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Investigating the role of BCL-2 family members in chemoresistance in adult and paediatric core-binding factor acute myeloid leukaemia

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Abstract

Paediatric and adult acute myeloid leukaemia (AML) are distinct diseases with different molecular and genetic signatures. Core-binding factor (CBF) AML is a subgroup of AML with chromosomal translocations [t(8;21)] or inversions [inv(16)]. Patients with CBF AML are considered to have favourable prognosis, despite this, relapse and chemoresistance is common in this subgroup.

Changes in apoptotic pathways have been demonstrated to contribute to resistance to established chemotherapies, such as cytarabine (Ara-C), in AML. BH3 mimetics have shown promise for the treatment of AML, however the use of BH3 mimetics in patients with CBF AML that are resistant to Ara-C remains unclear. Therefore, the broad aim of this project was to determine the role of apoptotic proteins in resistance to Ara-C using t(8;21) and inv(16) CBF AML cell lines. This project also sought to assess the use of BH3 profiling as a biomarker of response to BH3 mimetics in CBF AML.

To achieve this, Ara-C resistant models were generated using escalating doses of Ara-C. Baseline BH3 profiling was used to determine how acquisition of Ara-C resistance affected apoptotic priming of CBF AML cell lines. The dependency of these cell lines on anti-apoptotic proteins was assessed using intracellular staining and measurement of response to BH3 mimetics. Dynamic BH3 profiling was performed and compared to response to BH3 mimetics by apoptosis assay.

This project demonstrated that Ara-C resistance has different impacts on the apoptotic priming of paediatric t(8;21) Kasumi-1 and adult inv(16) ME-1 AML cell lines. In both Ara-C–resistant cell lines, dynamic BH3 profiling using cytochrome c release recapitulated the apoptotic response typically seen with BCL-2 inhibition. Particularly noteworthy was the up-regulation of MCL-1 expression in the resistant lines, suggesting increased reliance on this anti-apoptotic protein for survival. However, treatment with the selective MCL-1 inhibitor, S63845, failed to enhance cytochrome c release or induce apoptosis. In contrast, venetoclax, a BCL-2 inhibitor, induced greater cytotoxicity in Ara-C–resistant ME-1, but not in Kasumi-1.

Results of this project highlight the complexity of BCL-2 family member interactions and highlight that it may not be possible to infer functional apoptotic dependencies from the expression levels of anti-apoptotic proteins. These results suggest that there is a potential for pre-treatment with Ara-C to increase response to venetoclax. However, further work is needed to determine the most effective sequence of treatment with Ara-C and BH3 mimetics. This project adds to previous evidence which suggests that the use of dynamic BH3 profiling for prediction of response to BH3 mimetics may be helpful.

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This thesis is dedicated to the loving memory of my Dad, who never doubted I could achieve whatever I put my mind too.

Author's declaration

I declare that, except where noted, this thesis is the result of my own work. Where others have contributed to elements of the work, this is stated clearly in the text. No element of this work has been submitted for any other degree, award or professional qualification to this, or any other University.

Nicola Lander

Definitions

Abbreviation	Definition
ADE	Cytarabine, daunorubicin, etoposide
AML	Acute myeloid leukaemia
Ara-C	Cytarabine
BAD	BCL-2-associated death promoter
BAK	BCL-2 homologues antagonist/killer
BAX	BCL-2-associated protein x
BCL-2	B-cell lymphoma 2
BCL-W	B-cell lymphoma/leukemia 2-like protein 2
BCL-xL	B-cell lymphoma-extra large
BH	BCL-2 homology
BID	BH3 interacting-domain death agonist
BIM	BCL-2 interacting mediator of cell death
BSA	Bovine serum albumin
CBF	Core binding factor
CML	Chronic myeloid leukaemia
CR	Complete remission
DAPI	4′,6-diamidino-2-phenylindole
DAT	Daunorubicin, cytarabine, thioguanine
DMSO	Dimethyl sulphoxide
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FLAG-IDA	Fludarabine, cytarabine, granulocyte colony stimulating factor, idarubicin
FLT3	FMS-like tyrosine kinase 3
FSC-A	Forward scatter area
FSC-H	Forward scatter height
IV	Intravenous
LSC	Leukaemic stem cell
MCL-1	Myeloid cell leukemia-1
MFI	Median fluorescence intensity
mL	Microlitre
MOMP	Mitochondrial outer membrane permeabilisation
MRC	Medical Research Council
MSCs	Mesenchymal stromal cells
nM	Nanomolar
OS	Overall survival
pAML	Paediatric acute myeloid leukaemia
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
RPMI	Roswell Park Memorial Institute
SEM	Standard error the mean

SSC-A	Side scatter area
WBC	White blood cell
μM	Micromolar

1 Introduction

1.1 Acute Myeloid Leukaemia

1.1.1 Adult and paediatric AML

Acute myeloid leukaemia (AML) is characterised by clonal expansion and accumulation of myeloid blasts, which originate from leukaemic stem cells (LSCs), in the bone marrow and peripheral blood (1). In the UK, an average of 2495 people were diagnosed with AML each year between 2017 and 2019, with 41% of diagnoses occurring in patients ≥75 years old (2). The net survival of patients with AML in the UK is 32.0%, 17.5% and 13.6% at 1-, 3- and 5-years, respectively (3).

Paediatric acute myeloid leukaemia (pAML) accounts for 20% of leukaemia in children (4). AML is the most common acute leukaemia in adults, and accounts for approximately 80% of acute leukaemia cases (5). Adult AML more commonly occurs as a result of pre-existing myeloproliferative neoplasms or myelodysplastic syndromes, whilst the majority of pAML cases arise *de novo* (6,7). Cytogenetic and molecular abnormalities are generally more common in pAML than adult AML, however some alterations occur exclusively or more commonly in pAML (6). It has been shown that leukaemia development and genetic signature is impacted by the age of the cell of origin, therefore demonstrating biological and molecular differences between adult and pAML (8). Similarly, paediatric and adult AML have distinct epigenetic landscapes and differ in terms of occurrence and types of epigenetic mutations (9).

The 5-year overall survival (OS) rate for pAML remains at 60-70%. Survival outcomes are poorer in adult AML, with a 5-year OS rate of approximately 30% which decreases with age to 5% in patients ≥70 years old (10). Survival of patients with AML is heterogenous due to factors such as age, co-morbidities, genetics, and therapy received (11). Despite improvements in treatment options and patient stratification, relapse rates remain high in AML and are dependent on risk factors, such as age (12). For paediatric patients, relapse occurs in 30-

40% (13), while risk of relapse ranges from 30-35% in adult patients ≤60 years of age up to 70-80% in patients >60 years (14).

1.1.2 Diagnosis of AML

The signs and symptoms of AML are similar in children and adults and include fatigue, fever, and easy bruising or bleeding (15). Peripheral blood analysis of patients with AML may show a very low white blood cell (WBC) count, or a high number of abnormal WBCs (16). Other indications of AML in peripheral blood include elevated lactate dehydrogenase, uric acid, potassium, and phosphorus levels (17). The percentage of myeloid blasts is required to establish a diagnosis of AML and is obtained by cellular bone marrow aspirate smears and/or touch preparations and a peripheral blood smear (18). The presence of ≥10% myeloid blasts in the bone marrow or peripheral blood is the threshold required for diagnosis of AML, with the exception of AML with t(9;22)(q34.1;q11.2)/ *BCR::ABL1* AML (which requires a presence of ≥20% myeloid blasts(18). However, blast percentage may not be as important in the diagnosis of certain subtypes of AML, including core-binding factor (CBF) AML.

Once a diagnosis of AML has been established, cytogenetic evaluation is required to determine cytogenetic abnormalities and to allow risk classification and subsequently treatment stratification (17).

1.1.3 Core-binding factor AML

CBF AML is a distinct subgroup of AML which contains leukaemias with chromosomal translocations [t(8;21)] or inversions [inv(16)], which subsequently target the transcription factors *RUNX1 (AML1)* and *CBFB,* respectively. The resulting fusion products, *RUNX1/RUNX1T1/t(8;21)(q22;q22)* or *CBFB-MYH11/inv(16),* prevent myeloid differentiation. However, these fusion events alone are not sufficient to induce AML and secondary hit mutations, commonly within the *Ras* pathway, are required to propagate the disease (19).

CBF AML is more common in pAML than adult AML, with this subgroup accounting for approximately 30% of pAML cases and approximately 8% of *de novo* adult AML cases (20,21). Whilst both t(8;21) and inv(16) AML have high remission rates

(>90% in pAML (22) and >85% in adult AML (23)), they have distinct clinical outcomes. For example, in adult AML, patients carrying the t(8;21) translocation have higher relapse rates and poorer long-term outcomes than patients with inv(16) (23). Similarly, in pAML, patients with t(8;21) have lower 10-year OS and relapse rate than patients with inv(16) (22).

Although CBF AML is widely regarded as a favourable prognostic subgroup due to the high remission rate, 30-50% of these patients will go on to relapse and are a clinical conundrum (24). These statistics highlight the need for treatment options and strategy for patients with CBF AML that relapse after treatment.

1.2 Chemoresistance

1.2.1 Standard-of-care treatments

Despite pAML having distinct molecular and biological profiles compared to adult AML, there is a broad overlap between standard-of-care treatment in children and adults (8,25). The most common first line treatment for pAML is a 10-day treatment of cytarabine (Ara-C) given with daunorubicin on 3 of the days, with additional etoposide or thioguanine (26). The MRC AML 10 trial sought to compare the efficacy of treatment regimens used for pAML, showing that there were no significant differences in complete remission (CR) or death rates between the DAT (daunorubicin, cytarabine, thioguanine) and ADE (cytarabine, daunorubicin, etoposide) regimens (27).

Similar to pAML, standard-of-care treatment for adult AML in the first line is Ara-C and an anthracycline, such as daunorubicin or idarubicin (18). Usually, Ara-C is administered by continuous intravenous (IV) infusion over 7 days and the anthracycline is administered by IV infusion in a single bolus dose for 3 days during this week of treatment (28). Therefore, this combination and schedule of treatment for AML is referred to as the 7+3 regimen (28). Other treatment options for patients eligible for intensive chemotherapy include the FLAG-IDA regimen (fludarabine, Ara-C, granulocyte colony-stimulating factor, and idarubicin), gemtuzumab-ozogamicin and mitoxantrone-based cytarabine regimens (18).

1.2.2 Chemoresistance in AML

Patients who do not respond to two cycles of induction therapy are described as having refractory disease (29). This lack of response to established chemotherapy regimens can be due to primary resistance prior to treatment, or acquired resistance following treatment, ultimately leading to relapse (30). Several mechanisms of chemoresistance in AML have been identified, including increased ability of efflux pumps to export drugs out of leukaemic cells, changes in function/expression of drug targets and increased ability to repair DNA damage caused by chemotherapy (31). As an important mechanism of action of cytotoxic chemotherapy is to induce apoptosis, a crucial adaptation made by cancer cells is changes in expression and function of key players in the apoptotic pathways. This allows for the evasion of apoptosis and subsequent leukaemia cell survival.

1.3 Intrinsic apoptosis

1.3.1 The intrinsic apoptotic pathway

Evasion of apoptotic signalling is a well-established hallmark of cancer cells and has therefore become a target for treatment options (32). The BCL-2 family of proteins are defined regulators of the intrinsic apoptosis pathway and are split into three distinct categories. The anti-apoptotic proteins e.g., BCL-2, BCL-xL, MCL-1, possess four BCL-2 homology (BH) domains (BH 1-4) (33). Pro-apoptotic proteins such as BAX, BAK and BOK contain BH domains 1-3, whilst sensitiser/activator BH3-only proteins (e.g., BIM, BIK, BAD, NOXA), as their name suggest, contain only the BH domain 3 (34). Some pro-apoptotic proteins, such as BAD and NOXA selectively inhibit specific anti-apoptotic proteins, whereas other pro-apoptotic proteins inhibit all of the anti-apoptotic proteins (**Figure 1.3.1)** (35). The apoptotic pathway involves a balance between these three subgroups of proteins. A shift in this balance towards pro-apoptotic proteins pushes cells closer to the so-called “apoptotic threshold”, defined as the minimum amount of pro-apoptotic signalling required to induce apoptosis (36).

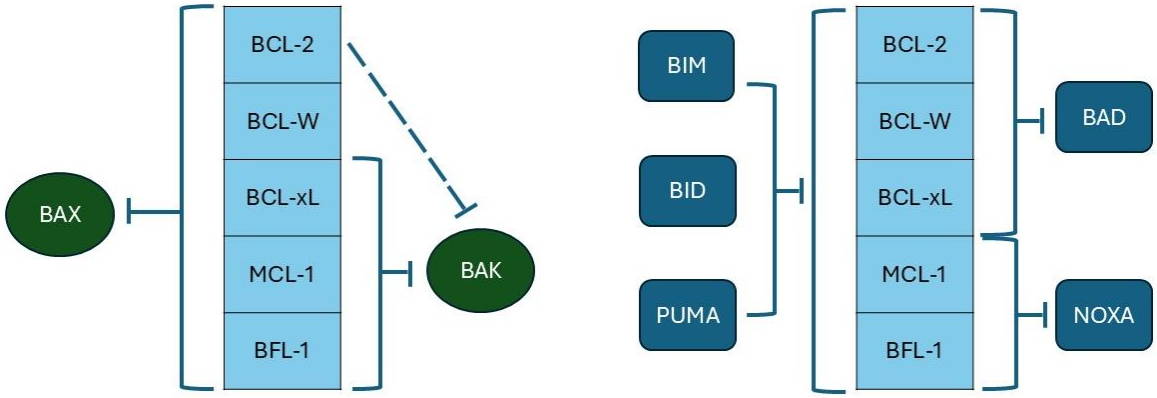

Figure 1.3.1 Selectivity of anti-apoptotic protein inhibition by pro-apoptotic proteins. BAX is inhibited by all anti-apoptotic proteins, while BAK is inhibited by BCL-xL, MCL-1 and BFL-1 in most instances, with occasional inhibition by BCL-2. The BH3-only proteins BIM, BID and PUMA probably inhibit all anti-apoptotic proteins. BAD inhibits BCL-2, BCL-W and BCL-xL selectively and NOXA inhibits MCL-1 and BFL-1 (37).

When a cell receives intracellular damage, activator BH3-only proteins stimulate pro-apoptotic proteins (**Figure 1.3.2**). The role of these pro-apoptotic proteins is to induce mitochondrial outer membrane permeabilisation (MOMP) which allows release of cytochrome c, formation of the apoptosome and subsequent cell death (38). The BH1-4 anti-apoptotic proteins actively oppose this process by inhibition of pro-apoptotic and activator BH3-only proteins. Sensitiser BH3-only proteins cannot directly activate the apoptotic pathway but function by inhibiting anti-apoptotic proteins, thereby sensitising cells to activator BH3-only and pro-apoptotic proteins (39). The homeostasis of the interplay of these proteins is crucial to healthy cell survival and damaged cell death.

