The complex regulatory mechanisms of the BCL-2 family of proteins in cancer

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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April 2019

DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

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This research was carried out in the Department of Molecular and Clinical Cancer Medicine, University of Liverpool, UK.

Contents

Acknowledgements

My thanks go out to everyone who has supported me along the treacherous path of a PhD. Of course, a special acknowledgement must go to Shankar Varadarajan, who, despite sometimes driving me up the wall, has been an incredible supervisor who gave me amazing opportunities, pushed me to develop an enormous range of skills, helped me publish papers and also made me laugh for the most outrageous reasons. I'm also greatly appreciative of the Varadarajan team who have provided endless support, both scientific and personal. Michael, Gerry, Rachel, Aoula, Govinda, Ahoud – thank-you! A special thank-you to Mateus Milani – you probably have no idea how much you've helped me along the way – thank-you for always being there for me, be it with the flow cytometer that was perpetually misbehaving or when I needed to have a good whinge.

A further enormous thank-you to North West Cancer Research for funding my project and also providing me with opportunities to share my research with the scientific and lay community. Additionally, I would like to thank the many members of the Cancer Medicine and Pharmacology departments who have provided facility use, support, and advice throughout my PhD.

I would also like to thank all my fellow PhD students and peers who provided me with incredible friendships, support and advice. Alex, Gurpreet, Sammie, Lucy, Mei, Lawrence – thank-you for your love! A further special acknowledgement to Lorna Bryant who will not stop following me around the country – you, Pez and Bonelli mean more to me than I can describe, thank-you for always being there for me. I would also like to extend my thanks to all my other friends for their support, especially my lovely online community!

Finally, I must of course thank my family, especially my parents for always driving me to pursue my scientific passion and cheering me on along the way, my grandmas for always being sweet and supporting and both of my late grandads who would always find scientific articles in the newspaper to talk to me about. A final thank-you to my brother, Eddy and our strange cat, Spike, who gave me lots of love whenever I was down. I would never have made the decisions that have led me to this point without any of you and I cannot thank you enough!

Publications

- **Greaves, G.,** Milani, M., Butterworth, M., Carter, R., Byrne, D. P., Eyers, P. A., Luo, X., Cohen, G. M, and Varadarajan, S. 'BH3-only proteins are dispensable for apoptosis induced by pharmacological inhibition of both MCL-1 and BCL-X_L.' Cell Death and Differentiation, September 2018. doi: 10.1038/s41418-018-0183-7

- Milani, M., Byrne, D. P., **Greaves, G.**, Butterworth, M., Cohen, G. M., Eyers, P. A., and Varadarajan, S. 'DRP-1 is required for BH3 mimetic-mediated mitochondrial fragmentation and apoptosis.' Cell Death and Disease, January 2017. doi:10.1038/cddis.2016.485

- Al-Zebeeby, A., Vogler, M., Milani, M., Richards, C., Alotibi, A., **Greaves, G.**, Dyer, M. J. S., Cohen, G. M., and Varadarajan, S. 'Targeting intermediary metabolism enhances the efficacy of BH3 mimetic therapy in haematological malignancies.' Haematologica, November 2018. doi: 10.3324/haematol.2018.204701.

- Carter, R., Butterworth, M., Schache A. G., Milani, M., **Greaves, G**., Alotibi, A. Al-Zebeeby, A., Jorgensen, A., Risk, J. M., Sacco, J. J., Shaw, R. J., Jones, T. M., Cohen, G. M., Varadarajan, S. 'Inhibition of MCL-1 and BCL-X_L as a potential therapy for patients with head and neck cancer.' Manuscript in preparation.

Abstract

Resistance to apoptosis is a key hallmark of most cancers. The BCL-2 family of proteins are the major arbiters of the cell death program and the interactions between pro- and anti-apoptotic members of this family are believed to shift the fate of a cell towards life or death. The anti-apoptotic BCL-2 family proteins (BCL-2, BCL-X_L, MCL-1, BCL-w and BCL-2A1) are often overexpressed in cancer, thereby promoting abnormal cell survival. As a result, these proteins are highly attractive targets for novel chemotherapeutic approaches. The recent clinical success of inhibitors against BCL-2 (Navitoclax and Venetoclax) has launched the compounds known as BH3 mimetics into the chemotherapeutic spotlight and has led to the successful development of several highly potent MCL-1 inhibitors. The targeting of MCL-1 in cancer is critical to restoring sensitivity to other chemotherapeutic approaches, as MCL-1 is strongly associated with abnormal cell survival and chemoresistance in many cancers.

The aims of this study were to (1) assess the specificity and potency of the novel MCL-1 inhibitor, S63845, as a single agent and in conjunction with other BH3 mimetics, to induce apoptosis in a range of cancer cell lines (2) characterise the dependence on BH3-only members for apoptosis induced by BH3 mimetics and (3) identify novel MCL-1 interacting partners which could explain BH3-only protein-independent apoptosis.

Using a panel of cancer cell lines with various dependencies on BCL-2 family members, it was shown that S63845 is an extremely potent MCL-1 inhibitor which can induce extensive apoptosis alone or in combination with other BH3 mimetics. Immunoprecipitation studies of the BCL-2 family during BH3 mimetic-induced apoptosis revealed that the interactions of a pro-survival protein with certain BH3-only proteins might not exclusively dictate sensitivity to certain BH3 mimetics. Moreover, extensive study of cells lacking the 8 key BH3-only proteins showed that BH3 mimetics can induce a BAX-dependent but BH3-only protein-independent apoptosis, going against the accepted dogma of BH3 mimetic action.

With the possibility of other BH3-only-like proteins existing to mediate the apoptosis seen in the absence of the 8 major BH3-only proteins, novel interacting partners of MCL-1 were explored by mass spectrometry. DRP-1, a mitochondrial fission regulator, was thus identified as an MCL-1-interacting protein. While knockdown of

DRP-1 could abrogate BH3 mimetic-induced apoptosis in H1299 cells, it did not interact with MCL-1 via a BH3 motif and exacerbated apoptosis in the cells lacking the BH3-only proteins. Thus, DRP-1, while confirmed as a regulator of apoptosis, was not proposed to function like a novel BH3-only protein which can activate BAX/BAK in the absence of the BH3-only proteins.

Overall this study reveals that S63845 is an important and highly effective MCL-1 inhibitor with strong potential for use in the clinic both as a monotherapy and in combination with other approaches. Moreover, the mechanism underlying BH3 mimetic-induced apoptosis and BAX activation has been revealed to be much more complicated than currently known, as it appears that the BH3-only proteins are dispensable for apoptosis following pro-survival protein neutralisation. Future studies should clarify how BAX can initiate MOMP without the BH3-only proteins, be it through activation of BAX by the outer mitochondrial membrane directly or, perhaps, the existence of novel BH3-only-like proteins which can activate BAX independently of the 8 key BH3-only proteins.

Abbreviations

8KO	Octa-knockout
AA	Amino acid(s)
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor 1
API	Annexin/PI
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATPB	ATP synthase subunit beta
AURKA	Aurora kinase A
BAD	BCL-2-associated death promoter
BAK	BCL-2-homologous antagonist killer
BAP31	B cell receptor associated protein 31
BAX	BCL-2 associated X
BC	Bead control
BCL-2	B cell lymphoma 2
BCL-2A1	BCL-2 related protein A1
BCL-X_L	B-cell lymphoma-extra large
BFL-1	BCL-2 related gene expressed in foetal liver
BH	BCL-2 homology
BID	BH3 interacting-domain death agonist
BIK	BCL-2 interacting killer
BMF	BCL-2 modifying factor
BSA	Bovine serum albumin
C	Centigrade
$CaCl_2$	Calcium Chloride
CAP	Catabolite activator protein
CDK1	Cyclin-dependent kinase 1
cDNA	Copy DNA
CHAPS	3-((3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate
CLL	Chronic lymphocytic leukaemia
CO_2	Carbon dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
CST	Cell Signalling Technology
C-terminal	Carboxy terminal
DAD1	Defender against apoptotic cell death 1
dH_2O	Distilled water
DISC	Death-inducing signalling complex
DKO	Double knockout
DMEM	Dulbecco's Modified Eagle medium
DMP	Dimethyl pimelimidate
DMSO	Dimethyl sulphoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRP-1	Dynamin related protein 1
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid
ER	Endoplasmic reticulum
ExPASy	Expert Protein Analysis System
FACS	Fluorescence-activated cell sorting
FADD	FAS-associated death domain
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FDA	Food and Drug Administration
FDR	False detection rate
Fig	Figure
FPLC	Fast protein liquid chromatography
FT	Flow-through
g	Grams
g	G force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hours
HCL	Hydrogen chloride
HDACi	Histone deacetylase inhibitor
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HMW	High molecular weight
HRK	Harakiri
HRP	Horseradish peroxidase
HSCs	Haemapoietic stem cells
HSP60	Heat shock protein 60
IAP	Inhibitor of apoptosis
IC_{50}	Half maximal inhibitory concentration
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IP	Immunoprecipitation
IRF1	Interferon Regulatory Factor 1
KCL	Potassium Chloride
kDa	Kilodalton
KO	Knockout
KOH	Potassium hydroxide

