upon withdrawal of ara-C from the cellular environment. We have also highlighted miR-196a and its target Bach1 have a fundamental role in this drug-resistance AML model. The interrelationship between Bach1, HO-1 and Nrf2 were uncovered. Moreover, the potential for pharmacological intervention with miR-mimics or anti-miRs that target HO-1, Nrf2 or Bach1 have been proven.

7.6 Future work

A number of areas of investigation have been highlighted during the course of this extended research project. The studies that I believe would shed most light on the drug resistance mechanisms that we were investigating include experiments to explore whether:

- THP1 cells also respond in a similar manner. One would develop a DNR-resistant THP1 cell line and repeat the key experiments, to uncover whether there are any similarities between DNR-resistant cells and the THP1ara-C(1) cells.

- Explore etoposide resistance. Etoposide is only used as an add on therapy in the clinic to treat AML, so these studies would be of less relevance.

- Branch out to other leukaemia cell lines regarding ara-C resistance for example U937, K562 and HL60.

- Find out what the role of miR-196a is in multiple myeloma cells. These studies are ongoing within the group currently.

- Further explore the role of Annexin A1 and miR-196a in regards to cell proliferation and apoptotic balance within AML cells.

- Explore the influence of bortezomib in more depth, and discover whether the miRNA changes that we observed here, are also true of brotezomib-resistance.

- Explore ara-C and DNR effects on the NF-κB pathways in both ara-C resistant and control non-resistant AML cells.

- Explore miR-181a involvement in ara-C resistance in the THP1ara-C(1) cell line. Additionally, find out why Bcl-2 regulation is indifferent between the wt THP1 and THP1ara-C(1) cell types.

Appendix

SBI QuantiMir Cancer Array

Well	MicroRNA	MicroRNA Sequence(s)
A1	let-7-family	ugagguaguagguuguauaguu, ugagguaguagguuguaugguu, agagguaguagguugcauagu, ugagguaguagauuguauaguu
A2	miR-7	uggaagacuagugauuuuguug
A3	miR-92a	uauugcacuugucccggccug
A4	miR-93	aaagugcuguucgugcagguag
A5	miR-9	ucuuugguuaucuagcuguauga
A6	miR-101	uacaguacugugauaacugaag
A7	miR-103	agcagcauuguacagggcuauga
A8	miR-106a	aaaagugcuuacagugcagguagc
A9	miR-106b	uaaagugcugacagugcagau
A10	miR-107	agcagcauuguacagggcuauca
A11	miR-10b	uacccuguagaaccgaauuugu
A12	miR-1	uggaauguaaagaaguaugua
B1	miR-122	uggagugugacaaugguguuugu
B2	miR-125a-5p	ucccugagacccuuuaaccugug
B3	miR-125b	ucccugagacccuaacuuguga
B4	miR-126	cauuauuacuuuugguacgcg
B5	miR-128	ucacagugaaccggucucuuuc
B6	miR-132	uaacagucuacagccauggucg
B7	miR-133a	uugguccccuucaaccagcugu
B8	miR-134	ugugacugguugaccagaggg
B9	miR-135b	uauggcuuucauuccuaugug
B10	miR-136	acuccauuuguuuugaugaugga
B11	miR-137	uauugcuuaagaauacgcguag
B12	miR-140	agugguuuuacccuaugguag
C1	miR-141	uaacacugucugguaaagaugg
C2	miR-142-3p	uguaguguuuccuacuuuaugga
C3	miR-143	ugagaugaagcacuguagcuca
C4	miR-145	guccaguuuucccaggaaucccuu
C5	miR-146a	ugagaacugaauuccauggguu
C6	miR-149	ucuggcuccgugucuucacucc
C7	miR-150	ucucccaacccuuguaccagug
C8	miR-151	acuagacugaagcuccuugagg
C9	miR-153	uugcauagucacaaaaguga
C10	miR-154	uagguuauccguguuugccuucg
C11	miR-155	uuaaugcuaaucgugauaggg
C12	miR-15a	uagcagcacauaaugguuugug
D1	miR-15b	uagcagcacaucauggguuuaca
D2	miR-16	uagcagcacguaaauauuggcg
D3	miR-17*	acugcagugaaggcacuugu
D4	miR-17	caaagugcuuacagugcagguagu
D5	miR-181a	aacauucaacgcugucggugagu
D6	miR-181b	aacauucauugcugucggugggg
D7	miR-181c	aacauucaaccugucggugagu
D8	miR-181d	aacauucauuguugucgguggguu
D9	miR-183	uauggcacugguagaauucacug
D10	miR-185	uggagagaaaggcaguuc
D11	miR-186	caaagaauucuccuuuugggcuu
D12	miR-188-5p	caucccuugcaugguggaggg