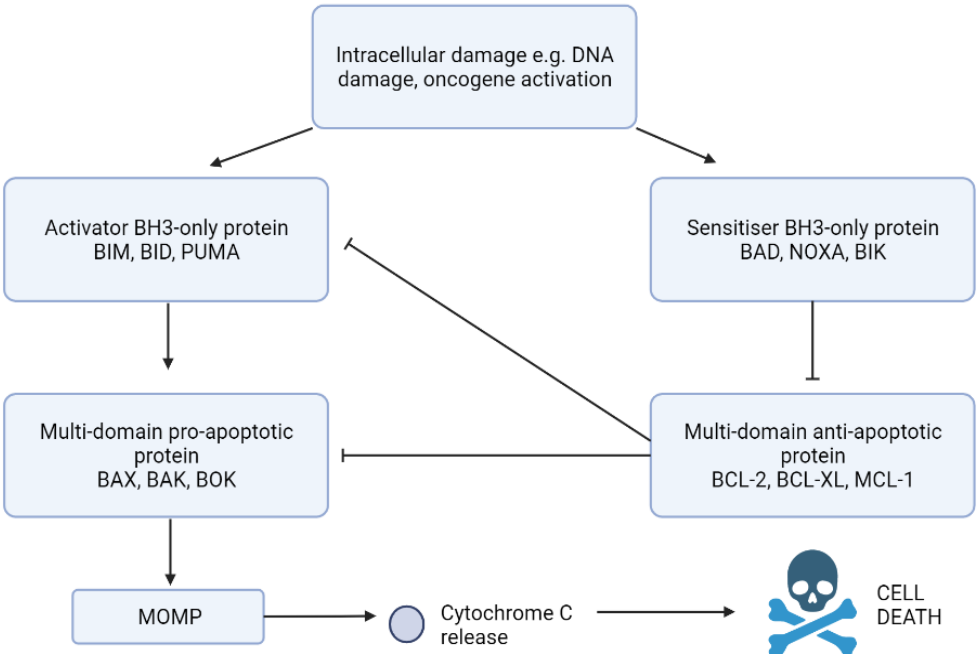

Figure 1.3.2. An overview of the intrinsic pathway of apoptosis. Receiving intracellular damage triggers both subtypes of BH3-only proteins. Activator BH3-only proteins directly activate multi-domain pro-apoptotic proteins to induce apoptosis, whereas sensitiser BH3-only proteins are antagonists to the anti-apoptotic BCL-2 family of proteins. Anti-apoptotic proteins function by inhibiting both activator BH3-only and pro-apoptotic proteins to aid cell survival. Activation of pro-apoptotic proteins is required to induce MOMP; this causes the release of cytochrome c from the mitochondria into the cytoplasm where it activates caspases, forming the apoptosome and resulting in cell death. Figure made using BioRender.

1.3.2 Involvement of anti-apoptotic proteins in AML

Since their discovery, the upregulation of members within the anti-apoptotic BCL-2 family has been linked to AML pathogenesis. A high percentage of BCL-2 expressing leukaemic cells has been shown to be present in patients who do not reach CR, and aberrant expression of BCL-2 associated with shorter survival (40). Further validating the power of BCL-2 expression as a marker for poor prognosis in AML, BCL-2 expression has been shown to correlate with the percentage of CD34+ LSCs (41).

Other pro-survival apoptotic proteins in the BCL-2 family have also been shown to play a role in AML. For instance, a high expression of MCL-1 has been shown to

correlate with low CR rate and poorer OS in *de novo* AML patients (42). This study also showed that the expression of MCL-1 fluctuates, with a high expression at diagnosis, decreasing after CR and then increasing again after relapse. This provides evidence for the use of MCL-1 for disease monitoring (42).

Importantly, upregulation of these anti-apoptotic proteins has been linked to chemotherapy resistance in AML. Treatment with chemotherapy has been shown to actively select for leukaemic CD34+ cells expressing BCL-2 and BCL-xL, therefore promoting a pro-survival, therapy-resistant phenotype (43). MCL-1 has also been shown to be upregulated in AML relapsing after chemotherapy (44). Due to these data showing the role of the anti-apoptotic members of the BCL-2 family in AML pathogenesis and chemoresistance, they have become a target for inhibition in AML therapy (45). This has resulted in the emergence of various BH3 mimetics, whose mechanism of action is to mimic the binding pattern of BH3-only proteins to anti-apoptotic proteins to inhibit their function and induce apoptosis (46).

1.3.3 Involvement of pro-apoptotic proteins in AML

Cancer cells have been shown to evade apoptosis, not only by the upregulation of anti-apoptotic proteins, but also by the downregulation or inhibition of pro-apoptotic proteins, such as BAX (47). For example, in FMS-like tyrosine kinase 3 (*FLT3*) mutant AML, upregulation of BCL-xL has been shown to activate the phosphoinositide 3-kinase (PI3K)-Akt signalling pathway in leukaemic blasts, leading to phosphorylation of the pro-apoptotic protein BAD, ultimately suppressing apoptosis (48).

1.3.4 BIM

BIM is an activator BH3-only protein which binds to and neutralises anti-apoptotic proteins and triggers the activation of BAX/BAK, causing subsequent cytochrome c release **(Figure 1.3.2)** (38). BIM plays a role as a tumour suppressor in chronic myeloid leukaemia (CML) and has been shown to be downregulated in BCR::ABL1-positive CML (49). In AML, the long non-coding RNA *MORRBID*, a repressor of *BIM*, is overexpressed and associated with poor survival (50). Increasing levels of BIM through the knockdown of *MORBIDD* induced cell

death in immature myeloid cells, reduced invasion of leukaemic blasts and prolonged survival in a murine model of AML (50). Similarly, elevation of BIM level using microRNA increased the efficacy of Ara-C towards killing of AML cells (51).

1.4 BH3 mimetics

BH3 mimetics targeting different members of the BCL-2 family have been developed, with varying success in terms of patient response (52). The mechanisms of action of each BH3 mimetic used in this project are described below and in **Figure 1.4.1.**

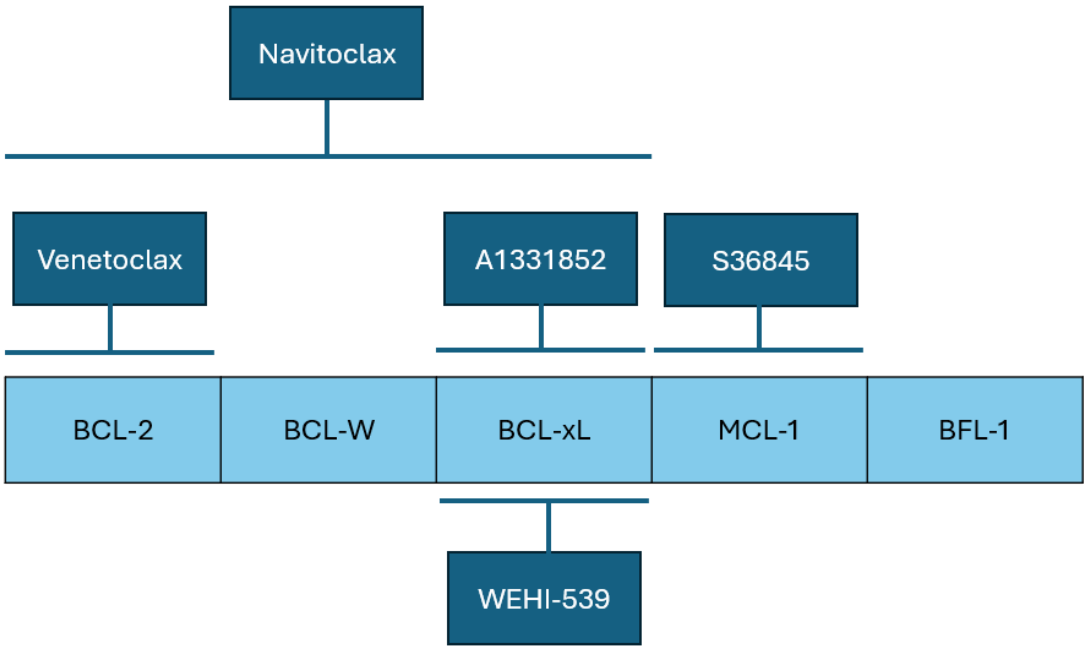

Figure 1.4.1. The mechanism of action for BH3 mimetics utilised in this project. Venetoclax inhibits the anti-apoptotic protein BCL-2, while navitoclax simultaneously inhibits BCL-2, BCL-W and BCL-xL. A1331852 and WEHI-539 are both inhibitors of BCL-xL. S63845 is a selective inhibitor of the anti-apoptotic protein MCL-1. There is currently no inhibitor for BFL-1 available.

1.4.1 Navitoclax

Navitoclax (ABT-263) is an inhibitor of BCL-2/BCL-W/BCL-xL (**Figure 1.4.1**) and was derived from ABT-737 to be orally bioavailable (53). Early studies

demonstrated that navitoclax administration caused complete tumour shrinkage in xenograft models of acute lymphoblastic leukaemia and B-cell lymphoma (53).

In a phase I clinical trial, navitoclax showed promise in its use as a monotherapy in chronic lymphocytic leukaemia, with 35% of patients achieving a partial response (54). However, some patients treated with navitoclax experienced high grade thrombocytopenia, which was later to shown to be due to the role of BCL-xL in platelet development (55). In a phase II clinical trial in small cell lung cancer, 40% of patients experienced grade III-IV thrombocytopenia in response to navitoclax (56).

Currently, navitoclax is being investigated for the treatment of myeloproliferative neoplasms. In initial results of the phase III, TRANSFORM-1 trial (NCT03222609), navitoclax, in combination with the JAK inhibitor ruxolitinib, demonstrated efficacy for the treatment of myelofibrosis (57). Although grade III thrombocytopenia occurred in 51% of patients receiving navitoclax + ruxolitinib, this adverse event was easily managed compared to previous studies (57).

1.4.2 Venetoclax

Venetoclax (ABT-199) is small molecule inhibitor of BCL-2 (**Figure 1.4.1**) and its mechanism of action is disruption of interaction between BCL-2 and pro-apoptotic proteins to induce MOMP, leading to subsequent cytochrome c release and apoptotic cell death (58). The first phase II clinical trial was a single-arm study investigating the efficacy of 800 mg venetoclax in high-risk relapsed or refractory AML (59). In this study, 19% of patients achieved CR and a further 19% of patients showed a partial bone marrow response (58). Overall, venetoclax demonstrated limited efficacy as a single agent but was well-tolerated by patients with relapsed/refractory AML. Therefore, subsequent clinical trials were performed to assess the efficacy of venetoclax in combination with other therapeutic agents in patients with AML.

An initial phase 1b study was conducted to assess the safety of venetoclax in combination with azacitidine in elderly patients with previously untreated AML (60). This combination was well-tolerated and 35/57 patients treated achieved

CR or CR with incomplete marrow recovery (60). Due to the promising results shown in this study, the phase III VIALE-A clinical trial was performed to assess the safety and efficacy of azacitidine in combination with venetoclax in previously untreated patients with AML who were ineligible for intensive therapy (61). In this study, patients treated with azacitidine + venetoclax had higher rates of CR compared to patients treated azacitidine + placebo (36.7% versus 17.9%; P<0.001) (61). Median OS was also higher in the azacitidine + venetoclax group than the control group (20.5 versus 9.6 months, respectively) (61).

The clinical trial VIALE-C sought to assess the efficacy and safety of venetoclax with low-dose Ara-C in patients with newly diagnosed AML ineligible for intensive chemotherapy. In patients receiving venetoclax + low-dose Ara-C the CR rate was 27% compared to 7.4% in patients treated with placebo + low-dose Ara-C (62). Duration of response was also longer in the venetoclax + low-dose Ara-C group than the placebo + low-dose Ara-C group (11.1 versus 8.3 months, respectively) (62).

Due to the success shown in these clinical trials, venetoclax, in combination with azacitidine or low-dose Ara-C is now approved for the treatment of patients with newly diagnosed AML that are ineligible for intensive induction therapy, due to age or comorbidities (63).

1.4.3 A1331852

A1331852 is an orally active BCL-xL inhibitor (**Figure 1.4.1**) which has been shown to induce apoptosis in BCL-xL dependent tumours (64). In an early study, A1331852 was shown to selectively disrupt BCL-xL-BIM complexes and induce apoptosis in a lymphoblastoid cell line (MOLT-4) and MOLT-4 xenograft models (64). In AML, a murine *MLL-AF9* cell line showed limited sensitivity on exposure to A1331852 and *MLL*-fusion human AML cell lines were resistant to treatment with A13318532 (65).

Further study into the mechanism of action of A1331852 in AML revealed that the inhibitor repressed transcription and expression of the anti-apoptotic protein MCL-1 leading to the death of U937 and HL-60 cells (66). This off-target effect of MCL-1 inhibition exhibited by A1331852 *in vitro* raised concerns about the

possible side effects should the inhibitor be used in clinical practice (66). Therefore, A1331852 has yet to be investigated for the treatment of AML in clinical trials.

1.4.4 S36845

S63845 is a selective inhibitor of the anti-apoptotic protein MCL-1 (**Figure 1.4.1**). S63845 has been shown to induce apoptosis in a range of haematological cancer cell lines and *in vivo* models (67). One study showed that, in primary AML samples, S63845 induces a higher or similar level of apoptosis as venetoclax (68). In primary AML samples co-cultured with mesenchymal stromal cells (MSCs) to mimic the bone marrow microenvironment, venetoclax-induced apoptosis decreased, while S63845-induced apoptosis did not decrease (68).

S63845 is the parent compound of S64315 (MIK665) which has been investigated in clinical trials for the treatment of haematopoietic cancers (69–71). S64315 showed limited activity in patients with relapsed/refractory lymphoma and multiple myeloma in a phase I clinical trial and ultimately the trial was stopped (71). Dose-limiting toxicities related to cardiovascular function were observed in a clinical trial investigating the use of S64315 for treatment of patients with AML or myelodysplastic syndromes (70).

1.4.5 WEHI-539

WEHI-539 is a selective BCL-xL inhibitor (**Figure 1.4.1**) which induced BAK-mediated apoptosis in BCL-xL-dependent mouse embryo fibroblast cells (72). Data are limited on the use of WEHI-539 for treatment of haematopoietic cancer, however one study investigated the use of WEHI-539 in combination with venetoclax and navitoclax (73). In this study, the WEHI-539 demonstrated synergistic activity with venetoclax in the U937 leukaemia cell line (73). Additionally, venetoclax-resistant U937 cells treated with WEHI-539 showed increased response to venetoclax through induction of NOXA-mediated MCL-1 degradation (73).