L	Litre
LMW	Low molecular weight
M	Molar
MCL-1	Myeloid cell leukemia 1
MDa	Megadalton
MEF	Mouse Embryonic Fibroblast
MEK	MAPK/ERK Kinase
METTL3	Methyltransferase Like 3
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minutes
mL	Millilitres
mM	Millimolar
MMW	Medium molecular weight
MOMP	Mitochondrial outer membrane permeabilisation
mRNA	messenger RNA
N	Number of replicates
NaCl	Sodium chloride
ng	Nanograms
nM	Nanomolar
nm	Nanometre
NMR	Nuclear magnetic resonance
NMT1	N-myristoyltransferase 1
NSCLC	Non-small cell lung cancer
N-terminal	Amino terminal
OD600	Optical density at 600 nanometres
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
p53	Tumour protein 53
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PEST	Proline (P), glutamic acid (E), serine (S), and threonine (T)
PFA	Paraformaldehyde
pH	Potential hydrogen
PHENGLAND	Public Health England
PI	Propidium iodide
PI-3K	Phosphoinositide 3-kinase
PMA	Phorbol 12 β-myristate 13 α-acetate
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
PUMA	p53 upregulated modulator of apoptosis

qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAi	RNA interference
RPM	Rotations per minute
RPMI	Roswell Park Memorial Institute
SAHBs	Stabilized Alpha-Helices of BCL-2 domains
SC	Santa Cruz
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	Seconds
SEM	Standard error of the mean
siRNA	Short interfering RNA
SMAC	Secondary mitochondrial activator of caspases
STAT3	Signal transducer and activator of transcription 3
STR	Short tandem repeat
SUFU	Suppressor of fused homologue
tBID	Truncated BID
TBS-T	Tris-buffered saline with Tween-20
TEA	Triethanolamine
TEB	Trehalose Experimental Buffer
TEMED	Tetramethylethylenediamine
TFEB	Transcription factor EB
TM	Transmembrane
TMRE	Tetramethylrhodamine ethyl ester
TNF	Tumour necrosis factor
UTR	Untranslated region
UV	Ultraviolet
V	Voltage
V600E	Valine to glutamic acid substitution at amino acid 600
VDAC2	Voltage-dependent anion channel 2
VLCAD	Very long-chain acyl-CoA dehydrogenase
WT	Wild-type
Z-VAD-FMK	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone
γ-H2AX	γ-H2A histone family member X
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar

List of figures

Chapter 1

Chapter 3

Chapter 4

4.1. HCT116 8KO cells do not appear to express the eight key BH3-only proteins.

4.2. Knockdown of pro-survival proteins can induce apoptosis in a BAX- and BAK-dependent but BH3-only protein-independent manner.

4.3. HCT116 WT and 8KO cells are equally primed for death following suppression of both MCL-1 and BCL-X_L but not BCL-X_L alone.

4.4. Pharmacological inhibition of BCL-X_L but not MCL-1 can induce apoptosis in a BH3-only protein-dependent manner.

4.5. Pharmacological suppression of BCL-X_L but not MCL-1 is sufficient to induce apoptosis in some cell lines at extended timepoints.

4.6. BH3 mimetics can induce apoptosis in a BH3-only protein-independent manner.

4.7. A combination of S63845 and A-1331852 induces BAX-dependent but BAK-independent death in HCT116 cells.

4.8. BAX and BAK activation are not detectable in HCT116 cells due to constant activation in all conditions

4.9. Pharmacological inhibition of MCL-1 and BCL-X_L can induce BH3-only protein-independent BAX translocation to the mitochondria.

4.10. BAX undergoes oligomerisation following S63845 and A-1331852 treatment independently of the BH3-only proteins.

4.11. Cytochrome *c* can be released by pharmacological inhibition of MCL-1 and BCL-X_L independently of BH3-only proteins

4.12. Pro-survival proteins do not interact directly with BAX in HCT116 cells to prevent its pro-apoptotic activity.

4.13 Apoptosis in HCT116 8KO cells may occur through non-conventional routes, perhaps involving an unknown BH3-only protein that activates BAX.

Chapter 5

5.1. MCL-1 exists in three major complexes and a monomeric form in MCL-1-addicted cell lines.

5.2. MCL-1 isoforms are detectable in all of the major subcellular compartments of HELA cells.

5.3. The presence of MCL-1 complexes varies in different subcellular compartments of HELA cells.

Chapter 1

General Introduction

Contents

1.1 Overview of apoptosis

Apoptosis is one of the major forms of cell death and is a highly orchestrated, genetically programmed process which continuously occurs from early development in many multicellular organisms. In one day, in a single human being, it is estimated that around 10 billion cells are made to replace those that have undergone apoptosis[1], indicative of the widespread and essential nature of the process to homeostasis. The term apoptosis comes from the Ancient Greek term 'ἀπόπτωσις' – which translates to 'separation' and 'falling off'[2] and was used because characteristics of an apoptotic cell resembled leaves falling away from a tree. While the concept of a programmed cell death was first remarked in a study of amphibian metamorphosis in 1842 by Carl Vogt[3], the term 'apoptosis' was not coined until 1972, when John Kerr distinguished apoptotic death from accidental cellular death and subsequently published a study with Alastair R. Currie and Andrew Wyllie, describing the key morphological stages of 'cell deletion', including neat condensation of the nucleus and cytoplasm and uptake of dying cells by other cells[4]. Almost 50 years later, the apoptotic process is considerably better understood and many new categories of programmed cell death have been described, however extensive research must still be done to fully comprehend the intricacies of apoptosis.

1.2 Apoptosis induced by a variety of stimuli is central to both physiological and pathological processes.

Apoptosis occurs at many developmental stages of an organism and continues in the adult, where it is central to homeostasis. The sculpting of organs is one of the best examples of the fundamental importance of apoptosis in development – demonstrated especially elegantly by digit formation, where cells undergo programmed cell death to allow distinct digits to form[5]. In adult organisms, apoptosis continues to play essential

roles throughout the body by maintaining cell numbers and removing aging, unwanted or dangerous cells. One well-studied example of apoptosis in the adult organism occurs in regulation of immune cell development. Lymphocytes undergo multiple differentiation steps and generate unique antigen receptors - integral units of the immune arsenal - in a stochastic manner. Consequently, some of the generated antigen receptors will recognise host antigens and the clones expressing these receptors must be removed by apoptosis to prevent autoimmunity[6]. With apoptosis being so pivotal to many processes, it comes as no surprise that signals to induce apoptosis exist in vast numbers and in many forms, including external signals such as death ligand-receptor binding[7] and internal signals, such as excessive DNA damage[8], aberrations in the cell cycle[9] and endoplasmic reticulum (ER) stress[10]. Apoptosis can also occur as a result of a lack of signalling, such as deprivation of growth factors[11–13]. Minute changes in any one of these environmental or internal factors can be enough to alter the life-death balance, therefore it is essential that there are intricate mechanisms in place to regulate apoptosis and its activation.