Well	MicroRNA	MicroRNA Sequence(s)
E1	miR-18a	uaaggugcaucuagugcagaua
E2	miR-190	ugauauguuugauauauuaggu
E3	miR-191	caacggaaucccaaaagcagcu
E4	miR-192	cugaccuaugaauugacagcc
E5	miR-194	uguaacagcaacuccaugugga
E6	miR-195	uagcagcacagaaauauuggc
E7	miR-196a	uagguaguuucauguuguuugg
E8	miR-197	uucaccaccuucuccacccagc
E9	miR-198	gguccagaggggagauagg
E10	miR-199a+b	cccaguguucagacuaccuguuc, cccaguguuuagacuaucuguuc
E11	miR-30b	uguaaacauccuacacucagcu
E12	miR-19a+b	ugugcaaaucuaugcaaaacuga, ugugcaaauccaugcaaaacuga
F1	miR-95	uucaacggguauuuauugagca
F2	miR-20a	uaaagugcuuauagugcagguag
F3	miR-200a	uaacacugucugguaacgaugu
F4	miR-200b	uaauacugccugguaaugaugac
F5	miR-200c	uaauacugccggguaaugaugg
F6	miR-202	agagguauagggcaugggaaaa
F7	miR-203	gugaaauguuuaggaccacuag
F8	miR-204	uucccuuugucauccuaugccu
F9	miR-205	uccuucauuccaccggagucug
F10	miR-206	uggaauguaaggaagugugugg
F11	miR-21	uagcuuaucagacugauguuga
F12	miR-210	cugugcgugugacagcggcuga
G1	miR-214	acagcaggcacagacaggcag
G2	miR-215	augaccuaugaauugacagac
G3	miR-372	aaagugcugcgacauuugagcgu
G4	miR-373	gaagugcuucgauuuuggggugu
G5	miR-218	uugugcuugaucuaaccaugu
G6	miR-219	ugauuguccaaacgcaauucu
G7	miR-22	aagcugccaguugaagaacugu
G8	miR-488	cccagauaauggcacucucaa
G9	miR-221	agcuacauugucugcuggguuuc
G10	miR-222	agcuacaucuggcuacugggucuc
G11	miR-223	ugucaguuugucaaauacccc
G12	miR-224	caagucacuagugguuccguuua
H1	miR-23a	aucacauugccagggauuucc
H2	miR-24	uggcucaguucagcaggaacag
H3	miR-25	cauugcacuugucucggucuga
H4	miR-26a	uucaaguaauccaggauaggc
H5	miR-26b	uucaaguaauucaggauagguu
H6	miR-27a+b	uucacaguggcuaaguuccgc, uucacaguggcuaaguucugc
H7	miR-30c	uguaaacauccuacacucucagc
H8	miR-29a+b+c	uagcaccaucugaaaucgguu, uagcaccauuugaaaucaguguu, uagcaccauuugaaaucggu
H9	miR-30a*	cuuucagucggauguuugcagc
H10	miR-30a	uguaaacauccucgacuggaag
H11	miR-296	agggcccccucaauccugu
H12	U6 snRNA	CGCAAGGAUGACACGCAAAUUC