WEHI-539 contains a hydrazone moiety which is labile and potentially toxic, which limited the use of this drug in subsequent studies (74). Additionally, the physicochemical properties of WEHI-539 meant that the use of this drug *in vivo*

was not possible (74). Therefore, A-1155463 was developed without the hydrazone moiety and has the ability to be used *in vivo* (74).

1.4.6 Proposed methods of use

Due to the identification of apoptotic protein dysregulation being key in primary and acquired resistance to chemotherapy, two models of chemotherapy/BH3 mimetic treatment have been proposed (**Figure 1.4.2**). One proposed method is to exploit pre-existing dependencies to anti-apoptotic proteins by selective targeting with a BH3 mimetic and simultaneous chemotherapy treatment. Whilst the other method involves initial treatment with chemotherapy to induce changes in apoptotic protein expression, and subsequent treatment with a BH3 mimetic to exploit these acquired changes. Although both proposed methods have benefits, they both require stratification of patients by their BCL-2 dependencies. BH3 profiling, a functional assay for BCL-2 dependency/apoptotic priming status would be a suitable candidate for this kind of patient stratification (52).

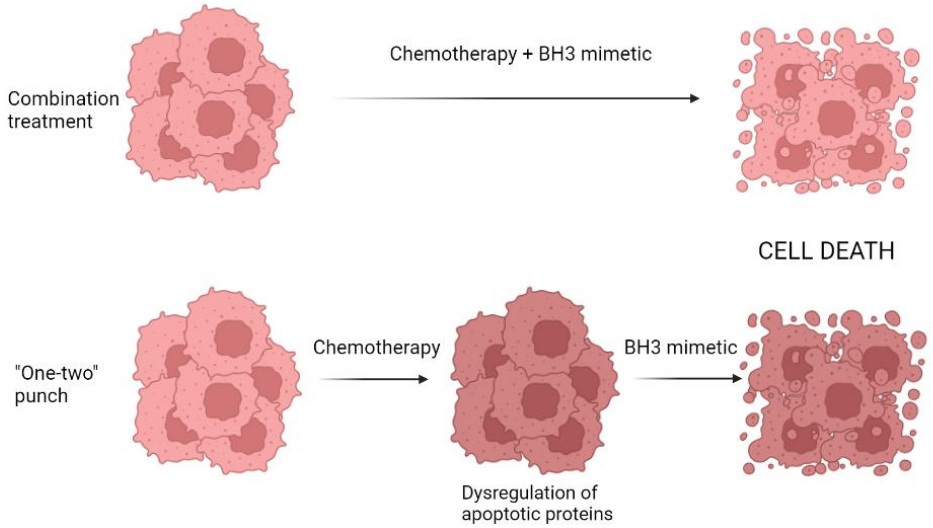

Figure 1.4.2. Proposed treatment options for the use of chemotherapy and BH3 mimetics to target existing and acquired dependencies on apoptotic proteins. Figure made using BioRender.

1.6 BH3 profiling

BH3 profiling is a functional approach which allows the measurement of the level of apoptotic priming of a cell, and can in turn highlight BCL-2 family dependencies (75). The method of baseline BH3 profiling involves the introduction of BH3-only peptides at varying concentrations and measuring the percentage of cytochrome c release (**Figure 1.6.1**), a direct surrogate for measuring MOMP. This allows the elucidation of how primed cells are for apoptosis i.e., how close are they to the apoptotic threshold. Using different BH3-only peptides can highlight dependencies upon anti-apoptotic proteins due to selective binding of different BH3-only peptides to specific BCL-2 family members, such as BCL-2, MCL-1 and BCL-xL (76). Dynamic BH3 profiling exposes cells to drug treatments and seeks to determine whether these treatments increase the apoptotic priming of cells (77).

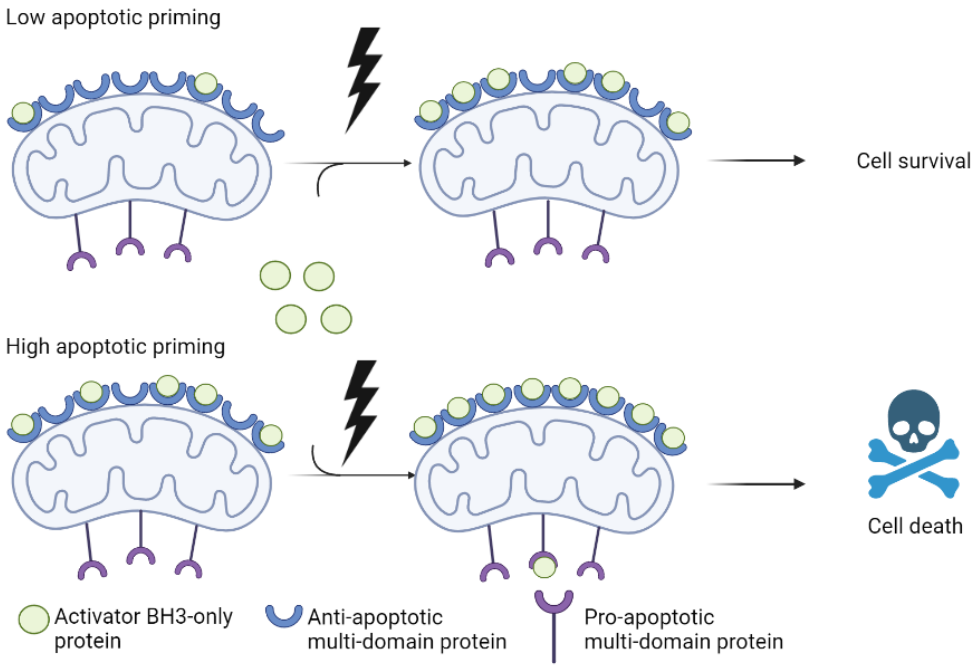

Figure 1.6.1 The mechanism of BH3 profiling allows the apoptotic priming status of a cell to be inferred. Cells that have low apoptotic priming (top) can withstand more cellular damage/stress than highly primed cells (bottom). BH3 profiling utilises this, by using BH3-only peptides as a substitute for intracellular damage. Cytochrome c release can then be calculated to determine how close cells were to the apoptotic threshold. Figure made using BioRender.

BH3 profiling has been shown to be a robust biomarker of pharmacodynamic activity of BH3 mimetics in AML cellular assays and pre-animal studies (78). Clinical trials investigating the use of venetoclax in combination with other treatments in AML have incorporated the use of BH3 profiling as a prognostic biomarker and indication of response (79,80). The use of BIM as a BH3 profiling marker has been shown to have high sensitivity and specificity for predicting patient response to Ara-C-based regimens and was therefore utilised for this project (81).

2. Aims

Traditional treatment of pAML using established chemotherapies is not sufficient to induce CR in many patients with AML. The CBF subgroup of pAML, including t(8;21) and inv(16) translocations, whilst regarded as having good prognosis, still has a high rate of relapse and chemoresistance. The use of BH3 mimetics could be an effective solution to this, however the response to these targeted therapies is varied. The timing of treatment with BH3 mimetics (either in combination with chemotherapy or following treatment with chemotherapy) remains under debate. The effect of BH3 mimetics in patients with AML that are resistant to chemotherapy also remains unclear. Therefore, the broad aim of this MSc(R) project is to investigate the role that the BCL-2 family plays in chemoresistance within CBF pAML by measuring and targeting BCL-2 dependencies, utilising baseline and dynamic BH3 profiling.

To investigate this, the work in this thesis addresses the following aims:

1. Generation of models for Ara-C resistance in t(8;21) and inv(16) AML cell lines;

2. Test dependencies of parental and chemoresistant t(8;21) and inv(16) AML cell lines on specific anti-apoptotic BCL-2 family proteins;

3. Measure expression of apoptotic proteins in parental and chemoresistant t(8;21) and inv(16) AML cell lines;

4. Assess the use of BH3 profiling as a biomarker to predict good response in patients treated with BH3 mimetics.

3. Materials and Methods

3.1. Materials

3.1.1. Tissue culture

3.1.1.1 Tissue culture plastics

Plastic	Supplier
Bottle vacuum 0.2μM filters (150 and 500ml)	Corning, Flintshire, UK
Cryovials	Scientific Laboratory Supplies, Nottingham, UK
Eppendorf tubes (0.5 and 1.5 ml)	Thermo Fisher Scientific, Paisley, UK
Falcon tube (15 and 50 ml)	Thermo Fisher Scientific, Paisley, UK
Flat bottom cell culture plates (6-, 12-, 24-, 48-, and 96-well)	Grenier Bio-One, Gloucestershire, UK
Haemocytometer	Hawksley, Sussex, UK
Mr Frosty™ Freezing Container	Thermo Fisher Scientific, Paisley, UK
Non-adherent tissue culture flasks (25, 75 and 175cm^3)	Grenier Bio-One, Gloucestershire, UK
Plastic syringes (5, 10, 20ml)	Fisher Scientific, Loughborough, UK
Serological pipettes	Fisher Scientific, Loughborough, UK
Sterile pipette tips (10, 200, 1000μl)	Grenier Bio-One, Gloucestershire, UK

3.1.1.2. Tissue culture reagents

Reagent	Supplier
Dimethyl Sulphoxide (DMSO)	Sigma-Aldrich, Dorset, UK
Ethanol (100%)	Thermo Fisher Scientific, Paisley, UK
Foetal Bovine Serum (FBS)	Thermo Fisher Scientific, Paisley, UK
L-glutamine	Thermo Fisher Scientific, Paisley, UK
Methanol (100%)	Sigma-Aldrich, Dorset, UK
Penicillin-Streptomycin (Pen-Strep)	Thermo Fisher Scientific, Paisley, UK
Phosphate Buffered Saline (PBS) tablets	Thermo Fisher Scientific, Paisley, UK
Resazurin	Thermo Fisher Scientific, Paisley, UK
Roswell Park Memorial Institute (RPMI) 1640 media	Thermo Fisher Scientific, Paisley, UK
Trypan blue solution (0.4%)	Thermo Fisher Scientific, Paisley, UK
Venor®GeM qOneStep (Mycoplasma Test)	Cambio Healthcare Systems, Reading, UK

3.1.1.3. Tissue culture equipment

Equipment	Supplier
GraphPad Prism 8	GraphPad, California, USA
Softmax Pro Software	Molecular Devices, California, USA
SpectraMax M5 plate reader	Molecular Devices, California, USA

3.1.1.4. Cell lines

Cell line	Origin	Characteristics	Supplier
Kasumi-1 **(82)**	Established from the peripheral blood of a 7-year-old with AML	Cells carry the *t(8;21)* leading to *RUNX1-RUNX1T1* (*AML1-ETO*) fusion gene	ATCC, Virginia, USA
ME-1 **(83)**	Established from the peripheral blood of a 40-year-old man with AML at second relapse	Cells carry the *inv(16)* (p13q22) leading to the fusion gene *CBFB-MYH11*	ATCC, Virginia, USA

3.1.1.5 Tissue culture media

3.1.1.5.1 20% RPMI

Component	Volume (ml)
RPMI 1640	390
FBS	100
L-glutamine	5
Pen-Strep	5

3.1.1.6 Tissue culture solutions

3.1.1.6.1. Cryopreservation solution for cell lines

Component	Volume (ml)
FBS	45
DMSO	5

3.1.1.6.2. 20% FBS/PBS washing solution

Component	Volume (ml)
Dulbecco's PBS	40
FBS	10

3.1.1.7 Drugs

Drug	Information	Supplier
A1331852	BH3 mimetic which inhibits BCL-xL	Stratech Scientific Ltd, Ely, UK
Cytarabine (Ara-C)	Cytotoxic chemotherapy	Stratech Scientific Ltd, Ely, UK
Navitoclax	BH3 mimetic which inhibits BCL-2 and BCL-xL	Stratech Scientific Ltd, Ely, UK
S63845	BH3 mimetic which inhibits MCL-1	Stratech Scientific Ltd, Ely, UK
Venetoclax (ABT-199)	BH3 mimetic which inhibits BCL-2	Stratech Scientific Ltd, Ely, UK
WEHI-539	BH3 mimetic which inhibits BCL-xL	Stratech Scientific Ltd, Ely, UK

3.1.2. Flow cytometry

3.1.2.1. Flow cytometry reagents

3.1.2.2. Flow cytometry antibodies

Antibody	Supplier	Concentration
Annexin V – FITC	BD Biosciences, Oxford, UK	1/300
BCL-2 - FITC	Biolegend, London, UK	1/200
BCL-xL - PE	Thermo Fisher Scientific, Paisley, UK	1/200
Cytochrome c – Alexa Fluor 647	Biolegend, London, UK	1/400
MCL-1 - APC	Thermo Fisher Scientific, Paisley, UK	1/200

3.1.2.3. Flow cytometry reagents

Reagent	Supplier
Alamethicin	Enzo Life Sciences, Exeter, UK
BD FACS Clean Solution	BD Biosciences, Oxford, UK
BD FACS Flow Solution	BD Biosciences, Oxford, UK
BD Perm/Wash™ Buffer	BD Biosciences, Oxford, UK
Bovine Serum Albumin (BSA) Fraction V	Sigma-Aldrich, Dorset, UK
DAPI	BD Biosciences, Oxford, UK
Digitonin	Sigma-Aldrich, Dorset, UK
EDTA	Sigma-Aldrich, Dorset, UK
EGTA	Sigma-Aldrich, Dorset, UK
Glycine	Sigma-Aldrich, Dorset, UK
Mannitol	Sigma-Aldrich, Dorset, UK
Methanol	Sigma-Aldrich, Dorset, UK
Potassium chloride	Sigma-Aldrich, Dorset, UK
Succinate	Sigma-Aldrich, Dorset, UK
Trizma® Base	Sigma-Aldrich, Dorset, UK

3.1.2.4. Flow cytometry peptides

Peptide	Sequence	Supplier
hBIM	Acetyl-MRPEIWIAQELRRIGDEFNA-Amide	Genscript, New Jersey, USA

3.1.2.4. Flow cytometry solutions

3.1.2.4.1 90% methanol permeabilization solution

Component	Volume (ml)
H_2O	5
Methanol	45

3.1.2.4.2 Mitochondrial buffer (MEB2)

Component	Volume/mass	Final concentration
dH_2O	Up to 500mL	
Mannitol	13.65g	150mM
HEPES-KOH ph7.5	1.19g	10mM
Potassium chloride	5.59g	150mM
EGTA (0.5M)	1ml	1mM
EDTA (0.5M)	1ml	1mM
BSA	0.5g	0.1%
Succinate	0.295g	5mM
Note: Filtered using a 0.22μM filter before use.		