1.3 Apoptosis and disease

As previously mentioned, a programmed cell death system allows for a precise balance of cells within an organism. If the balance of apoptosis is altered within a tissue, disease is likely to ensue. In the case of excessive apoptosis, neurodegenerative disorders (such as Alzheimer's disease) can occur as a result of massive death of neuronal cells[14]. Excess apoptosis (in combination with necrosis) has also been described in cardiomyocytes during ischemia/reperfusion injury following myocardial infarction[15] as well as in pancreatic β-islets in diabetes[16]. In contrast, failure of cells to undergo apoptosis is also believed to be the driving factor of multiple diseases, including autoimmune diseases where the body generates antibodies against its own

tissues, leading to their destruction. The generation of auto-antibodies is linked to failure of their associated lymphocytes to undergo apoptosis during their development, leading to dissemination of auto-reactive antigen receptor lymphocytes throughout the body and subsequent auto-immune response[17]. Perhaps one of the most striking diseases where the lack of apoptosis plays a central role is cancer. Abnormal cell survival is, in fact, one of the original hallmarks of cancer as defined by Hanahan and Weinberg[18]. Cancers arise when cells accumulate mutations in their DNA which permit them to grow and divide uncontrollably. In most circumstances, following detection of cellular abnormalities (especially DNA damage), various signalling pathways are initiated, often converging on the key tumour suppressor p53, to determine cell fate[19,20]. In a simple model, if the damaged DNA can be repaired, p53 arrests the cell cycle until everything is repaired and the cell no longer poses a threat. However, when a cell accumulates too much DNA damage and may harbour potentially dangerous mutations, p53 no longer tries to instigate repair of the cell and instead, promotes transcription of proteins which will promote cell death (reviewed in [21]). The pivotal role of p53 in restraining abnormal growth and sending potentially cancerous cells to their death means that p53 is one of the most commonly mutated genes across all cancers (reviewed in [22]). In the case of cancer cells, the loss of tumour suppressors like p53 results therefore results in excessive proliferation. However, simply having the capacity to wildly grow is not enough to allow a tumour to form, therefore the apoptotic machinery must somehow be altered to resist cell death. As the programmed cell death field has grown, it has become evident that resistance to apoptosis is essential to oncogenesis. In the case of the cancer cells which grow out to become tumours, these cells resist death, often by acquiring mutations which can either increase the levels of proteins which promote cell survival or decrease those

which promote apoptosis[18]. It is interesting to note that, despite cancer cells often being described as being more resistant to apoptosis than other cells, they are relatively 'primed' for apoptosis induction, as remarked following the development of the BH3 profiling technique. BH3 profiling uses synthetic peptides resembling the BH3 domains of pro-apoptotic proteins to determine how 'ready' or 'primed' a cell is to undergo apoptosis[23]. This technique can be used to predict the response of cancer cells to chemotherapeutic responses[24]. Surprisingly, in addition to upregulating pro-survival proteins, cancer cells are so genetically unstable that they often also upregulate pro-apoptotic proteins, leaving them 'primed' on a knife-edge of life or death. Moreover, cells of many healthy tissues are relatively un-primed as they tend to express low levels of pro-apoptotic proteins and could thus be considered resistant[24]. Thus, while cancer cells do indeed evade apoptosis, their inherent genetically instability places them in a precarious position which can be targeted using therapies which target arms of the apoptotic machinery, as discussed later.

Overall, it is evident that alterations to apoptosis induction are central to multiple diseases, therefore understanding the process and developing potential therapeutics to target the arms of the apoptotic pathway is essential.

1.4 Hallmarks of apoptosis

The process of apoptosis is well-organised and causes minimal interruption of surrounding cells or tissue by limiting any inflammatory response. This well-structured process means that apoptotic cells have a very defined morphology which was originally outlined in the first paper describing apoptosis[4]. It was shown that during the onset of apoptosis, a cell will shrink and round-up as the cytoplasm and chromatin condense. Furthermore, the nuclear envelope disintegrates and chromatin

bodies form from the condensed chromatin. Towards the later stages of apoptosis, the cell becomes fragmented, leaving apoptotic bodies for immune cells to recognise, engulf and safely dispose of to prevent inflammation[4,25]. These morphological changes are a result of distinct biochemical changes within the cell[4]. These major biochemical changes are facilitated by the activation/inactivation of numerous signalling proteins which result in activation of a specific family of enzymes called the caspases. The caspase family of proteins are the executioners of apoptosis which cleave specific targets within the cell to ultimately lead to its demise. Apoptotic caspases exist as inactive zymogens under non-apoptotic conditions and are typically split into two major groups – apical (or initiator) caspases and effector caspases. Apical caspases involved in apoptosis include caspases-2, -8, 9 and 10 while effector caspases are -3, -6 and -7. Caspases -1, -4 and -5, while associated with cell death, are often designated as non-apoptotic caspases as they are linked to the inflammatory response and subsequent pyroptosis, a form of cell death which is typical following intracellular infection (reviewed in [26] and [27]). The distinct morphology of an apoptotic cell is a result of the caspases targeting specific proteins and structures within the cell. Examples include focal adhesion kinase (FAK), which regulates interactions of a cell to the extracellular matrix via integrins. FAK is cleaved by caspases, leading to loss of its activity, detachment of cells from the extracellular matrix and the rounded-up morphology of apoptotic cells[28]. Other proteins that are targeted during apoptotic signalling include core members of the cytoskeleton, such as actin, which is cleaved at the cell surface - this likely contributes to the shrinking and rounded up morphology of the apoptotic cell[29], as well as proteins which maintain nuclear structure, such as lamins[30], leading to the organised breakdown of the nucleus. Poly ADP ribose polymerase (PARP), a DNA repair enzyme[31], is another key target of caspases.

Cleavage of PARP abrogates its ability to detect and label sites of DNA damage, meaning DNA can no longer be repaired effectively, thereby further committing the cell to apoptosis[32,33]. Cleavage of PARP has also been proposed to maintain cellular ATP levels during apoptosis, as PARP activity requires extensive use of ATP. Blocking PARP activity therefore aids the energy-dependent apoptotic process and allows a cell to avoid necrosis as a result of ATP depletion[34]. In addition to cleaving targets to downregulate their activity, caspases can also cleave proteins to activate them. This includes other caspases within the family as well as enzymes such as scramblase, which induces the irreversible exposure of the phospholipid phosphatidylserine on the outer leaflet of the plasma membrane, which serves as an 'eat me' signal for immune cells[35].

These targets of the apoptotic signalling pathway are just a few of the many proteins which are altered or degraded to induce a programmed cell death, allowing for an inflammation-free and consistent cell death.

1.5 Major pathways of apoptosis.

Programmed cell death can occur via two major pathways, namely the intrinsic and extrinsic pathways, with the pathway taken depending upon the apoptotic stimulus. The major steps of each pathway are summarised in Figure 1.1. While the extrinsic pathway occurs at the level of the cell surface, is activated by external factors and typically involves engagement of transmembrane 'death receptors' by their respective ligands, the intrinsic pathway of apoptosis detects issues within the cell and delivers a response as a result of changes to mitochondrial integrity. Typically, when we think of the mitochondria, we consider them as the powerhouse of the cell, providing vast amounts of energy through oxidative phosphorylation in order to maintain normal

cellular metabolism. However, they are also critical to cell death, particularly in the case of apoptosis, and could even be considered as the gatekeepers of life or death within the cell[36].

The intrinsic apoptotic pathway

In order to understand how the mitochondria plays a role in apoptosis and therefore understand the intrinsic apoptotic pathway, it is first necessary to understand mitochondrial structure. Mitochondria are double membraned organelles, with the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM), the matrix and the cristae being the major features[37]. Each of these features play their own important roles in cell death regulation, withholding multiple apoptotic promoters from the rest of the cell until it is forced to undergo apoptosis. The key protein of the mitochondria which activates the cell death pathway is cytochrome *c*[38]. Cytochrome *c* normally functions as part of the electron transport chain, acting to transfer electrons at the junction of complexes III and IV[39]. However, it is also a life or death switch. Cytochrome *c* exists in the intermembrane space (between the OMM and IMM) and is believed to be sequestered by the folds of the IMM, known as the cristae (as shown in Figure 1.1), by proteins such as OPA1 (optic atrophy 1). OPA1 is believed to act like the drawstring of a bag, withholding cytochrome *c* within the cristae folds to prevent it from escaping the mitochondria. During apoptosis, mitochondrial membrane integrity is disrupted and OPA1 is proteolysed, leading to re-modelling of the cristae and cytochrome *c* release from the IMM folds[40]. Subsequently, for the newly released cytochrome *c* to escape the mitochondria, members of the BCL-2 family (as discussed in detail later) form pores in the outer mitochondrial membrane[41]. Cytochrome *c* thus escapes through the BAX/BAK pores in the outer mitochondrial membrane[42,43] into the cytosol, where it