SBI QuantiMir Stem cell array

Well	MicroRNA	MicroRNA assay
A1	miR-18a	TAAGGTGCATCTAGTGCAGATA
A2	miR-19a	TGTGCAAATCTATGCAAAACTGA
A3	miR-19b	TGTGCAAATCCATGCAAAACTGA
A4	miR-24	TGGCTCAGTTCAGCAGGAACAG
A5	miR-25	CATTGCACTTGTCTCGGTCTGA
A6	miR-17-5p	CAAAGTGCTTACAGTGCAGGTAGT
A7	miR-30c	TGTAAACATCCTACACTCTCAGC
A8	miR-34a	TGGCAGTGTCTTAGCTGGTTGTT
A9	miR-106a	AAAAGTGCTTACAGTGCAGGTAGC
A10	miR-106b	TAAAGTGCTGACAGTGCAGAT
A11	miR-130b	CAGTGCAATGATGAAAGGGCAT
A12	miR-141	TAACACTGTCTGGTAAAGATGG
B1	miR-150	TCTCCCAACCCTTGTACCAGTG
B2	miR-199a	CCCAGTGTTCAGACTACCTGTTC
B3	miR-200b	TAATACTGCCTGGTAATGATGAC
B4	miR-200c	TAATACTGCCGGGTAATGATGG
B5	miR-301	CAGTGCAATAGTATTGTCAAAGC
B6	miR-302a	TAAACGTGGATGTACTTGCTTT
B7	miR-302b	ACTTTAACATGGAAGTGCTTTCT
B8	miR-302c	TTTAACATGGGGGTACCTGCTG
B9	miR-302d	TAAGTGCTTCCATGTTTGAGTGT
B10	miR-367	AATTGCACTTTAGCAATGGTGA
B11	miR-368	ACATAGAGGAAATTCCACGTTT
B12	miR-369-5p	AGATCGACCGTGTTATATTCGC
C1	miR-369-3p	AATAATACATGGTTGATCTTT
C2	miR-370	GCCTGCTGGGGTGGAACCTGG
C3	miR-371	GTGCCGCCATCTTTTGAGTGT
C4	miR-372	AAAGTGCTGCGACATTTGAGCGT
C5	miR-373	ACTCAAAATGGGGGCGCTTTCC
C6	let-7a	TGAGGTAGTAGGTTGTATAGTT
C7	let-7b	TGAGGTAGTAGGTTGTGTGGTT
C8	miR-10a	TACCCTGTAGATCCGAATTTGTG
C9	miR-10b	TACCCTGTAGAACCGAATTTGT
C10	miR-16	TAGCAGCACGTAAATATTGGCG
C11	miR-17-3p	ACTGCAGTGAAGGCACTTGT
C12	miR-20a	TAAAGTGCTTATAGTGCAGGTAG
D1	miR-20b	CAAAGTGCTCATAGTGCAGGTAG
D2	miR-23a	ATCACATTGCCAGGGATTTCC
D3	miR-23b	ATCACATTGCCAGGGATTACC
D4	miR-26a	TTCAAGTAATCCAGGATAGGC
D5	miR-26b	TTCAAGTAATTCAGGATAGGTT
D6	miR-30b	TGTAAACATCCTACACTCAGCT
D7	miR-30d	TGTAAACATCCCCGACTGGAAG
D8	miR-32	TATTGCACATTACTAAGTTGC
D9	miR-33	GTGCATTGTAGTTGCATTG
D10	miR-92	TATTGCACTTGTCCCGGCCTG
D11	miR-93	AAAGTGCTGTTCGTGCAGGTAG
D12	miR-99a	AACCCGTAGATCCGATCTTGTG