3.1.2.4.3 TRIS-Glycine buffer

Component	Volume/mass	Final concentration
dH_2O	Up to 500mL	
Trizma® base	15.14	0.25M
Glycine (1.25M)	Variable	Up to pH 8.3
Note: Filtered using a 0.22μM filter before use.		

3.1.2.5. Flow cytometry equipment

Equipment	Supplier
BD FACSCanto™ II	BD Biosciences, Oxford, UK
BD FACSDIVA™ Software	BD Biosciences, Oxford, UK
FlowJo v.10.8.1	FlowJo LLC, Oregon, USA

3.2. Methods

3.2.1. Cell culture

3.2.1.1. Standard cell culture conditions

Cells were cultured in an incubator set to 37°C with 5% CO_2 in RPMI/20% FBS (**3.1.1.5.1**). All cell culture manipulations were performed in a sterile hood using aseptic techniques. Mycoplasma testing was performed regularly using Mycoalert™ Detection Kits.

3.2.1.2. Cell line thawing and cryopreservation

Frozen cell lines were thawed rapidly in a 37°C water bath. Thawed cells were diluted by dropwise addition of pre-warmed media. Cells were centrifuged at 300g for 5 minutes with pre-warmed 20% FBS/PBS. Trypan Blue exclusion was used to determine the number of viable cells, then cells were incubated overnight in 20% FCS/RPMI at a concentration of $1x10^6$ cells/mL. Thereafter, cells were re-cultured at the recommended density for each individual cell line ($0.5x10^6$ cells/mL for Kasumi-1 (84) and $0.5\text{-}1.0x10^6$ cells/mL for ME-1 (85)) . Cells were split to approximately 70% confluency every 3-4 days in fresh media.

Prior to freezing, cells were washed in PBS and then resuspended in cryopreservation solution at a concentration of $5x10^6$ cells per mL. Cell suspensions were moved into cryovials and placed in a Nalgene® Mr Frosty freezing container at -80°C overnight. For long-term storage, cells were stored in liquid nitrogen tanks.

3.2.1.5. Drugs and reagents

Drug stocks were dissolved in sterile DMSO in a sterile hood, according to manufacturer instructions. BH3 mimetics were maintained at a concentration of 1μM and kept at -20°C for short-term storage and -80°C for long-term storage. Drugs were aliquoted for single use and drug dilutions were freshly prepared for experiments as required.

3.2.1.6. Resazurin assay

The resazurin assay was utilised to assess cell viability and metabolic activity. Resazurin was prepared according to manufacturer instructions and made into a 25mM solution using distilled water and syringed through a 0.45μM filter. Aliquots were stored at -20°C until required.

Stock resazurin (25 mM) was diluted to a concentration of 500 μM in pre-warmed serum-free media (1:50). 20 μL of 500 μM solution was added to each well and cells were incubated for 4 hours at 37°C. Fluorescence was measured at 535_{ex} and 590_{em} using a SpectraMax M5 plate reader and analysed using SoftMax Pro software.

3.2.2. Flow cytometry

3.2.2.1. Apoptosis assay

Cells were plated in triplicate at a density of $0.1x10^6$ cells/mL in 200μL of media in each well of a 96-well plate. Cells were treated with appropriate concentrations of drug and incubated at 37°C for 24, 48 or 72 hours, depending on the planned experiment.

Flow cytometric analysis (BD FACS Canto II and FlowJo v10.8.1) was used to determine the viability of the cells after treatment. Annexin V staining detects the presence of extracellular phosphatidylserine, a marker of early apoptosis (86). 4′,6-diamidino-2-phenylindole is a fluorescent stain which inefficiently passes through intact cell membranes and is therefore used to identify dead cells (87). Therefore, cells were stained with annexin V (annexin V-FITC; **3.1.2.2**) and DAPI. Cells were deemed to be viable if they were negative for annexin V and/or DAPI. This assay was used to determine the degree of cell death in response to treatment, therefore cell death was considered to be equal to 100% - (DAPI-negative/annexin V-negative cells %).

3.2.2.2. Baseline and dynamic BH3 profiling

Baseline and dynamic BH3 profiling were utilised in this project to determine how primed cell lines were for apoptosis and how exposure to BH3 mimetics effected the apoptotic priming of the cell lines, respectively. The protocol used

for BH3 profiling in this thesis was adapted from the Letai group for use at the Paul O'Gorman Leukaemia Research Centre by Dr Narissa Parry (88,89).

The day prior to the experiment, cells were split to their seeding density. On the day of the experiment, plates were prepared with escalating doses of BIM (for baseline BH3 profiling) and BH3 mimetics (for dynamic BH3 profiling) in 0.002% w/v digitonin in MEB2 buffer and were added to the treatment wells. The role of digitonin in BH3 profiling is permeabilisation of the cell membrane to ensure even exposure of peptides to cell mitochondria and was prepared in DMSO (88). For controls, final concentrations of 25μM alamethicin (positive control for cytochrome c release) or 1% DMSO (negative control for cytochrome c release) were prepared in 0.002% w/v digitionin in MEB2 buffer.

Cells were washed in PBS and resuspended in MEB2 buffer (without digitonin) at a concentration of $2x10^6$ cells/mL. Cells were added at an equal volume to wells containing peptides prepared in MEB2 (as described above), resulting density of $1x10^6$ cells/mL, and incubated for 30 minutes at 25°C. 4% v/v paraformaldehyde was added to each well to fix the cells and terminate peptide/inhibitor exposure, then incubated at room temperature for 10 minutes. To neutralise the paraformaldehyde, 33 μL TRIS-glycine buffer was added to each well and left at room temperature for 5 minutes. Antibodies for cytochrome C (cytochrome C-Alexa Fluor 647) and DAPI were diluted in BD Perm/Wash™ and used to stain the cells for flow cytometric analysis and left overnight at 4°C.

The following day, FACS analysis was performed. Cells treated with alamethicin were considered to have complete cytochrome c release and cells treated with DMSO were considered to have no cytochrome c release. Gating for FACS analysis was determined using these positive and negative controls for cytochrome c release. An example of the gating strategy used for BH3 profiling is shown in **Figure 3.2.1.**

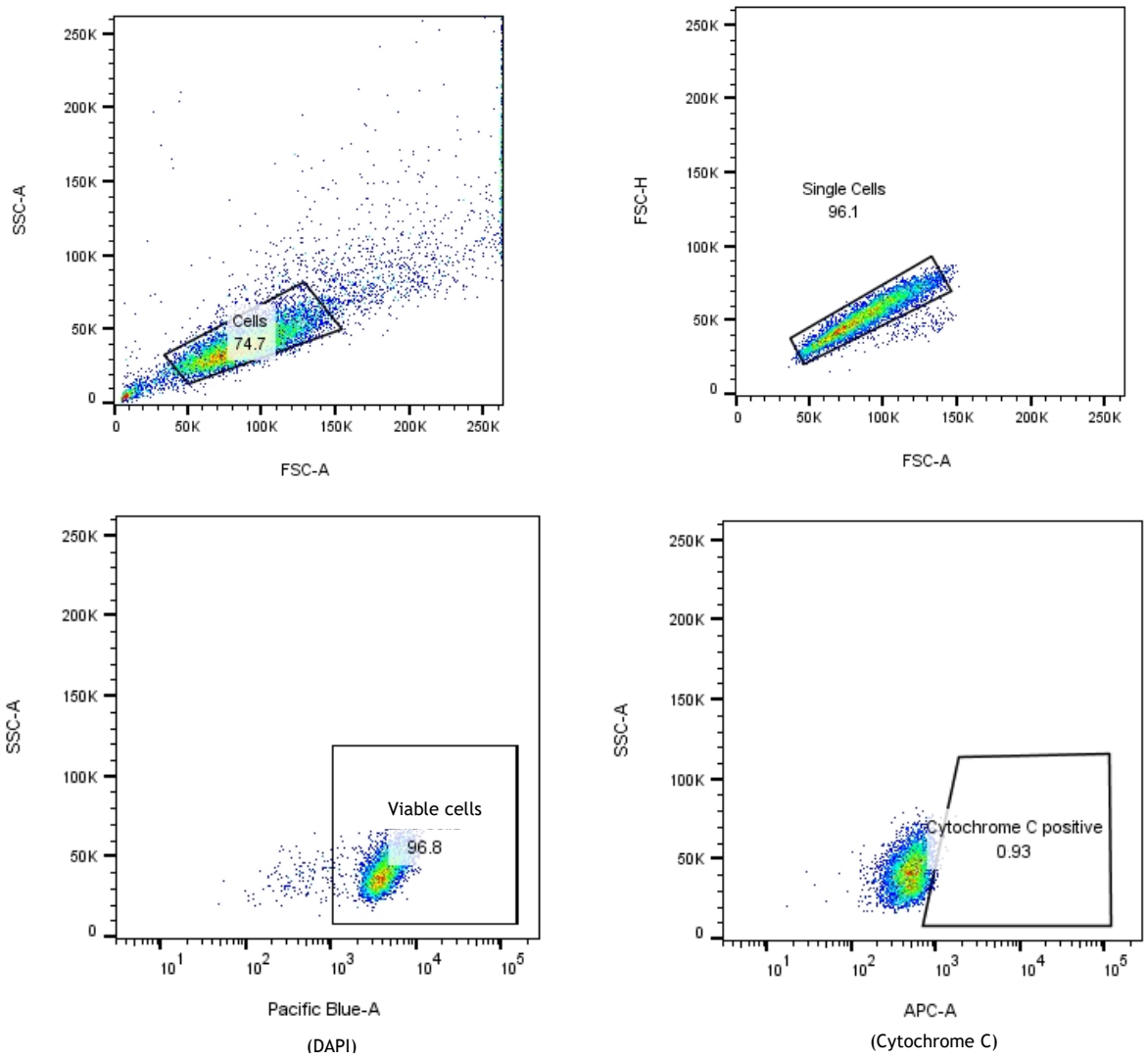

Figure 3.2.1: Gating strategy used to assess the percentage of cytochrome c release. Events were gated using the SSC-A x FSC-A plot and the cell populations for further analysis were selected using the FSC-H x FSC-A plot. From this cell population, viable cells (DAPI-positive) were selected using the SSC-A x Pacific Blue-A plot. Cytochrome c positive cells were gated for using the alamethicin control for complete cytochrome c release using the SSC-A x APC-A plot.

Once the gating strategy had been applied, the percentage of cytochrome c release could be determined using the following formula.

$$Cytochrome\ c\ release\ (\%) = 100 - cytochrome\ c\ positive\ (\%)$$

3.2.2.3. Intracellular staining of BCL-2 family proteins

The expression of BCL-2 family proteins (BCL-2, MCL-1, BCL-xL) in parental and Ara-C resistant cell lines was determined using intracellular staining.

Cells were washed twice with PBS and fixed by exposure to 4% v/v paraformaldehyde in PBS at room temperature for 20 minutes. Cells were then washed twice again with PBS to remove any remaining paraformaldehyde. Cells were permeabilised by drop-wise addition of ice-cold 90% methanol and incubated on ice with regular vortexing over a period of 30 minutes. Cells were washed twice again to remove methanol and stained with antibodies against the proteins of interest (BCL-2, MCL-1, BCL-xL) and DAPI. Cells were washed with PBS a final time and FACS analysis was performed. Median fluorescence intensity (MFI) for each cell line was normalised to unstained controls.

3.2.3. Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8. Half maximal effective concentration (EC_{50}) values were calculated using lines of best fit for means of replicate using GraphPad Prism 8. Where possible, all experiments have been performed in triplicate, however this was not the case for all experiments due to time constraints. Results are presented as mean values, with error bars representing the standard error the mean (SEM). Student t-tests were used to compare results from two groups, with statistical significance defined as *$p<0.05$; **$p<0.01$ and p-values ≥ 0.05 were deemed as not significant.

4. Results

4.1 Effect of Ara-C on CBF cell lines

In order to measure the EC_{50} for Ara-C, the cell lines ME-1 and Kasumi-1 were treated with a range of concentrations of Ara-C for 24, 48, and 72 hours. Cell viability was assessed using resazurin and FACS apoptosis assays, as described previously, to calculate the apoptotic EC_{50} (apoptosis assay) and metabolic EC_{50} (resazurin assay).

In both cell lines, the optimal timepoint for cell death was 72hrs (**Figure 4.1.1.A**), therefore this timepoint was used for all further treatments with Ara-C. There was not a complete reduction in cell viability in the resazurin assay (**Figure 4.1.1.B**), even at the highest concentrations of Ara-C. This could have been due to a number of reasons, including incubation time and cell-to-volume ratio. However, due to time constraints, this experiment was not troubleshooted and FACS apoptosis assay was used to assess cell viability in this project in subsequent experiments. Using a FACS apoptosis assay, EC_{50} concentrations for Ara-C were calculated for each cell line, which would be used for further experiments (**Table 4.1.1**, highlighted values).

Table 4.1.1. Calculated EC_{50} values for 3 cell lines treated with Ara-C, assessed by resazurin and FACS apoptosis assay. Highlighted values indicate EC_{50} concentrations used in further experiments. n=3 for all experiments. NC, non-calculable. nM, nanomolar.

Cell line	Apoptotic EC_{50} (nM)			Metabolic EC_{50} (nM)		
	24h	48h	72h	24h	48h	72h
ME-1	96470.00	1823.00	299.60	NC	1865.00	939.40
Kasumi-1	3784.00	48.14	101.70	16.54	101.80	31.90

The response to Ara-C was varied between the two cell lines. ME-1 was the more resistant to Ara-C, with the highest EC_{50} concentration (299.60 nM; **Table 4.1.1**). EC_{50} concentrations were used to calculate EC_{25} and EC_{75} concentrations at 72 hours for generation of Ara-C resistant cell lines (**Table 4.1.2**). The error bars at some concentrations are wide and given further time, these experiments would need to be repeated to be less variable between triplicate experiments. As EC_{50} values were calculated using the normalised mean of triplicate experiments, the statistical significance of the difference in EC_{50} concentration could not be calculated.