performs the apical steps of the post-mitochondrial apoptotic signalling pathway. This process is thought to be the terminal step in the life or death decision process, as it is believed that release of cytochrome *c* is an irreversible process, although some studies have suggested otherwise, if caspase activity is blocked[44]. Once cytochrome *c* enters the cytosol, it binds to and activates apoptotic protease activating factor 1 (APAF1), inducing oligomerisation of APAF1 and subsequent recruitment and activation of the apical pro-caspase-9. This complex of proteins is often referred to as the apoptosome and activated caspase-9, at the peak of the caspase signalling network, can initiate downstream apoptotic signalling via activation of other caspases, such as caspase-3 and caspase-7[45–47]. Once activated, these enzymes begin to target structures and proteins within the cell to lead to its death in an organised manner as previously described[26,48,49]. In addition to cytochrome *c*, the mitochondria also sequester other pro-apoptotic factors, including SMAC (secondary mitochondrial activator of caspases), also known as DIABLO. SMAC is localised to the IMS and, like cytochrome *c*, is released to the cytosol during apoptosis. Once released, SMAC binds to inhibitors of apoptosis (IAPS), which are believed to bind to and prevent the activity of caspases. Release of SMAC inhibits IAP activity, thereby relieving the restrain on the caspases and bolstering the apoptotic signalling pathway[50,51]. Though both cytochrome *c* and SMAC are released from the mitochondria during apoptosis, they are very distinct in function and activation - knockout studies suggest that SMAC release occurs downstream of cytochrome *c* and requires caspase activity[52,53]. Thus, cytochrome *c* is the major apoptotic promoter withheld by the mitochondria that activates caspases, with SMAC existing to further fine-tune apoptotic signalling and caspases are the major effectors of the apoptotic program.

The extrinsic apoptotic pathway

The intrinsic pathway of apoptosis can convert a wide variety of internal stress stimuli into an appropriate apoptotic response. The extrinsic pathway, meanwhile, can do the same and shares of a number of elements with the intrinsic pathway, but the initial processes of the extrinsic pathway take place at the plasma membrane and involves an array of distinct proteins. Following engagement of a death ligand and its receptor, a death-inducing signalling complex (DISC) is recruited to the receptor. The DISC is made of up multiple proteins including FADD (FAS-associated death domain), an adaptor protein which forms the link between cell surface death receptors and downstream signalling elements, such as caspases[54,55]. Caspases-8 and -10 are required for the extrinsic pathway and are activated by the DISC complex following their homodimerisation[54,56]. Once activated, caspase-8 and caspase-10 can initiate extrinsic apoptotic signalling via two distinct pathways. The first involves caspase-8/-10-mediated activation of the executioner caspases (namely caspase-3) which go on to execute the demolition of the cell. In some cases, however, cells require the extrinsic pathway to converge on the intrinsic mitochondrial pathway. This occurs via cleavage of the pro-apoptotic BCL-2 family protein, BID, which is cleaved to truncated BID (tBID) by caspases-8[57] and -10[58]. The highly promiscuous tBID can then translocate to the mitochondrial membrane and disrupt interactions of the BCL-2 protein family, namely by binding to and activating BAK[59] and BAX[60], leading to MOMP, apoptosome formation and, eventually, activation of the executioner caspases[57]. Thus, the extrinsic pathway of apoptosis can function independently of the mitochondria by directly activating caspases or may converge on the intrinsic apoptotic when a more robust apoptotic signalling is required.

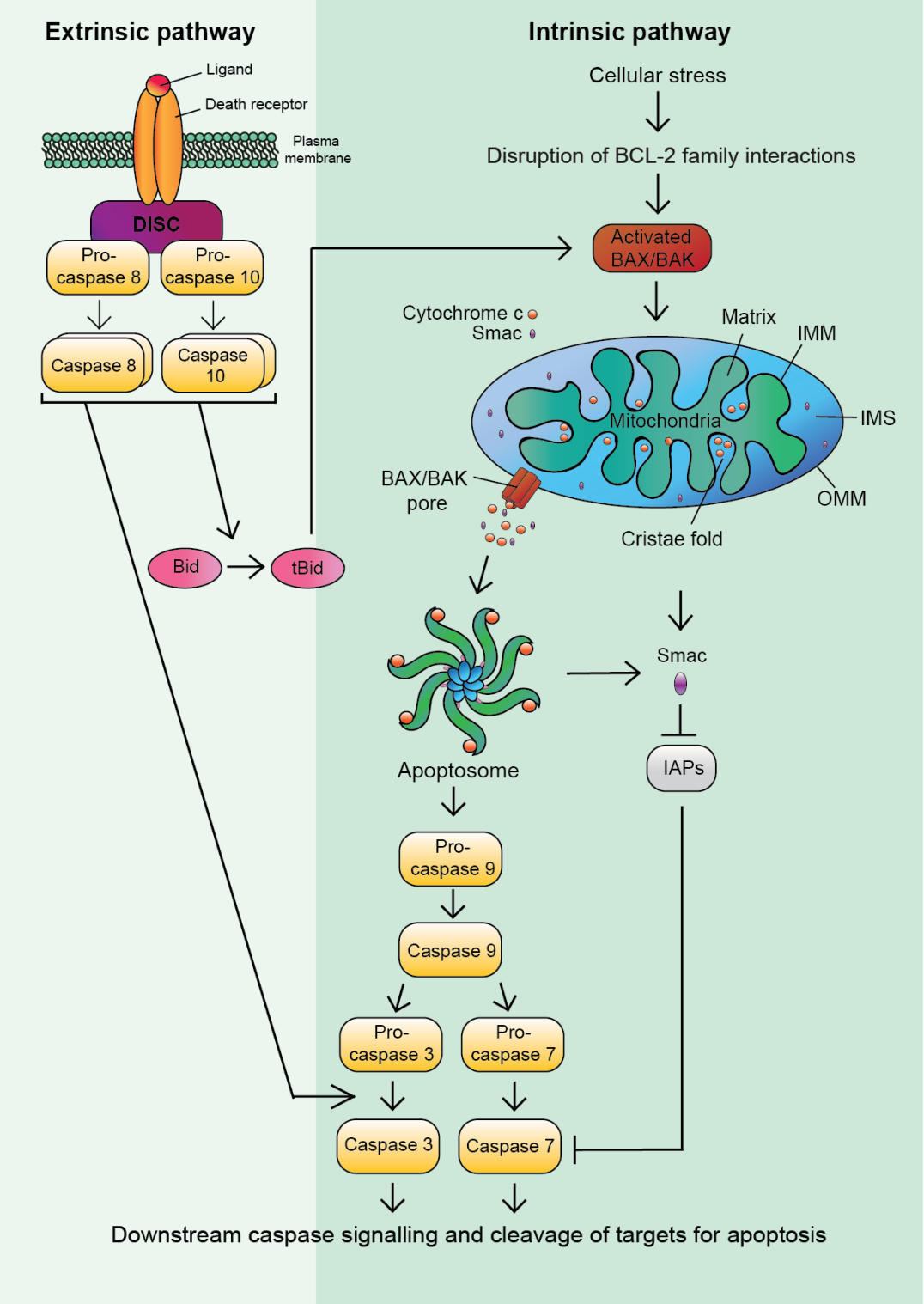


Figure 1.1 Both major pathways of apoptosis converge on activation of the caspases. The intrinsic apoptotic pathway functions via the mitochondria and apoptosome, while the extrinsic pathway can activate caspases independently of the mitochondria. The two pathways can converge via tBID, which translocates to the mitochondria to facilitate BAX/BAK translocation and activation. Ultimately, activation of either pathway will lead to cleavage of effector caspases, driving the organised breakdown of the cellular structure and death.