Well	MicroRNA	MicroRNA assay
E1	miR-101	TACAGTACTGTGATAACTGAAG
E2	miR-107	AGCAGCATTGTACAGGGCTATCA
E3	miR-126	CATTATTACTTTTGGTACGCG
E4	miR-130a	CAGTGCAATGTTAAAAGGGCAT
E5	miR-142-5p	CATAAAGTAGAAAGCACTAC
E6	miR-142-3p	TGTAGTGTTTCCTACTTTATGGA
E7	miR-146a	TGAGAACTGAATTCCATGGGTT
E8	miR-146b	TGAGAACTGAATTCCATAGGCT
E9	miR-155	TTAATGCTAATCGTGATAGGGG
E10	miR-181a	AACATTCAACGCTGTCGGTGAGT
E11	miR-181b	AACATTCATTGCTGTCGGTGGG
E12	miR-181c	AACATTCAACCTGTCGGTGAGT
F1	miR-181d	AACATTCATTGTTGTCGGTGGGTT
F2	miR-191	CAACGGAATCCCAAAAGCAGCT
F3	miR-193a	AACTGGCCTACAAAGTCCCAG
F4	miR-193b	AACTGGCCCTCAAAGTCCCGCTTT
F5	miR-197	TTCACCACCTTCTCCACCCAGC
F6	miR-221	AGCTACATTGTCTGCTGGGTTTC
F7	miR-223	TGTCAGTTTGTCAAATACCCC
F8	miR-339	TCCCTGTCCTCCAGGAGCTCA
F9	miR-9	TCTTTGGTTATCTAGCTGTATGA
F10	miR-103	AGCAGCATTGTACAGGGCTATGA
F11	miR-124a	TTAAGGCACGCGGTGAATGCCA
F12	miR-125a	TCCCTGAGACCCTTTAACCTGTG
G1	miR-125b	TCCCTGAGACCCTAACTTGTGA
G2	miR-127	TCGGATCCGTCTGAGCTTGGCT
G3	miR-128a	TCACAGTGAACCGGTCTCTTTT
G4	miR-128b	TCACAGTGAACCGGTCTCTTTC
G5	miR-132	TAACAGTCTACAGCCATGGTCG
G6	miR-134	TGTGACTGGTTGACCAGAGGG
G7	miR-135a	TATGGCTTTTTATTCCTATGTGA
G8	miR-135b	TATGGCTTTTCATTCCTATGTG
G9	miR-136	ACTCCATTTGTTTTGATGATGGA
G10	miR-138	AGCTGGTGTTGTGAATC
G11	miR-149	TCTGGCTCCGTGTCTTCACTCC
G12	miR-153	TTGCATAGTCACAAAAGTGA
H1	miR-154	TAGGTTATCCGTGTTGCCTTCG
H2	miR-183	TATGGCACTGGTAGAATTCACTG
H3	miR-218	TTGTGCTTGATCTAACCATGT
H4	miR-219	TGATTGTCCAAACGCAATTCT
H5	miR-222	AGCTACATCTGGCTACTGGGTCTC
H6	miR-1	TGGAATGTAAAGAAGTATGTA
H7	miR-122a	TGGAGTGTGACAATGGTGTTTGT
H8	miR-133a	TTGGTCCCCTTCAACCAGCTGT
H9	miR-133b	TTGGTCCCCTTCAACCAGCTA
H10	miR-195	TAGCAGCACAGAAATATTGGC
H11	miR-206	TGGAATGTAAGGAAGTGTGTGG
H12	U6	CGCAAGGAUGACACGCAAAUUC

SBI QuantiMir Cancer Array

Plate Array Arrangement

	1	2	3	4	5	6	7	8	9	10	11	12
A	let-7-family	miR-7	miR-92a	miR-93	miR-9	miR-101	miR-103	miR-106a	miR-106b	miR-107	miR-10b	miR-1
B	miR-122	miR-125a-5p	miR-125b	miR-126	miR-128	miR-132	miR-133a	miR-134	miR-135b	miR-136	miR-137	miR-140
C	miR-141	miR-142-3p	miR-143	miR-145	miR-146a	miR-149	miR-150	miR-151	miR-153	miR-154	miR-155	miR-15a
D	miR-15b	miR-16	miR-17*	miR-17	miR-181a	miR-181b	miR-181c	miR-181d	miR-183	miR-185	miR-186	miR-188-5p
E	miR-18a	miR-190	miR-191	miR-192	miR-194	miR-195	miR-196a	miR-197	miR-198	miR-199a+b	miR-30b	miR-19a+b
F	miR-95	miR-20a	miR-200a	miR-200b	miR-200c	miR-202	miR-203	miR-204	miR-205	miR-206	miR-21	miR-210
G	miR-214	miR-215	miR-372	miR-373	miR-218	miR-219	miR-22	miR-488	miR-221	miR-222	miR-223	miR-224
H	miR-23a	miR-24	miR-25	miR-26a	miR-26b	miR-27a+b	miR-30c	miR-29a+b+c	miR-30a*	miR-30a	miR-296	U6 snRNA