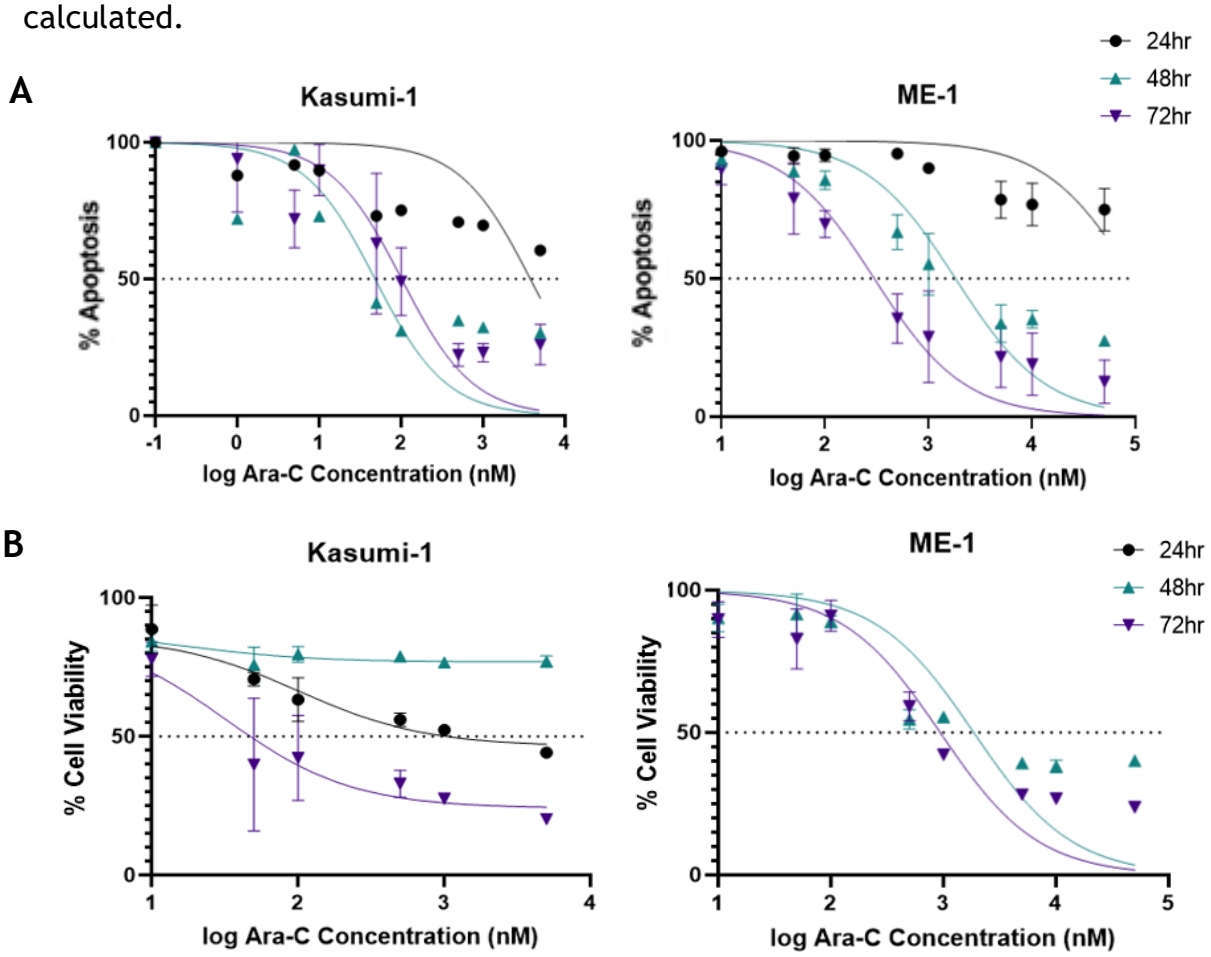

Figure 4.1.1. Effect of Ara-C on viability of Kasumi-1 and ME-1 cell lines as assessed by A) FACS apoptosis assay and B) resazurin assay after 24-, 48- and 72-hour treatment (n=3). 24-hour time point experiment was not performed for ME-1 in the resazurin assay. Error bars represent SEM. nM, nanomolar.

Table 4.1.2. Calculated EC_{25}, EC_{50} and EC_{75} Ara-C concentrations at 72 hours for two cell lines used for the generation of Ara-C resistant cell lines.

Cell line	EC_{25} (nM)	EC_{50} (nM)	EC_{75} (nM)
ME-1	99.87	299.60	898.8
Kasumi-1	33.9	101.70	305.10

4.2 Generation of chemoresistant CBF cell lines

For the Ara-C resistant cell lines, parental cell lines were treated for 72 hours with escalating doses of Ara-C (EC_{25}, EC_{50}, and EC_{75} concentrations; **Table 4.1.2**). The treatment of cell lines with escalating doses of chemotherapy for the generation of treatment-resistant cell lines has been described previously (90). Cell lines were allowed to recover by leaving cells in drug-free media until they had reached a confluency of double the minimum density recommended for each line before treating with the next highest dose (**Figure 4.2.1**). Once cells had been treated with the EC_{75} concentration and had recovered, cells were kept in drug-free media and maintained as per a normal cell line, in drug-free media. These cell lines were generated to simulate, *in vitro*, patients that had been continually treated with Ara-C and had become resistant over time.

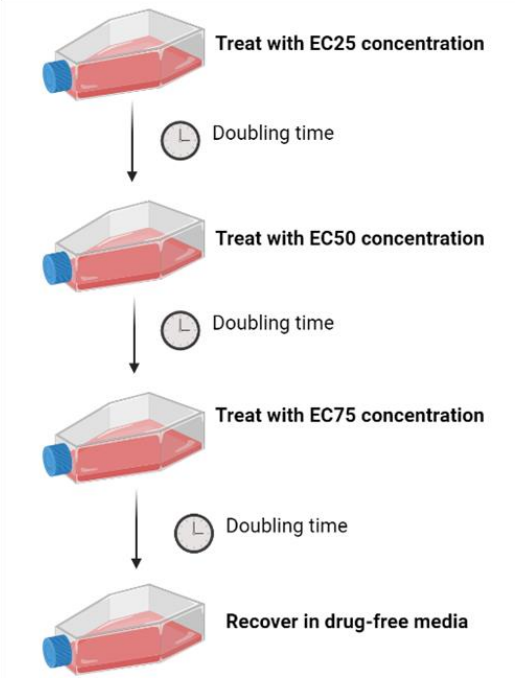

Figure 4.2.1. The method used to generate models of resistance to Ara-C for this project. To generate the Ara-C resistant model, AML cell lines were treated with the Ara-C EC_{25} concentration calculated for each cell line for 72 hours. Cells were washed with PBS/FBS and spun in a centrifuge at 300xg for 5 minutes. Cells were transferred to drug-free media at a high confluency to recover to a confluency which was double that recommended for each cell line. This process was then repeated for the EC_{50} and EC_{75} concentration of Ara-C. The generated Ara-C resistant model was then kept in drug-free media long-term and maintained as per recommendations for the paternal cell lines.

4.3 Baseline BH3 profiling of parental CBF cell lines

To determine the apoptotic priming of the cell lines used in this study, baseline BH3 profiling was performed. Parental and Ara-C resistant cell lines were treated with a range of concentrations of BIM peptide, as previously described **(Section 3.2.2.2)**. Apoptotic priming of the cell lines by baseline BH3 profiling was assessed using flow cytometry and the FlowJo gating profile shown in **Figure 3.2.1**.

The BIM peptide EC_{50} concentrations for the parental Kasumi-1 and ME-1 cell lines were 69.0 and505.9 nM, respectively (**Figure 4.3.1**). Kasumi-1 had the lower BIM peptide EC_{50} concentration (69.0 nM), indicating that this cell line was

the more primed for apoptosis, as a lower concentration of BIM peptide was required to induce cytochrome c release **(Figure 4.3.1.A)**.

ME-1 had the higher BIM EC_{50} (505.9 nM; **Figure 4.3.1.B**), showing that more BIM peptide was required for these cells to cross the apoptotic threshold and induce cytochrome c release. This shows that the parental ME-1 cell line is less primed for apoptosis of the two cell lines used in this study, and indicates that this cell line contains less primed BCL-2 pro-apoptotic family members. ME-1 was also the more resistant of the two parental cell lines to treatment with Ara-C (**Table 4.1.2**), which further supports that ME-1 has low apoptotic priming.

As EC_{50} values were calculated using the normalised mean of triplicate experiments, the statistical significance of the difference in EC_{50} concentration between Kasum-1 and ME-1 could not be calculated.

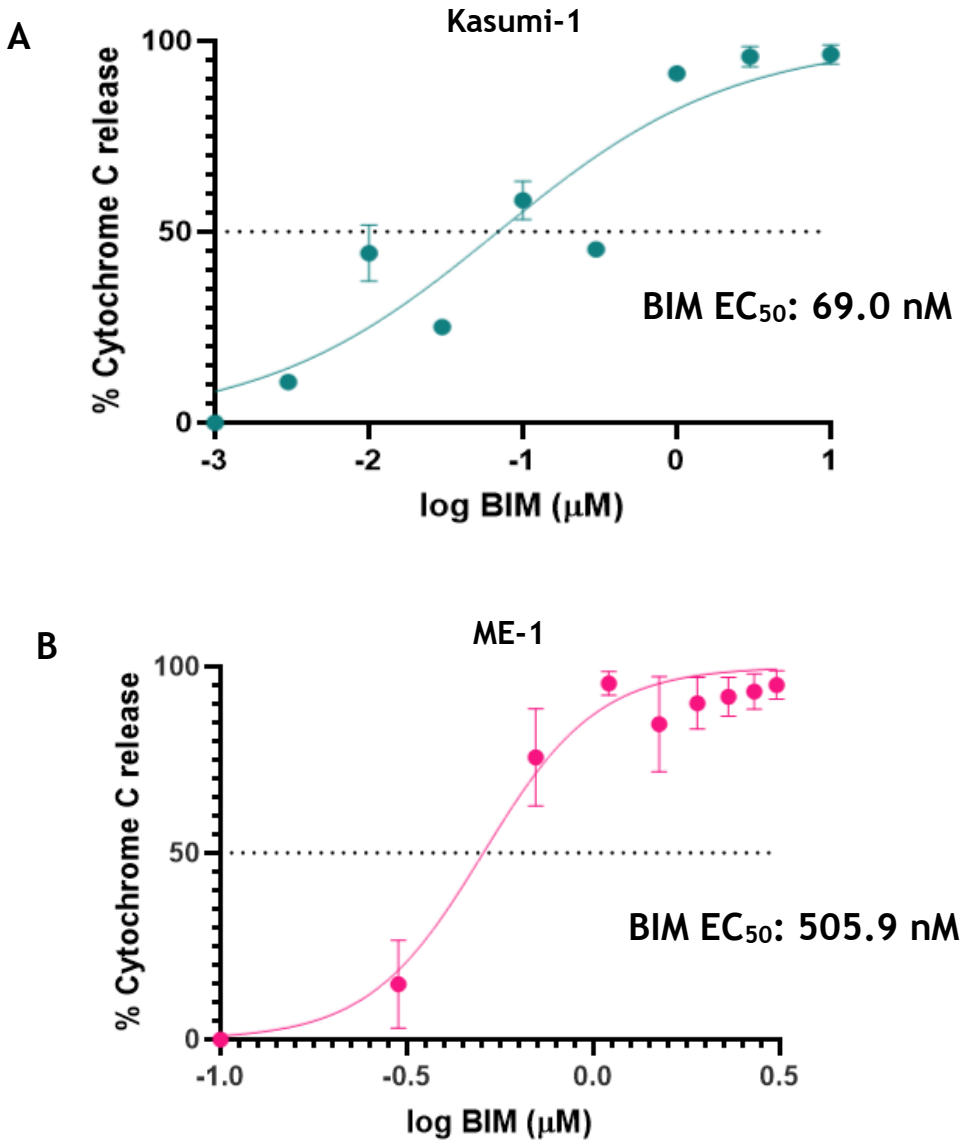

Figure 4.3.1. BIM curves demonstrate that Kasumi-1 has a higher degree of apoptotic priming than ME-1 (n=3). Release of cytochrome c in response to BIM peptide are shown here for **(A)** Kasumi and **(B)** ME-1 cell lines. n=3. Data points represent the mean of data from experiments in triplicate and error bars represent SEM.

4.4 Baseline BH3 profiling of chemoresistant CBF cell lines

To assess the effect of Ara-C resistance on the apoptotic priming of cell lines, baseline BH3 profiling was also carried out on the generated Ara-C resistant Kasumi-1 and ME-1 cell lines (referred to hereafter as Kasumi-1R and ME-1R, respectively).

The comparison of apoptotic priming of parental and Ara-C resistant cell lines showed variability. For ME-1, the parental cell line had a BIM EC_{50} concentration of 530.6 nM, while ME-1R had a BIM EC_{50} concentration of 155.9 nM (F**igure 4.4.1.A**). This shows that in the case of ME-1, acquired resistance to Ara-C increased apoptotic priming, as less BIM peptide was required to induce cytochrome c release.

Conversely, for Kasumi-1, the parental cell line had a BIM EC_{50} concentration of 69 nM and Kasumi-1R had a BIM EC_{50} concentration of 930 nM (**Figure 4.4.1.B**). Kasumi-1R was less primed for apoptosis than the parental Kasumi-1 cell line as more BIM peptide was required to induce cytochrome c release.

These experiments were not performed as matched pairs, i.e., the EC_{50} values of the parental cell lines were not re-evaluated alongside respective resistant cell lines. To improve confidence in these results, these experiments should be repeated as matched pairs, with apoptosis assays to calculate EC_{50} values of parental and resistant cell lines performed in parallel.