1.6 The BCL-2 family proteins are the master regulators of apoptosis

Mitochondria withhold several proteins which promote apoptosis from the rest of the cell, thereby preventing cell death under normal circumstances. However, the complexity of the life-death balance begins much earlier in the hierarchy of the apoptotic signalling pathway and involves members of the BCL-2 protein family. This family, consisting of over 25 proposed members, can be grouped into either pro- or anti-apoptotic proteins, based on their functions. The anti-apoptotic members prevent a cell from dying, while the pro-apoptotic members, which are further split into activator, sensitiser and effector subgroups, promote activation of the apoptotic signalling pathway, with some capable of directly disrupting mitochondrial integrity. The balance and interactions of these different pro- and anti-apoptotic BCL-2 proteins within a cell is thought to be what determines its fate[61].

The BCL-2 protein family shares a strong degree of structural homology in the form of BCL-2 homology (BH) domains (Fig. 1.2). Phylogenetic analysis has revealed that these short (~20 amino acid) sequences[62], first identified in BCL-2[63] and subsequently BAK[64], typically contain distinct, conserved residues that dictate the binding of the pro- and anti-apoptotic proteins. While initially named BH domains, the literature is moving towards calling these sequences 'motifs', as they are short amino acid sequences with only small number of conserved residues rather than distinct conserved domains[65]. Traditionally, it is proposed that anti-apoptotic BCL-2 family proteins contain BH1, BH2, BH3 and BH4 motifs in addition to a transmembrane domain, allowing membrane insertion. Some of the well-characterised anti-apoptotic BCL-2 family members are BCL-2, BCL-X_L and MCL-1, and these are generally found to be highly expressed in several cancers, conferring resistance to apoptosis. Meanwhile, pro-apoptotic BCL-2 family members contain distinct groups of BH motifs which set

the basis for the different subgroups – effectors and BH3-only proteins. The effector proteins BAX and BAK, that form pores in the outer mitochondrial membrane to facilitate the release of cytochrome *c*, have BH1, BH2 and BH3 motifs, plus a BH4-like region, which shares some homology with the anti-apoptotic BH4 motif. In contrast, the other pro-apoptotic BCL-2 family members, categorised as activators and sensitisers, contain only BH3 motifs and are thus termed BH3-only proteins (extensively reviewed in [66]). BH3-only proteins are highly promiscuous in binding as described below and this is partly attributed to the fact that most of them are intrinsically disordered proteins, meaning that their three-dimensional structure can vary greatly, permitting diverse binding[67–70].

Current evidence suggests that the BH3 motif is the minimum requirement for a protein to be classed as a member of the BCL-2 family. Thus, the BH3 motif is often considered as the most important motif of the BCL-2 protein family and is arguably the best understood. Sequence analysis of several BCL-2 family members revealed that a signature LXXXGDE sequence is the standard format for a BH3 motif, with only the aspartate residue being completely conserved across all BH3-only proteins[71]. This sequence has since been re-defined multiple times to a 13-residue motif, which represents the more general structures of the BH3 motifs of different BCL-2 family proteins, with distinct types of residues (though not necessarily distinct amino acids) present at certain points[72]. This highlights the massive potential variation within the BH3-only proteins and supports the notion of diverse interactions. Remarkably, despite the high sequence variability, BH3-only proteins largely adopt the same structure - a helical BH3 motif with its hydrophobic features typically buried in the groove of the anti-apoptotic protein that it is in complex with[73]. Moreover, the BH3 domain has a dual function within the BCL-2 family – it functions as a ligand in the

BH3-only proteins while functioning like a receptor within the pocket of multi-motif BCL-2 family members. This interaction is believed to be central to the two-way inhibitory effects that pro- and anti-apoptotic BCL-2 family members exert on each other.

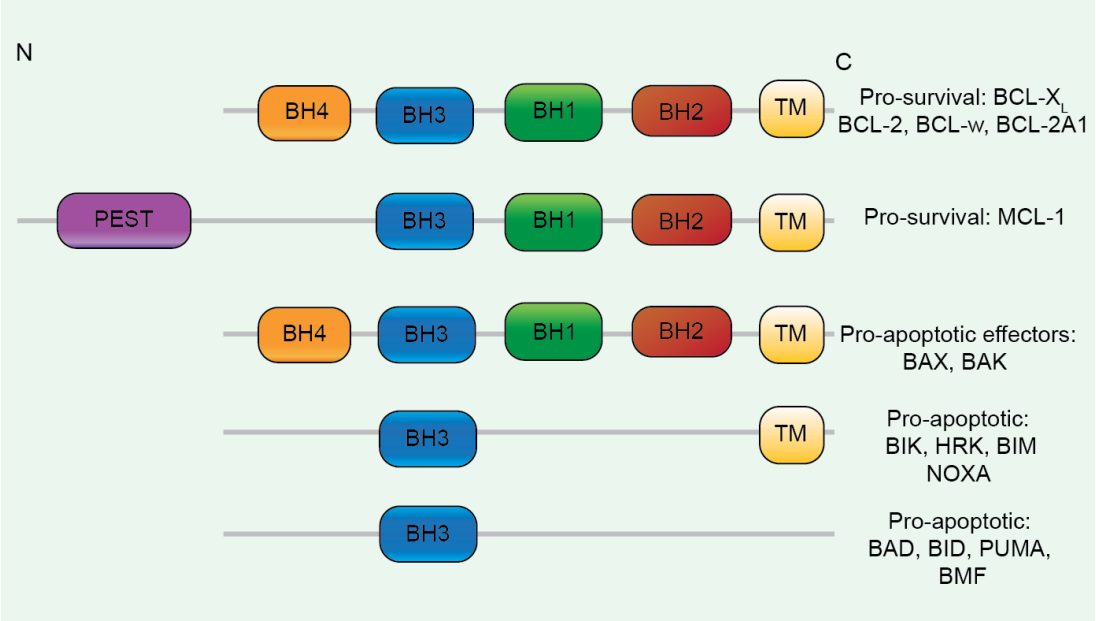


Figure 1.2 The BCL-2 protein family shares a high degree of homology in the form of BH motifs. All members of the BCL-2 protein family contain at least one BCL-2 homology domain, with the BH3 motif being common to all members. Some BCL-2 family members also contain transmembrane (TM) domains which allow membrane localisation. MCL-1 has a unique structure among the major anti-apoptotic BCL-2 proteins, with no evident BH4 motif and an elongated N-terminal tail containing regulatory PEST (proline, glutamic acid, serine, threonine) sequences which are associated with its rapid turnover[74–76]. 'N' and 'C' represent the amino and carboxy terminals of the proteins respectively.

1.7 The pore-forming, effector BCL-2 proteins, BAX and BAK.

BAX and BAK are the keys to unlocking the mitochondrial death gates – they are the two proteins proposed to form the pores in the mitochondria, thereby disrupting mitochondrial integrity and releasing cytochrome *c* into the cytosol.

Initially, BAX was identified as a BCL-2-associated protein (and thus termed BCL-2 associated X) which, when overexpressed, can dramatically increase apoptosis induced by loss of interleukin-3 signalling, even in the presence the pro-survival BCL-2. Thus, it was initially proposed that the balance of BCL-2 versus BAX was what determined cell fate[77], though how this could actually lead to the destruction of the cell was unclear. Subsequent studies showed that BAX can form high order oligomers and translocate from the cytosol to the mitochondria under stress conditions[78,79] which could cause the release of cytochrome *c* from the mitochondria to the cytosol and induce apoptosis[80,81]. Based on the structural similarity of BCL-2 family members to pore-forming bacterial toxins, it was hypothesised that BAX could potentially form pores in the mitochondrial membrane once oligomerised to initiate cytochrome c release[82]. This was confirmed in later studies and the pore-forming activity of BAX was shown to be antagonised by BCL-2[83]. Since then, there have been extensive studies of the intricacies of the formation of BAX pores in the mitochondria (reviewed in [41]). Following identification of BAX, a second pro-apoptotic BCL-2 family member, BAK (BCL-2-homologous antagonist killer), was found simultaneously by two groups[84,85] and was proposed to function in the same manner as BAX – able to induce apoptosis when overexpressed alone. BAK was shown to be able to release cytochrome *c* following cleavage of BID in the extrinsic apoptotic pathway[59], affirming it as a protein with some degree of functional redundancy to BAX.