SBI QuantiMir Stem cell Array

Plate Array Arrangement

|	1	2	3	4	5	6	7	8	9	10	11	12
A	miR-18a	miR-19a	miR-19b	miR-24	miR-25	miR-17-5p	miR-30c	miR-34a	miR-106a	miR-106b	miR-130b	miR-141
B	miR-150	miR-199a	miR-200b	miR-200c	miR-301	miR-302a	miR-302b	miR-302c	miR-302d	miR-367	miR-368	miR-369-5p
C	miR-369-3p	miR-370	miR-371	miR-372	miR-373	let-7a	let-7b	miR-10a	miR-10b	miR-16	miR-17-3p	miR-20a
D	miR-20b	miR-23a	miR-23b	miR-26a	miR-26b	miR-30b	miR-30d	miR-32	miR-33	miR-92	miR-93	miR-99a
E	miR-101	miR-107	miR-126	miR-130a	miR-142-5p	miR-142-3p	miR-146a	miR-146b	miR-155	miR-181a	miR-181b	miR-181c
F	miR-181d	miR-191	miR-193a	miR-193b	miR-197	miR-221	miR-223	miR-339	miR-9	miR-103	miR-124a	miR-125a
G	miR-125b	miR-127	miR-128a	miR-128b	miR-132	miR-134	miR-135a	miR-135b	miR-136	miR-138	miR-149	miR-153
H	miR-154	miR-183	miR-218	miR-219	miR-222	miR-1	miR-122a	miR-133a	miR-133b	miR-195	miR-206	U6 snRNA
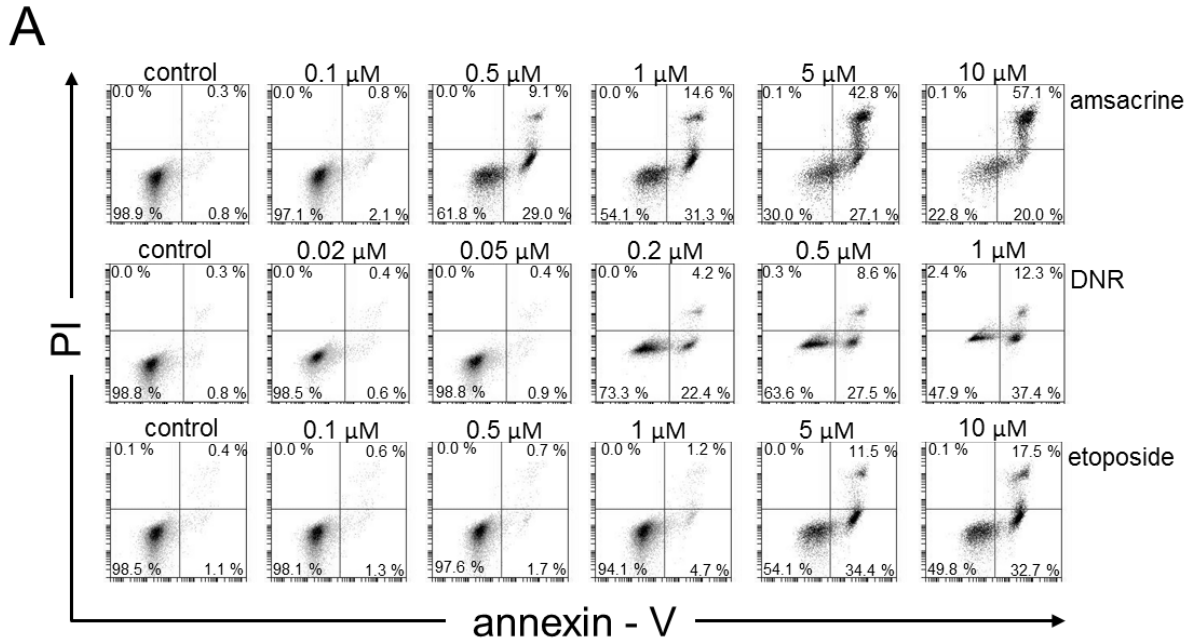
Figure 8.1. wt THP1 resistance to various chemotherapeutics. (A) wt THP1 cell were treated with amsacrine (0, 0.1, 0.5, 1, 5, 10 µM), DNR (0, 0.02, 0.05, 0.2, 0.5, 1 µM) and etoposide (0, 0.1, 0.5, 1, 5, 10 µM). Cells were stained with annexin-V and P1, number of viable cells was established using flow cytometry, (n = 1).

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