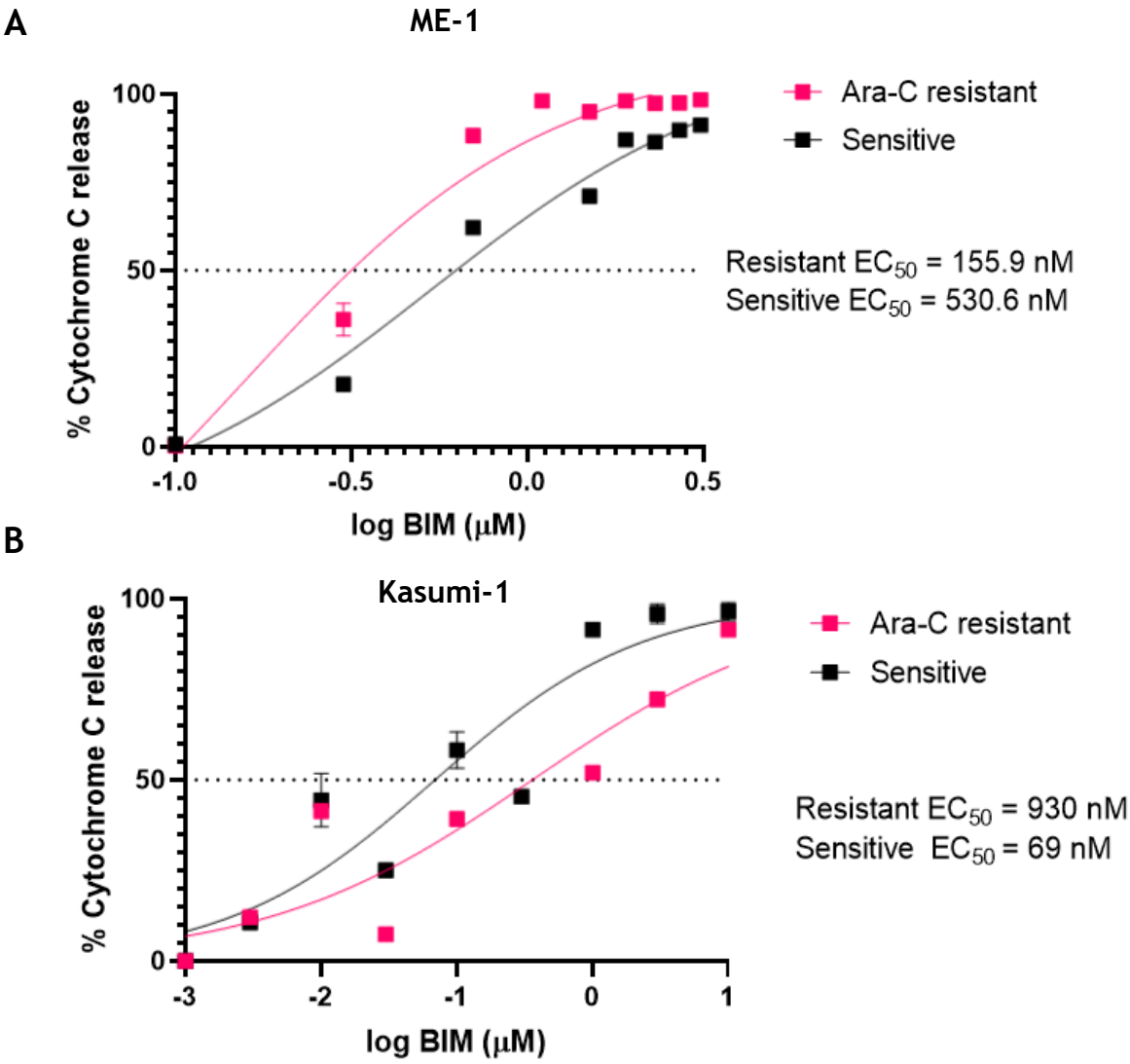

Figure 4.4.1. Effect of Ara-C resistance on apoptotic priming differs between Kasumi-1 and ME-1 (n=3). Kasumi-1R is less primed for apoptosis than parental Kasumi-1 cells as less BIM peptide is required to induce cytochrome c release **(A)**. ME-1R cells were more primed for apoptosis than parental ME-1 cells, as higher concentrations of the BIM peptide are required to induce cytochrome c release **(B).** Error bars represent SEM.

4.5 Expression of BCL-2 family proteins in parental CBF cell lines

The expression of BCL-2 family member proteins (BCL-2, MCL-1, BCL-xL) was assessed using intracellular flow cytometry staining **(Figure 4.5.1)**.

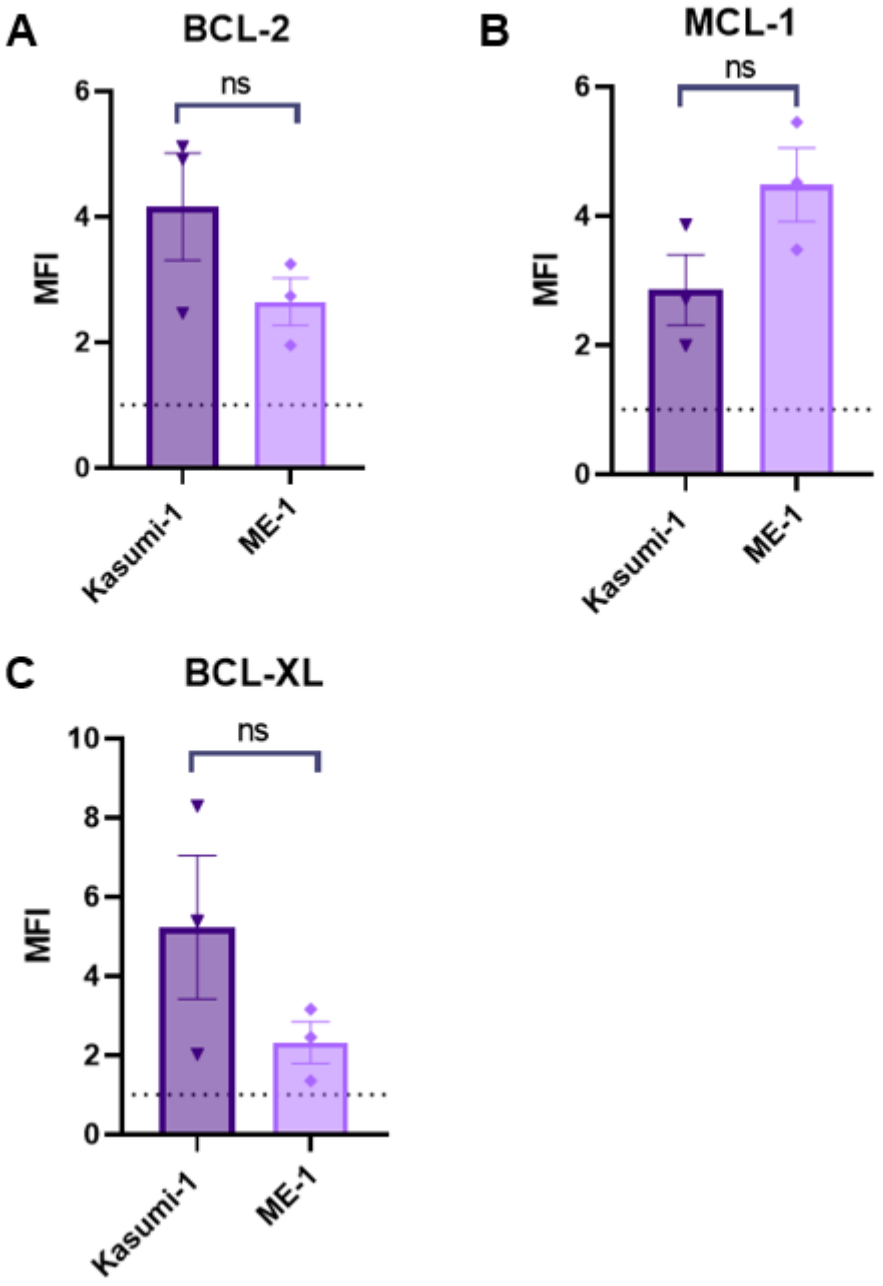

Figure 4.5.1. BCL-2, MCL-1 and BCL-xL were expressed in both cell lines (n=3). The expression of BCL-2 **(A)**, MCL-1 **(B)**, and BCL-xL **(C)** are shown. Data were normalised to the median fluorescence intensity (MFI) of unstained controls which are represented by the dotted line.. Statistical analysis was performed using an unpaired t test. Error bars represent SEM. MFI, median fluorescence intensity. ns, not significant.

The expression of BCL-2 was lower in ME-1 than in Kasumi-1 **(Figure 4.5.1.A)**, however this did not reach statistical significance. Kasumi-1 showed a lower level of expression of MCL-1 **(Figure 4.5.1.B)** and BCL-xL expression than ME-1 **(Figure 4.5.1.C)**. However, the differences in expression of MCL-1 and BCL-xL

between the two cell lines did not reach statistical significance. More replicates may have increased confidence in these results, as the error bars were very wide for these data, specifically for BCL-xL expression in Kasumi-1.

4.6 Effect of Ara-C resistance on expression of BCL-2 family proteins

Once the expression of BCL-2 family proteins had been determined in the parental cell lines, the effect of Ara-C resistance on the expression of these proteins was assessed using the generated models of Ara-C resistance **(Figure 4.6.1)**.

In Kasumi-1, expression of MCL-1 was significantly higher in Kasumi-1R than the sensitive parental cell line (P=0.0253; **Figure 4.6.1.A**). Similarly, the expression of MCL-1 was significantly higher in ME-1R than ME-1 cells (P=0.0049; **Figure 4.6.1.B**). Expression of BCL-2 and BCL-xL were numerically higher in Kasumi-1R than the sensitive parental line, however these results were not statistically significant **(Figure 4.6.1.A).** Similarly, in ME-1, the expression of BCL-2 and BCL-xL were numerically higher in ME-1R than in the sensitive, parental cell line **(Figure 4.6.1.B)**, but these differences in protein expression did not reach statistical significance.

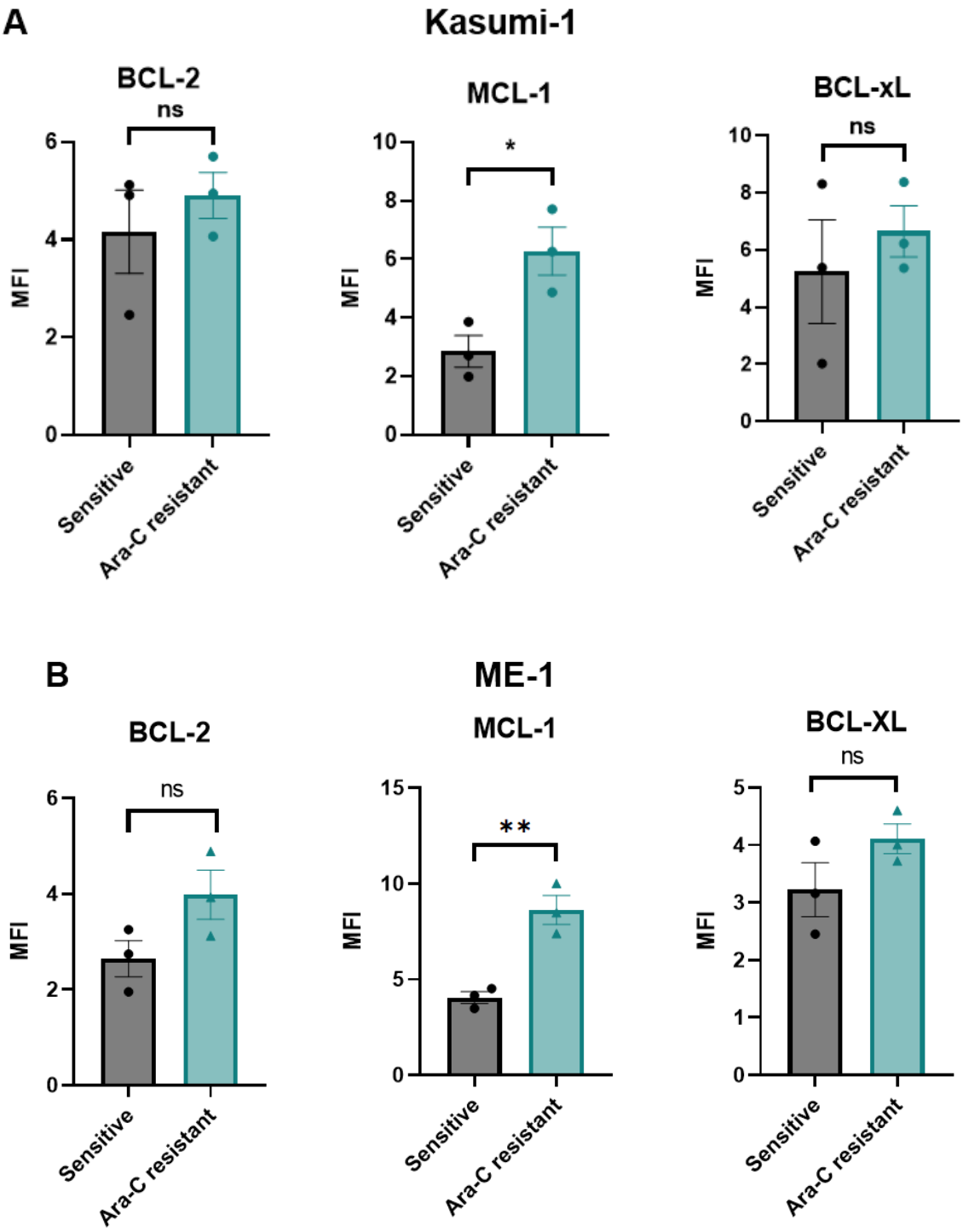

Figure 4.6.1. Expression of BCL-2, MCL-1, and BCL-xL are higher in Ara-C resistant CBF cell lines than in parental cell lines (n=3). The expression of BCL-2 family proteins of interest are presented for **A)** Kasumi-1 and **B)** ME-1. Statistical analysis was performed using an unpaired t test. Error bars represent SEM. *p<0.05; **p<0.01. MFI, median fluorescence intensity. ns, not significant.

4.7 Response of BH3 mimetic treatment in parental and Ara-C resistant CBF cell lines

As the expression of all anti-apoptotic proteins of interest was higher in the Ara-C resistant models of both Kasumi-1 and ME-1 than the parental cell lines, it was hypothesised that response to BH3 mimetics which target these anti-apoptotic would be enhanced in the Ara-C resistant models. To assess this, parental and Ara-C resistant cell models of Kasumi-1 and ME-1 were treated with 250 nM of BH3 mimetics of interest for 24 hours and percentage cell death was ascertained through an apoptosis assay. As the aim of this experiment was to produce a snapshot of the response to BH3 mimetics in parental and Ara-C resistant cell lines, a concentration of 250 nM was chosen as the half-maximal inhibitory concentration (IC_{50}) of the selected drugs in these cell lines ranged from 5-500nM and therefore some response could be expected in each cell line (91).

Due to time constraints, it was not possible to perform triplicate experiments to assess the response of Kasumi-1 (parental and Ara-C resistant) to BH3 mimetics of interest. Although statistical analysis cannot be performed on data from one experiment, as these data are utilised for comparisons with other data in the next section, they are presented here for completeness **(Figure 4.7.1)**. In Kasumi-1, response to A1331852 was numerically higher in Kasumi-1R compared to parental Kasumi-1 cells. For all other BH3 mimetics utilised, cell death was decreased in Kasumi-1R compared to parental Kasumi-1. Despite the lack of triplicate repeats in this experiment, it is noteworthy that in Kasumi-1R there was no cell death in response to venetoclax or navitoclax .