Though some aspects of how both BAK and BAX end up as pores in the mitochondrial membrane remain controversial, it is known that both must undergo multiple stages of conformational changes to become activated. BAX must transform from its cytosolic, inactive, globular structure within which its pro-apoptotic regions (the hydrophobic BH3 binding groove and the transmembrane domain) are internalised[86]. Upon activation by BH3-only proteins, reportedly at the 'rear' activation site of BAX, these internalised domains are exposed, permitting insertion at the mitochondrial membrane and its oligomerisation with other BAX molecules. This has been suggested to allow for transient interactions between the BCL-2 family members and BAX, therefore permitting a two-step activation process – with BAX translocating to the mitochondrial membrane first before exposure of the hydrophobic BH3-binding groove which is critical for pore-formation[60,87,88]. This allows for a finer control of apoptotic signalling and allows for cycling of BAX back to the cytosol when apoptosis is not required. Activation of BAK may involve a little less fine control as it is believed that the major activation site is the BH3-binding groove. However recent work supports the idea of separate activation sites on BAK, suggesting that, like BAX, it may also have fine layers of regulation[89–91]. Notably, while BAX has a cytosolic localisation under basal conditions and moves to the OMM during apoptosis, BAK has been proposed to be predominantly mitochondrially localised under basal conditions[92], further differentiating these proteins and suggesting that their regulation mechanisms may be a little different.

It is worth noting that, as the major effectors of the apoptotic pathway, both BAK and BAX can be considered as tumour suppressors and in turn, have been identified as proteins which are likely to be mutated in cancers to drive abnormal cell survival. Indeed, it has been shown that DNA repair defects in colon and gastric cancers can

leads to mutations in both the BAX and BAK genes and this can promote abnormal cell survival[93–95].

In addition to BAX and BAK, there is a third proposed pore-forming BCL-2 family protein, BOK (BCL-2-related ovarian killer), whose roles in classical apoptosis are controversial. BOK can form pores in the OMM, however it is believed to do so independently of the known BCL-2 family proteins and is strongly associated with endoplasmic reticulum stress. Instead of modulation by BCL-2 family proteins, it has been suggested that the constant degradation of BOK inhibits its activity[96], highlighting the idea that not all BCL-2 family proteins may function in the same way.

1.8 The activator BH3-only proteins

As previously mentioned, the pro-apoptotic BCL-2 proteins can be categorised into effector (BAX/BAK) and activator and/or sensitiser proteins. Three of the BH3-only proteins, namely BIM, BID and PUMA, are proposed to be capable of interacting with and altering the activity of multiple pro- and anti-apoptotic BCL-2 family members, which means that they are designated as both 'activator' and 'sensitiser' proteins. While this could suggest that these proteins are functionally redundant, studies have shown that each of these BH3-only proteins has its own unique regulation and activity.

BIM

BIM, first identified in a screen to search for BCL-2 -interacting proteins[97] is considered to be especially promiscuous in its ability to bind multiple members of the BCL-2 family (Fig. 1.3.). BIM exists as multiple isoforms - extra-long (BIM_{EL}), long (BIM_L) short (BIM_S)), all of which can induce apoptosis but with different potencies[98,99]. BIM is highly regulated at transcriptional, epigenetic, post-transcriptional and post-translational levels, with many phosphorylation sites

identified (reviewed in [100]). Of note, it has been proposed that BIM is normally sequestered by components of the cytoskeleton (namely LC8 of the motor protein dynein) and, upon apoptotic signalling, is released from the cytoskeleton and translocates to the anti-apoptotic proteins, where it may quench their activity and initiate apoptosis[101]. These types of regulation, along with many others, allow intricate control of the highly promiscuous BIM under healthy and cellular stress conditions and maintains a precise ratio between BIM and its anti-apoptotic counterparts, thereby regulating apoptosis. It is therefore not surprising that, as one of the major pro-apoptotic proteins, BIM is often altered in some cancers, conferring protection from apoptosis. For example, multiple reports suggest that BIM expression is reduced in lung cancers and that the presence of deleterious BIM polymorphisms negatively impacts patient survival[102–104]. BIM has also found been found to undergo homozygous deletion in various lymphomas[105], suggesting it is likely an important tumour suppressor in multiple types of cancer.

PUMA

PUMA (p53 upregulated modulator of apoptosis) was originally identified as one of the BCL-2 family proteins upregulated by p53 following cellular stress, such as DNA damage[106,107]. PUMA is also highly responsive to many other forms of cellular stress in both p53-dependent and -independent scenarios, including cytokine deprivation and both glucocorticoid and staurosporine exposure[108,109]. Like BIM, PUMA is thought to be promiscuous in binding multiple members of the anti-apoptotic BCL-2 family (summarised in Figure 1.3). With the high incidence of p53 mutations in many cancer types, PUMA expression can be vastly altered in cancer cells as p53 can no longer upregulate its expression following DNA damage, thereby allowing cancer cells to resist apoptosis[110].

BID

Meanwhile, BID (BH3 interacting-domain death agonist) was identified in a search for proteins that could heterodimerise with other BCL-2 family proteins, including both effector proteins (BAX) and pro-survival proteins (BCL-2)[111]. Subsequent studies demonstrated that BID could regulate release of cytochrome *c* from the mitochondria to the cytosol following activation of the various death receptors[112]. This highlighted BID as the mediator of cross-talk between the intrinsic and extrinsic apoptotic pathways. As described earlier, BID can be cleaved by caspase 8/10 to tBID. tBID is proposed to translocate to the mitochondrial membrane where it is believed to facilitate the translocation, insertion and activation of the BCL-2 effector proteins, BAX[60]/BAK[59], at the mitochondrial membrane, leading to apoptosis[57,112,113]. While tBID is a remarkably potent activator of apoptosis, its full-length, non-truncated form is also believed to be associated with apoptosis[111], as mutation of the caspase-cleavage site does not fully attenuate its pro-apoptotic activity[114].

1.9 The 'sensitiser' BH3-only proteins

Sensitiser BH3-only proteins are distinct from the activators as they generally have more specific subsets of pro-survival binding partners and are not believed to be capable of binding to and activating the effector proteins directly. Instead, it is proposed that they add a finer layer of control over apoptosis and each protein has its own distinct regulation mechanisms.

BAD

The BH3-only protein BAD (BCL-2-associated agonist of cell death) was identified in a screen for BCL-2-interacting proteins and has been shown to be capable of heterodimerising with both BCL-2 and BCL-X_L (but not MCL-1) to antagonise their

cell survival activity[115]. Like other BH3-only proteins, BAD can undergo post-translational modifications to alter its activity. For instance, phosphorylation of two serine residues in BAD prevents its interaction with BCL-X_L but instead facilitates its interaction with another protein, 14-3-3, following exposure of a cell to certain survival factors, such as interleukin-3. This in turn sequesters BAD in the cytosol, where it cannot promote apoptosis, allowing BCL-X_L signalling to promote survival[116–118].

BMF

BMF (BCL-2-modifying factor), identified as a BH3-only protein in a yeast-two-hybrid screen using MCL-1 as bait, is a pro-apoptotic protein which, like BIM, might have its activity sequestered by components of the cytoskeleton. Thus, when the cell undergoes stress (for example detachment from the extracellular matrix), the cytoskeleton undergoes changes and BMF is released and can activate apoptosis[119]. Further studies have also showed that BMF expression can be upregulated following inhibition of CAP-dependent protein synthesis as a result of various factors, including loss of growth factor signalling and hypoxia[120]. Elevated or free BMF can therefore promote apoptosis under multiple stress conditions by binding to and altering the activity of its BCL-2 family partners.

NOXA

In addition to PUMA, p53 also upregulates another BH3-only protein called NOXA (also known as Phorbol-12-myristate-13-acetate-induced protein 1 or PMAIP) which was identified as a BCL-2 family member following a screen for elevated proteins in mRNA expression profiles in wild-type versus IRF-1 (interferon regulatory factor 1)/p53-deficient (both tumour suppressors), irradiated cells[121]. Later studies showed

that NOXA mediates apoptosis via regulation of the BCL-2 protein family, thereby activating MOMP[122]. NOXA has been suggested to perform the partner function of BAD, binding to the two major anti-apoptotic proteins that BAD does not – MCL-1 and BCL-2A1, as outlined in Figure 1.3. NOXA is highly labile therefore it is not surprising that several studies have highlighted tight post-translational regulation of NOXA – notably NOXA is a key target of the proteasome[123–125]. It was presumed that the targeting of NOXA to the proteasome was a result of the ubiquitination system, however data seems to indicate that proteasome-dependent degradation of NOXA is ubiquitination-independent[126] and may in fact rely on its unstable C-terminal structure[127].

BIK

Meanwhile BIK (BCL-2-interacting killer), originally identified as an interacting partner of both BCL-2 and BCL-X_L, was one of the first BH3-only proteins identified. While BIK was shown to be important to apoptosis, its interaction with anti-apoptotic proteins alone is not enough to induce apoptosis, allowing for categorisation of BIK as a sensitiser BH3-only protein[128]. Interestingly, BIK has been shown to have a very strong endoplasmic reticulum localisation and is thought to even function from the ER to modulate mitochondrial integrity during apoptosis[129].

HRK

Similar to BIK, Harakiri (HRK) was identified in a yeast two-hybrid screen as a protein that could interact with BCL-2 and BCL-X_L but not the effector pro-apoptotic proteins and was therefore proposed to be another member of the sensitiser BH3-only protein group, with the BH3 motif being found to be required for its pro-apoptotic capacity[130]. HRK, like the other BH3-only proteins, can be induced by various stimuli,

including lack of growth factor signalling[131]. Interestingly, HRK has been identified as a key tumour suppressor that is altered in gastric cancers, with possible epigenetic silencing of HRK contributing to a malignant phenotype[132]. This further highlights the importance of assessing the levels of pro-apoptotic BCL-2 proteins in cancer.

1.10 The pro-survival BCL-2 family members

Pro-survival members of the BCL-2 family are the endogenous antagonists of the pro-apoptotic subgroups, existing in dual functions to prevent activity of both BH3-only and effector BCL-2 family proteins and thereby preventing apoptosis. As such, the pro-survival proteins are overexpressed or altered in a vast number of cancers, allowing tumour cells to survive abnormally.

BCL-2

The first and founding member of the BCL-2 family identified, as the name might suggest, was BCL-2 (B-cell lymphoma 2). BCL-2 was identified and named following the seminal article describing the study of an acute B-cell leukaemia cell line established from a patient with follicular lymphoma[133]. In this cell line, it was remarked that a chromosomal translocation (14;18) between a highly active immunoglobulin heavy chain enhancer and a putative oncogene was one of the driving factors of the malignancy developed in a multi-step process of oncogenesis. The chromosomal translocation permitted hyperexpression of the putative oncogene, BCL-2, and this hyperexpression augmented B-cell survival, thereby promoting oncogenesis[133]. Despite extensive further study into the BCL-2 chromosomal translocation in leukaemia, it was not until four years later that the pro-survival function of BCL-2 was suggested, following the observation that forced expression of BCL-2 could mediate resistance to cell death induced by growth factor withdrawal in

multiple cell lines[12]. Subsequent generation of knockout mice revealed that BCL-2 is a pro-survival protein which is essential to the survival of multiple types of cells, including various haematopoietic lineages and cells of the kidneys[134]. Later, identification of a number of proteins with structural homology to BCL-2 revealed the true nature of this protein family, which are able to hetero- and homodimerize with each other to mediate apoptosis. Indeed, BCL-2 has been suggested to interact with a number of other BCL-2 family proteins (as summarised in Figure 1.3) to regulate apoptosis. Moreover, since its identification, many studies have demonstrated that overexpression of BCL-2 was not limited to specific subsets of lymphoid malignancies and in fact, multiple cancers could be driven by BCL-2, including chronic lymphocytic leukaemia[135], non-Hodgkin's lymphoma[136] and acute myeloid leukaemia[137]. BCL-2 overexpression has also been detected in many non-haematological tumours including breast[138], prostate[139], lung[140], bladder[141], colorectal[142] and brain malignanices[143].

BCL-X_L

B-cell lymphoma extra-large (BCL-X_L) was identified in an effort to identify BCL-2-related genes and was first described as a 'BCL-2-independent regulator of programmed cell death'[144]. BCL-X_L is expressed in various tissues including the central nervous system while also being highly expressed in cells undergoing development as part of the immune system[144] and was later shown to be functionally and structurally similar, though not functionally redundant to, BCL-2[145]. BCL-X_L has very similar binding preferences for BH3-only proteins as BCL-2, though does indeed have some unique interaction partners (Fig. 1.3), further exemplifying it as a pro-survival protein distinct from BCL-2. Like BCL-2, BCL-X_L has been identified as overexpressed or possessing aberrant activity in various types of cancer, including

colorectal[146], prostate[147,148], chronic myeloid leukaemia (CML)[149,150] and head and neck malignancies[151].

BCL-2A1

There are also several pro-survival proteins which are less well understood than those originally identified. This includes BCL-2A1, also known as BCL-2 related gene expressed in foetal liver (BFL-1), an anti-apoptotic protein which has been shown to interact with many of the pro-apoptotic BCL-2 family members, as summarised in Figure 1.3 BCL-2A1 is associated with multiple types of cancers, including acute lymphoblastic leukaemia[152], CLL[153], stomach[154] and breast cancers[154,155]. BCL2-A1 may also play a central role in resistance to chemotherapy, particularly therapy targeting other arms of the BCL-2 family[156,157].

BCL-w

BCL-w is another pro-survival protein which is relatively unexplored but with vast potential implications for disease, especially cancer[158]. While it was demonstrated that induced expression of BCL-w can promote survival of various haemapoietic cell lines under stress conditions, knockout of BCL-w in mice did not significantly impact development of the organism and was shown to only be essential to spermatogenesis[159] However other studies have claimed important roles for BCL-w in the epithelium of the small intestine[160] as well as in the survival of B cells during their development[161], supported by the notion that BCL-w is overexpressed in various B cell malignancies[162]. Furthermore, BCL-w has been found to be overexpressed or important to various other types of cancers, including in melanoma[163], colorectal[164] and breast cancer cell malignancies[165].

MCL-1

Myeloid cell leukaemia 1 (MCL-1), named after the cell in which it was first identified (ML-1 human myeloid leukaemia) is quite a distinct member of the BCL-2 family for multiple reasons. While it does contain the standard BH1-3 motifs, it is unusual in that it lacks an identifiable BH4 motif[76] (Figure 1.3). Moreover, MCL-1 has a long, unstructured N-terminus with two regulatory PEST (proline, glutamic acid, serine, threonine) sequences which regulate the rapid turnover of short-lived proteins, such as MCL-1. The rapid turnover of MCL-1 is also attributed to its intricate regulation by various post-translation modifications including ubiquitination. It has been shown that MCL-1 is ubiquitinated by multiple E3 ligases, including MULE – a BH3 motif-containing E3 ligase which targets MCL-1 for degradation by the proteasome[166,167]. Thus, MCL-1 has a vast number of potential sites for modification and unsurprisingly, multiple signalling pathways (including the STAT3[168] and MEK/MAPK and PI-3K/AKT[169] pathways) can therefore regulate MCL-1 expression at the level of transcription, with further post-transcriptional, post-translational and epigenetic control mechanisms over MCL-1 existing to finely regulate its activity.

Another distinct feature of MCL-1 and its regulation comes in the form of the distinct MCL-1 isoforms. When analysed by immunoblotting, MCL-1 appears to exist as multiple isoforms, that can be detected by the presence of multiple bands between the 28 and 40 kDa range, depending on the antibody used. There has been much debate about what these isoforms are and how they come to be, however, as no single study has suggested what the distinct isoforms are and how they arise. Alternative splicing has been attributed to the presence of two major MCL-1 isoforms, MCL-1_L and MCL-1_S which have mitochondrial and cytosolic localisation respectively. MCL-1_L is proposed to be a mitochondrial-localised, anti-apoptotic protein[76,170] whose expression

is associated with poor clinical outcome in a number of cancers[171,172] while it has been proposed that the short MCL-1 protein, containing only the BH3 domain, is pro-apoptotic[173,174].

To further complicate matters, the shortest isoform of MCL-1 (proposed to be detectable in immunoblotting at 36 kDa) has been shown to localise to the inner mitochondrial membrane to perform non-apoptotic mitochondrial roles, including but not limited to regulation of mitochondrial bioenergetics[175]. Furthermore, some reports also claim other forms of MCL-1 exist, including MCL-1 extra short (MCL-$1_{ES)}$ and MCL-1 short nuclear (MCL-$1_{SN)}$ which are claimed to have pro-apoptotic[176] and cell cycle regulatory functions respectively[177]. It remains unclear if all these variants are directly represented by the bands seen in immunoblotting and some of these isoforms of MCL-1 may arise under very distinct circumstances, however these studies clearly highlight the complex biology of MCL-1.