ME-1R cells showed a significantly higher response to the BCL-2 inhibitor venetoclax than parental ME-1 cells (P=0.0339; **Figure 4.7.2**). Response to the BCL-xL inhibitor A1331852 at 250nM was low in ME-1 cells overall, however ME-1R had reduced response to A1331852 compared to parental ME-1 which was approaching statistical significance (P=0.0558). The response to the MCL-1 inhibitor S63845 was numerically higher in parental ME-1 compared to ME-1R, however this difference did not reach statistical significance. A similar response to the dual BCL-2/BCL-xL inhibitor navitoclax was seen in parental and ME-1R . The response to the BCL-xL inhibitor WEHI-539 at the concentration used was low in both ME-1 cell lines, with no significant difference between them.

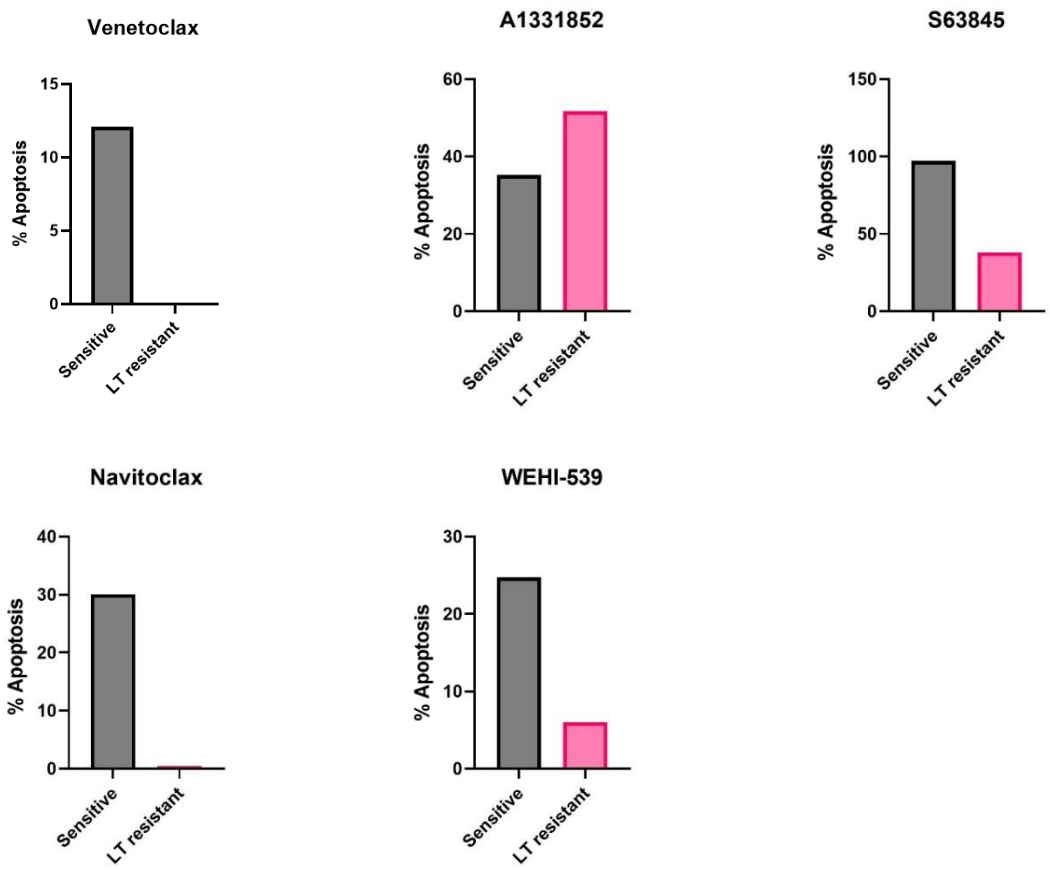

Figure 4.7.1. Kasumi-1R had reduced response to most BH3 mimetics utilised compared to parental Kasumi-1 (n=1). Percentage cell death was assessed by apoptosis assay and normalised to DMSO controls. LT, long-term.

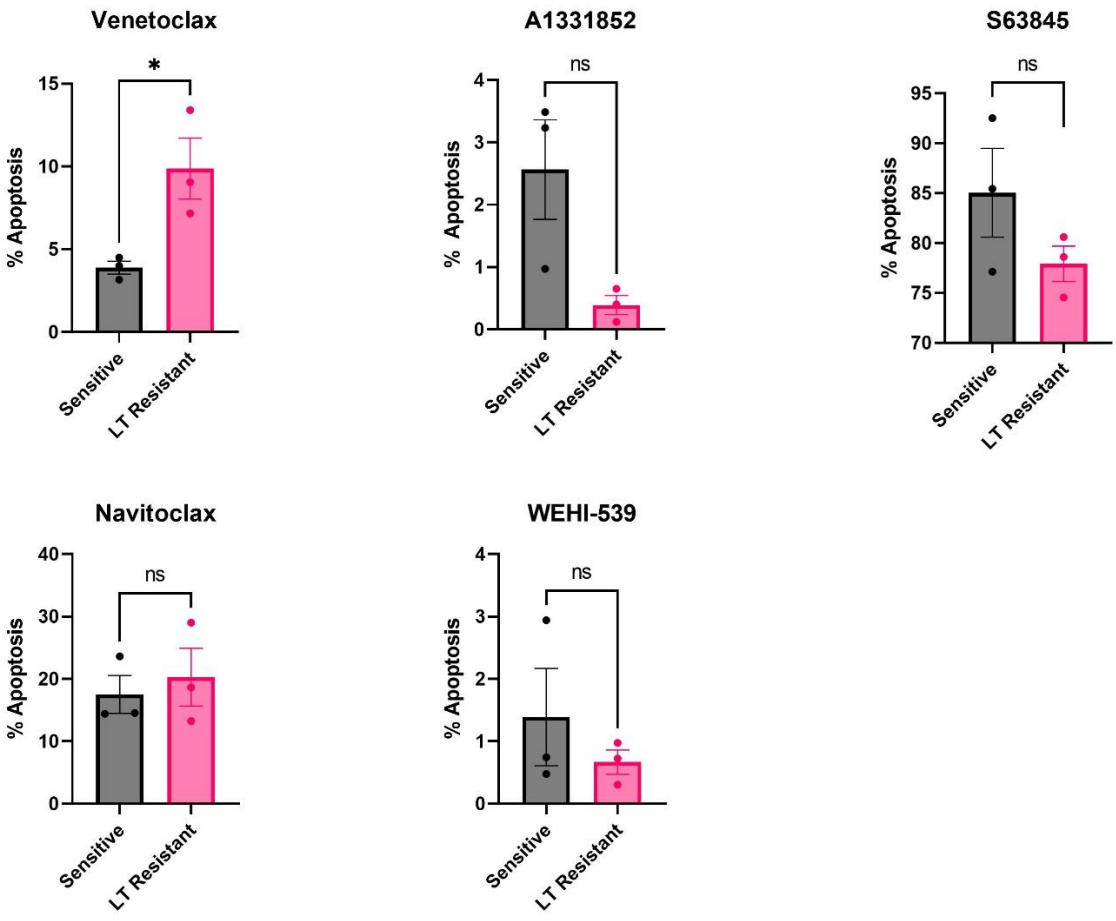

Figure 4.7.2. There is variable response to BH3 mimetics between sensitive parental ME-1 and ME-1R cell lines (n=3). Percentage cell death was assessed by apoptosis assay and normalised to DMSO controls. Statistical analysis was performed using an unpaired t test. Error bars represent SEM. LT, long-term. ns, not significant.

4.8 Assessing the use of dynamic BH3 profiling to predict response to BH3 mimetics

To assess the use of dynamic BH3 profiling to predict response to BH3 mimetics, dynamic BH3 profiling was performed as previously described (**Section 3.2.2.2**). Due to the time constraints of this project, this experiment was only performed using Kasumi-1 parental and Ara-C resistant cell lines. The results of dynamic BH3 profiling in these cell lines were compared to previously reported (**Section 4.7**) data demonstrating the response of these cell lines to a fixed concentration of BH3 mimetics.

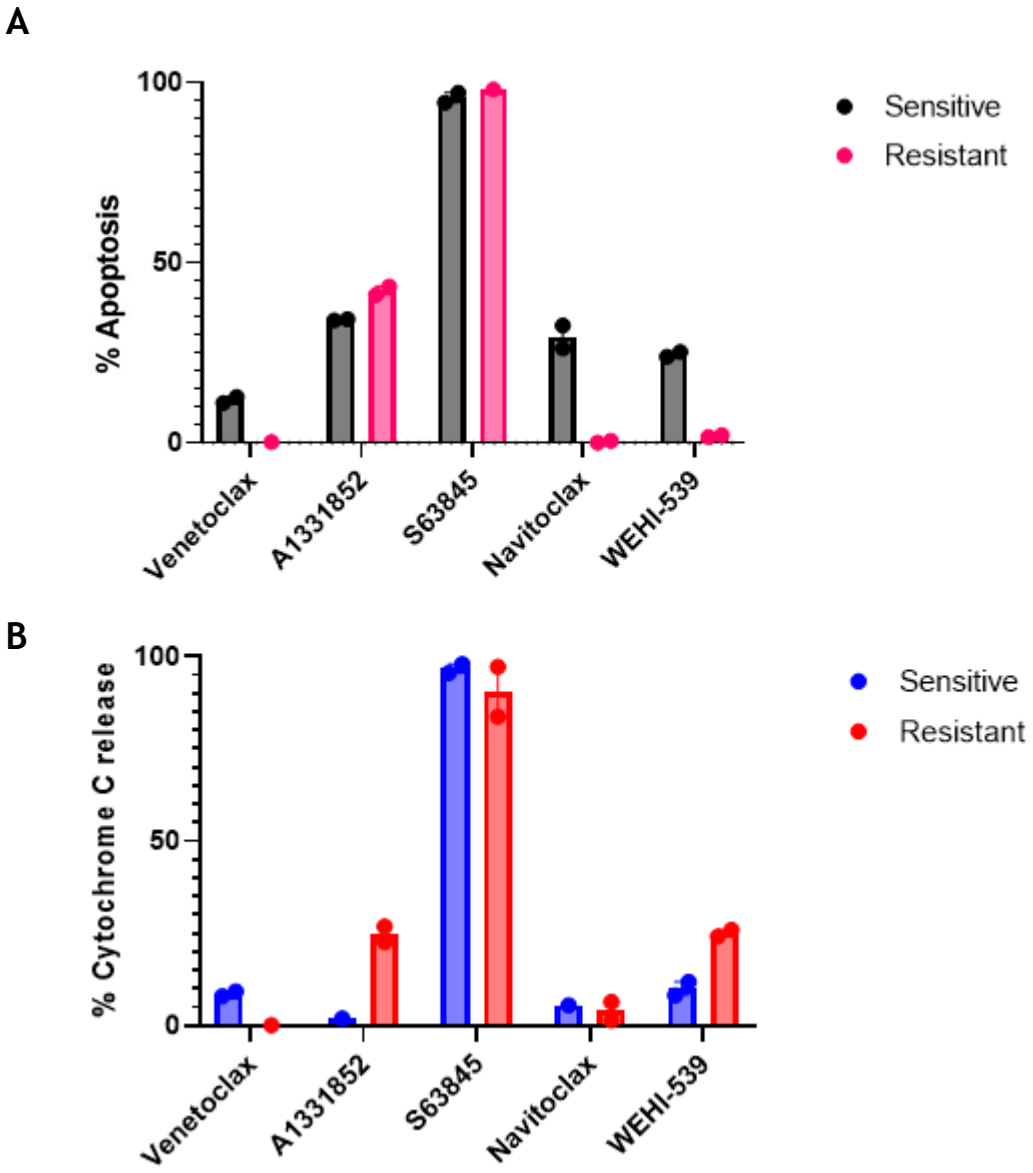

Figure 4.8.1 Dynamic BH3 profiling using BH3 mimetics predicts the broad response to BH3 mimetics in Kasumi-1 and Kasumi-1R cell lines (n=2). Exposure to a fixed concentration of multiple BH3 mimetics as assessed by apoptotic assay **(A)** are compared here to the percentage of cytochrome c release assessed by dynamic BH3 profiling using the same BH3 mimetics **(B)**.

The results of dynamic BH3 profiling broadly mimic the response to treatment in parental and Ara-C resistant Kasumi-1 cells **(Figure 4.8.1)**. Parental and Ara-C resistant Kasumi-1 cell lines both had low levels of cell death, measured by the apoptosis assay **(Figure 4.8.1.A)**, which was captured by the level of cytochrome c release measured by dynamic BH3 profiling **(Figure 4.8.1.B)**. Kasumi-1R was more resistant to venetoclax treatment than parental Kasumi-1 cells when assessed by apoptosis assay **(Figure 4.8.1.A)** and this difference

between Kasumi-1R and parental Kasumi-1 cells was captured by dynamic BH3 profiling **(Figure 4.8.1.B)**. Both parental Kasumi and Kasumi-1R cell lines were sensitive to S63845 treatment when assessed by apoptosis assay **(Figure 4.8.1.A)**, which was also shown by measurement of cytochrome c release by dynamic BH3 profiling **(Figure 4.8.1.B)**.

However, there are a few instances where the response to BH3 mimetics is not replicated in the dynamic BH3 profiling results. For example, response to A1331852 was similar in Kasumi-1 and Kasumi-1R cells **(Figure 4.8.1.A)**, however dynamic BH3 profiling showed that A1331852 induced less cytochrome c release in both cell lines **(Figure 4.8.1.B)**. Also, dynamic BH3 profiling showed that more cytochrome c release occurred in Kasumi-1R compared to parental Kasumi-1 cells in response to A1331852 **(Figure 4.8.1.B)**, which is the same pattern observed in response to A1331852 in the apoptosis assay **(Figure 4.8.1.A)**. However, overall the pattern of cytochrome c release in response to A1331852 is broadly similar to the corresponding apoptotic assay even if the magnitude of response is different, and a single time-point assay for two different cellular events may not correlate perfectly. The response to navitoclax as assessed by apoptosis assay **(Figure 4.8.1.A)** did not match the percentage of cytochrome c release assessed by dynamic BH3 profiling **(Figure 4.8.1.B)**. In the apoptosis assay, parental Kasumi-1 cells had increased response to navitoclax compared to Kasumi-1R cells **(Figure 4.8.1.A)**. However, results of the dynamic BH3 profiling showed a similar, low level of cytochrome c release in response to navitoclax **(Figure 4.8.1.B)**. Similarly, the response to navitoclax and WEHI-539 as assessed by apoptosis assay **(Figure 4.8.1.A)** did not match the percentage of cytochrome c release assessed by dynamic BH3 profiling **(Figure 4.8.1.B)**.

5. Discussion

5.1 Model of Ara-C resistance in CBF cell lines

Previously, models of Ara-C resistance in AML have been generated, namely a *FLT3*-ITD positive Ara-C resistant line, which was generated by treating the cell line MV4-11 with increasing doses of Ara-C (ensuring that the growth rate of the parental line was reached before each dose increase) (92). However, there have been no reported models of Ara-C resistance in t(8;21) or inv(16) paediatric AML cell lines. The method used to generate Ara-C resistant models of Kasumi-1 (derived from a child with AML) and ME-1 (of adult origin) cell lines in this project were similar to those in the validated Ara-C resistant MV4-11 cell line (92). However, another model of Ara-C resistance in HL60 was generated by constant exposure to the same concentration of Ara-C (93). The method of intermittent, escalating Ara-C exposure to generate resistant cell lines over constant Ara-C exposure was chosen to mimic cycles of chemotherapy received by patients in clinic.

5.2 Apoptotic priming of parental and Ara-C resistant CBF cell lines

Through the use of baseline BH3 profiling, it was shown that the parental Kasumi-1 cell line was more primed for apoptosis than ME-1 (**Figure 4.3.1)**. This may be due to the origins of these cell lines, as apoptotic priming has been shown to be higher in pAML than adult AML, as measured by BH3 profiling (94). This correlated to the sensitivity of these cell lines to treatment with Ara-C, as ME-1 had a higher EC_{50} Ara-C concentration than Kasumi-1 **(Table 4.1.1)**. A previous study showed that BIM, as a BH3 profiling biomarker, and patient age had the potential to predict patient response to Ara-C when stratified by cytogenetic risk (81). The relationship between response to Ara-C and apoptotic priming demonstrated here supports the potential use of baseline BH3 profiling with BIM as an *in vitro* tool to predict response to Ara-C.

ME-1R cells were shown to be more primed for apoptosis than parental ME-1 cells **(Figure 4.4.1.A)**. It has previously been reported that treatment with chemotherapy is most effective at inducing apoptosis in cells that are the most

primed for apoptosis and selects for survival of a population of cells that are poorly primed for apoptosis (95). This has been suggested to be a cause of relapse and resistance to chemotherapy (96). While the results seen in Kasumi-1R cells seem to support this finding (resistance to conventional chemotherapy comes from reduced apoptotic priming), ME-1R was surprisingly more primed for apoptosis than the ME-1 parental line **(Figure 4.4.1.B).** However, one study showed that a loss of mitochondrial priming was a consistent mechanism of resistance to a range of targeted therapies in AML (97), which could explain the reduced apoptotic priming of Kasumi-1R compared to parental Kasumi-1. Additionally, the acquisition of somatic mutations in response to treatment has also been identified as a mechanism of drug resistance in AML (98). In fact, Ara-C has been shown to have a mutagenic effect by induction of C>G transversions, the incidence of which are higher at relapse (99). While the mechanism of Ara-C resistance cannot be determined for certain from these results, the variance of the effect of acquired Ara-C resistance on apoptotic priming highlights an avenue for further work. The results presented here highlight the heterogeneity and complexity of resistance mechanisms in AML.

5.3 Expression of anti-apoptotic proteins in parental and Ara-C resistant CBF cell lines

In this project, although not statistically significant, the expression of BCL-2 and BCL-xL was numerically higher in the Ara-C resistant cell lines compared to the parental lines of ME-1 and Kasumi-1 **(Figure 4.6.1)**. The association between overexpression of BCL-2 and poor prognosis and response to chemotherapy has been well-documented (40,100). Similarly, increased expression of BCL-xL has been associated with poor response to chemotherapy, poor prognosis and resistance to apoptosis (101). The increase in BCL-2 expression in the Ara-C resistant models of ME-1 and Kasumi-1 cells observed in this study could indicate that long-term treatment with Ara-C selects for cells which upregulate pro-survival BCL-2 family members to mediate Ara-C resistance.

In both Kasumi-1 and ME-1 cell lines, there was a significant increase in MCL-1 expression in the Ara-C resistant models compared to the parental cell lines **(Figure 4.6.1)**. This is consistent with a previous study which established an Ara-C-resistance *FLT3*-ITD-positive cell line, in which, the expression of MCL-1 was

upregulated (92). Previous studies have shown that overexpression of MCL-1 is associated with poor prognosis in AML and resistance to cytarabine (42,102). Additionally, in matched diagnostic and relapse AML samples, an increase of MCL-1 expression has been identified at relapse (44). The results presented in this project suggest that therapeutic targeting of MCL-1 may induce apoptosis in Kasumi-1R and ME-1R cell lines. Overall, these results indicate that the Ara-C resistant cell lines may be an appropriate model of relapse for *in vitro* study of anti-apoptotic proteins, as MCL-1 overexpression is present in these models, as in relapse AML samples.

Targeting of MCL-1 for the treatment of AML has been investigated through the use of BH3 mimetics. As discussed previously **(Section 1.4.4)**, S63845 is the parent compound of S64315 which is under clinical investigation for use in haematopoietic cancers (69–71). However, S64315 has shown dose-limiting toxicities in clinical trials, particularly in patients with AML or myelodysplastic syndromes (70). Cardiotoxicity has also been present among other MCL-1 inhibitors which have been developed and investigated (103). Indirect inhibition of MCL-1 have been explored through the use of CDK9 inhibitors, such as AZD4573, which transcriptionally regulate MCL-1 (104). AZD4573 has shown promising results in haematological cancer models and has been investigated in a phase I clinical trial in relapsed/refractory haematological malignancies, however results of this study have not yet been published (105,106). This alternate way of targeting MCL-1 via indirect inhibition may provide a solution to the cardiotoxicity demonstrated with MCL-1 inhibitors in clinical trials thus far.

5.4 Effect of acquired Ara-C resistance on response to BH3 mimetics in CBF AML

While it is clear that the response of the cell lines to BH3 mimetics were affected by their acquired resistance to Ara-C, the changes in response differed between the BH3 mimetics. A previous study showed that patients with increased expression of MCL-1 and BCL-xL were less sensitive to treatment with venetoclax (59). While in this study, the expression of BCL-2, MCL-1 and BCL-xL were increased in the ME-1R, the response to venetoclax was increased in this cell line **(Figure 4.7.2)**. The significant increase in response to venetoclax seen in ME-1R compared to parental ME-1 presents evidence towards the ‘one-two

punch' model of BH3 mimetics use which has been presented **(Figure 1.4.2)** (52). The acquisition of Ara-C resistance by ME-1 cells and the subsequent dependence on anti-apoptotic protein expression may be responsible for this increase in susceptibility to venetoclax treatment. However, further work is needed to assess if the increase in response to venetoclax is greater than the response of parental ME-1 cells to a combination of Ara-C and venetoclax to determine the most efficacious sequence of treatment.

While statistical analyses could not be performed for Kasumi-1, the response to BH3 mimetics in the Ara-C resistant model for this cell line seemed to decrease compared to the parental cell line **(Figure 4.7.1)**. This difference in response to BH3 mimetics between Ara-C resistant Kasumi-1 and ME-1 cells and their parental counterparts could be due to the origins of these cell lines. ME-1 is an adult CBF AML cell line, while Kasumi-1 is a paediatric CBF AML cell line (82,83). It has been shown previously that pAML and adult AML are distinct diseases with key differences in molecular profiles (8). Adult patients with AML have a higher degree of somatic mutations than pAML patients, which is a process linked to ageing (108,109). It has been suggested that, in adult AML, pre-existing haematopoietic cells harbouring leukaemic somatic mutations may be present, which over time accumulate further somatic mutations which ultimately lead to development of leukaemia (110). This difference between adult and paediatric leukaemias could play a pivotal role in the response to treatment demonstrated here, as acquisition of somatic mutations is associated with decreased response to treatment (111).

Ara-C resistance in ME-1 cells caused a significant increase in response to venetoclax compared to parental ME-1 cells, while in Kasumi-1 cells Ara-C resistance seemed to decrease sensitivity of the cell line to venetoclax compared to parental Kasumi-1 cells. This difference in response to venetoclax could be due to the different translocations in these cell lines. ME-1 carries an inv(16) translocation, while Kasumi-1 is a t(8;21) cell line (82,83). A previous, retrospective study investigating the use of venetoclax in combination with hypomethylation agents in CBF AML showed that patients with t(8;21) had poor response to this treatment combination, while venetoclax + hypomethylating agents showed promising results in patients with inv(16) AML (112). However, this study included a small number of patients who were ineligible for intensive

therapies and therefore had not been pre-treated with chemotherapy (112). Further work is needed to determine if there is a difference in the efficacy of venetoclax between patients with inv(16) and t(8;21) AML that are resistant to chemotherapy.

Although the development of Ara-C has had different impacts on the response to BH3 mimetics in this project, conclusions cannot be drawn from these data due to the lack of triplicate repeats in the experiments with Kasumi-1 cells.

5.5 Use of dynamic BH3 profiling to predict response to BH3 mimetics

In this project, the use of dynamic BH3 profiling captured the pattern of response to BH3 mimetics in both Kasumi-1 and Kasumi-1R **(Figure 4.8.1)**. This supports previous work which has shown that dynamic BH3 profiling can predict response in patient-derived xenografts and human patients (97). Despite the labelling of CBF AML as a favourable prognostic subgroup, there is still a high risk of relapse in both adult and pAML suggesting that the use of other risk stratification is needed. Previous studies have shown that the stratification of patients by their mitochondrial priming phenotype may provide a refinement for risk assessment within AML (94). Most recently, refined risk stratification has been shown to improve prognostication for patients following venetoclax-based therapies (113). This highlights the importance of consistent updates to risk stratification and the need for more tools to achieve this.

Although, as discussed **(Section 4.8)**, the output of cytochrome c release measured by dynamic BH3 profiling did not exactly match that of cell death measured by apoptosis assay. It has been discussed that any stratification technique used in AML is unlikely to be perfect due to the heterogeneity of the disease, patient-specific factors and treatment-related differences (114). Therefore, the results presented here suggest a role for dynamic BH3 profiling for the stratification of patients with paediatric t(8;21) AML for BH3 mimetic treatment.

5.6 Limitations

After generation of the Ara-C resistant cell line models used in this project, no experiments were performed to ascertain how resistant these cell lines were to subsequent treatments with Ara-C. The rationale behind this was that even if cell death occurred in response to Ara-C after the chosen method had been completed for the cell line, changes to apoptotic priming and other molecular or genetic changes would have already occurred. Despite this, the lack of data to determine how resistant the generated cell lines were to subsequent treatments with Ara-C is a major limitation of this study and represents an avenue for further work. Another limitation of this study is the absence of genetic testing to confirm that a CBF translocation was still present in the parental and resistant cell lines.

A limitation of the results in Section 4.4 is that there was a high degree of variance between triplicate repeats when performing intracellular staining for the anti-apoptotic proteins of interest. This may account for the lack of statistical significance of the difference in expression of BCL-2 and BCL-xL between parental and Ara-C resistant cell line models. Further repeats of this experiment were required to ensure that the method used for intracellular staining generated repeatable, robust results. However, due to time constraints, repeats of this experiment were not possible and represent an opportunity for further investigation.

The concentrations of BH3 mimetics used in this project were chosen to produce a snapshot of the response to BH3 mimetics in parental and Ara-C resistant cell lines, using IC_{50} concentrations of the selected drugs in these cell lines to elicit some response in each cell line (91). However, further work using concentrations of BH3 mimetics that are physiologically relevant to paediatric and adult AML would be needed to confirm the results.

Some results presented here did not have three biological replicates (n=3), due to time constraints surrounding this project. This affects the interpretation of these results and further work would be needed to increase the number of biological replicates for each experiment. This would increase the statistical power of results and the robustness of conclusions drawn.

5.7 Future work

Further work is needed to assess the validity of the Ara-C resistant cell lines generated in this project. To do this, apoptosis assays could be utilised to compare the EC_{50} concentration of Ara-C in parental and the Ara-C resistant cell line models. If the Ara-C resistant models generated in this project are truly resistant to Ara-C, it would be expected that the EC_{50} concentrations of Ara-C would be significantly higher in the Ara-C resistant cell lines compared to the parental lines. The experiments performed in this project should be repeated across different time points after Ara-C resistance has been acquired to establish if resistance to Ara-C is maintained and for how long, with the aim to optimise the experimental use of these cell models.

The resazurin assay was not successful to determine the EC_{50} concentration of Ara-C in this project. Due to time constraints, it was not possible to troubleshoot these experiments. However, future work could utilise ATP-based assays, such as CellTiter-Glo®, to assess the viability of cells and subsequently calculate EC_{50} concentrations of Ara-C for cell lines.

Results of this project have shown that the mechanism of Ara-C resistance in the generated cell line models is unknown. To assess the genetic changes which may be contributing to Ara-C resistance, the use of whole exome sequencing would be appropriate to highlight any polymorphisms present in the Ara-C resistant lines which are not present in the parental cell lines. In addition, the use of bulk RNA sequencing would be beneficial to determine which genes are differentially expressed in the Ara-C resistant lines compared to the parental cell lines.

Intracellular staining of anti-apoptotic proteins (BCL-2, MCL-1 and BCL-xL) was performed in this project and showed that expression of MCL-1 was significantly higher in both Ara-C resistant cell lines compared to parental cell lines **(Figure 4.6.1)**. To put these data into a wider context future work could include intracellular staining of other pro- and anti-apoptotic proteins. Intrinsic apoptosis is controlled by a fine balance between these protein classes (36), so inclusion of a wider range of proteins would provide a better understanding of

changes to the apoptotic pathway as a whole. These data could also be complemented by Western blot analysis.

The results shown here demonstrate potential for the use of dynamic BH3 for investigation of primary AML samples. Matched patient samples taken at diagnosis, post-treatment, and relapse could be used to determine changes in response to BH3 mimetics. This would provide evidence to determine when patients are most likely to respond to treatment with BH3 mimetics and if pre-treatment with chemotherapy, such as Ara-C, is increases the sensitivity of patients to BH3 mimetics.

5.8 Conclusions

Overall, the results of this thesis show that the acquisition of Ara-C resistance has a different impact on the apoptotic priming between paediatric t(8;21) Kasumi-1 and adult inv(16) ME-1 AML cell lines. The expression of the apoptotic protein MCL-1 was significantly increased in the Ara-C resistant cell lines compared to parental cell lines, confirming the potential of MCL-1 inhibition for the treatment of AML. Response to venetoclax was significantly increased in ME-1R compared to parental ME-1 cells, which suggests that initial treatment with Ara-C prior to treatment with venetoclax could increase response overall. However, further work is needed to ascertain the most effective sequence of treatment with Ara-C and other BH3 mimetics. This project adds to previous evidence which suggests that the use of dynamic BH3 profiling could be a valuable tool for the prediction of response to BH3 mimetics, which could in turn aid patient stratification and improve responses.

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