At the subcellular level, MCL-1 can, unsurprisingly, be found at high levels at the mitochondrial membrane[76] due to the anchoring of its N-terminal region on the OMM[75]. Its localisation is, in fact, much more widespread than just the OMM however, indicative of its multi-faceted behaviour. Studies have reported MCL-1 expression in the nuclear envelope[178], nucleus[177–180], the cytosol[180], the inner mitochondrial membrane[175,181,182] and the endoplasmic reticulum[76]. Some of these localisations have also been linked to unique and unexpected functions of MCL-1, including regulation of oxidative phosphorylation[175], lipid metabolism[183], the cell cycle[177,180] and the DNA damage response[179,184].

Meanwhile, at the tissue level, MCL-1 is also widely expressed. While first identified as an immediate-early gene induced by PMA in ML-1 cells[76], MCL-1 is now known

to be essential to the survival of many cell types in a non-functionally redundant manner. Tissues and cell types which MCL-1 is essential to include B and T cells[185,186], granulocytes[187], neutrophils[188], haematopoietic stem cells (HSCs)[189], neuronal cells[190] and fibroblasts[191]. The exact number of cell types that MCL-1 is essential to remains unclear as MCL-1 is also important at the developmental stage, as knockout of MCL-1 in mice is embryonic lethal before E3.5[192]. While this has made identifying tissue-specific requirements for MCL-1 difficult, it further highlights the non-redundant functions of the BCL-2 family members, particularly as BCL-X_L knockout only induces embryonic death by E13.5[193]. In further support of the non-redundant nature of pro-survival proteins, MCL-1 has a unique set of BCL-2 family interaction partners, as shown in Figure 1.3. While MCL-1 can bind to the three main 'activator' BH3-only proteins, it also has very strong affinity for NOXA[194] while the major other BCL-2 family members do not bind to NOXA (Fig 1.3).

Like the other pro-survival BCL-2 family members, MCL-1 has been described as an oncogene which, when aberrantly overexpressed, promotes the key cancer hallmark of resistance to apoptosis. MCL-1 is one of the most highly amplified genes in most cancers[195] and is often overexpressed or mutated in both solid and haematological cancers[195], including multiple myeloma[196], acute myeloid leukaemia (AML)[197], lung[198,199] breast[200–202], oral[171] and oesophageal cancers[203], hepatocellular[204], thyroid[205], ovarian[206], prostate[207] and pancreatic cancers[208]. The wide expression of MCL-1 in many cancers has highlighted it as both as an important therapeutic candidate to target in tumours and also a chemoresistance factor for other therapies. Indeed, many studies have shown that targeting MCL-1 is imperative to sensitising cells to various types of chemotherapy, as tumour cells can upregulate MCL-1 (or already have high levels of MCL-1) that mediates resistance to death even in high

stress conditions. High MCL-1 expression levels have also been linked to resistance of cancers to various conventional chemotherapeutic approaches, including cisplatin[171,203] and radiotherapy[172,208] as well as targeted therapies like BRAF inhibitors[209].

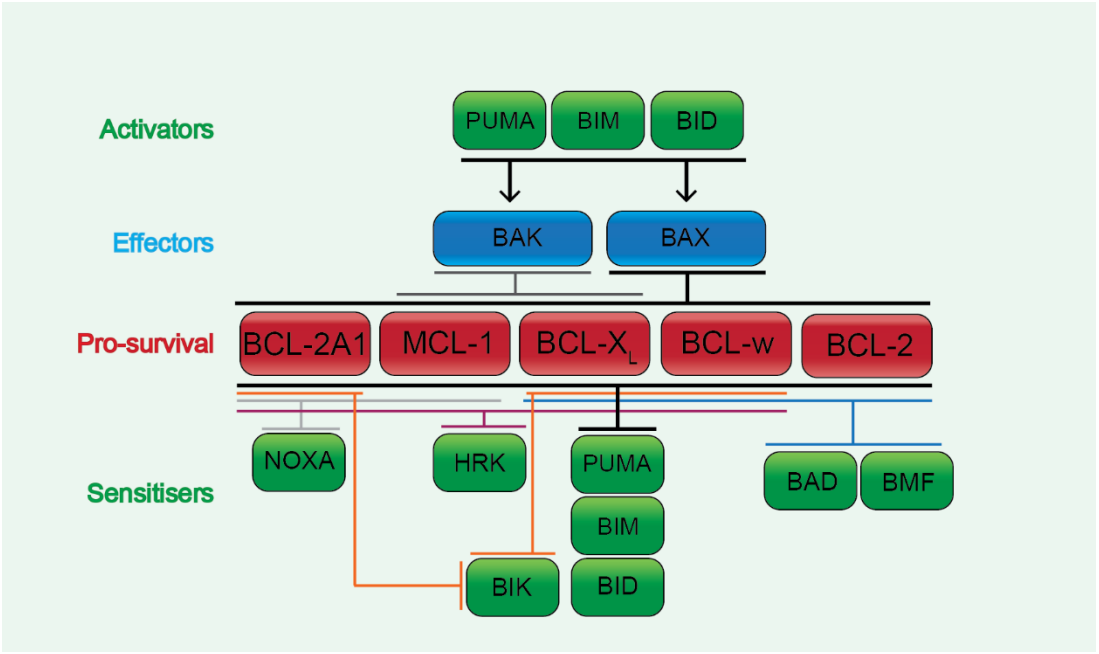


Figure 1.3 The complex interaction network of the major BCL-2 protein family members. Pro-survival BCL-2 family proteins (red) are proposed to interact with both activator/sensitiser and effector BCL-2 family members (green/blue) to regulate induction of apoptosis within a cell. The promiscuous activator BH3-only proteins can bind to the major pro-survival and effector proteins, while the sensitiser BH3-only proteins have more specific binding patterns for each pro-survival protein. For example, BCL-2, BCL-w and BCL-X_L can bind to and inhibit BAD, while MCL-1 and A1 cannot. Instead, MCL-1 and BCL-2A1 can uniquely bind to NOXA. Thus, the network of interactions is highly intricate. While some of these interactions have been shown *in vivo*, some have only been inferred by studies in *in vitro* binding models and therefore remain to be clarified[23,60,185,210–216].

1.11 The BCL-2 family and apoptosis induction

While it has been established that it is the interactions between the pro- and anti-apoptotic BCL-2 family members that dictate the apoptotic status of a cell, the precise molecular mechanisms underlying these events have proven to be both complicated and controversial. Early models first proposed that anti-apoptotic BCL-2 proteins

functioned simply by directly binding to and inhibiting BAX[216]. However, this kind of model was not compatible with the distinct localisations of the BCL-2 family proteins or the ever-expanding BCL-2 protein family. Further studies led to the proposal of direct and indirect activation models.

The direct model proposes that, that upon receipt of signals for death upstream in the cell, 'activator' BH3-only proteins bind to and directly activate BAX/BAK, leading to apoptosis. In this model, it is proposed that the pro-survival proteins function to inhibit the activator BH3-only proteins (and vice-versa), while the sensitiser BH3-only proteins relieve this inhibition[23,210–212]. Meanwhile, the indirect model proposes that anti-apoptotic proteins directly sequester the effector proteins (BAX/BAK) thereby stopping their insertion into the mitochondrial membrane and ensuing apoptosis. In this case, on receipt of death stimuli, the BH3-only proteins interact with and displace the anti-apoptotic proteins from BAX/BAK, freeing them to disrupt mitochondrial integrity[213,215,217]. These two models highlight the nature of the pro- and anti-apoptotic protein interactions during apoptosis but do not capture the more complex interaction network of the BCL-2 family. For example, the direct activation model does not explain why MCL-1 binds to and potently inhibits the activity of BAK[215,218].

Thus, a more refined and complex model - the 'unified' model - of activation was proposed, which integrates both the direct and indirect activation models by classifying different 'modes' of apoptosis which depend on cellular stress levels. In mode 1 (the direct model), anti-apoptotic proteins, such as MCL-1, bind to and inhibit the activity of pro-apoptotic BH3-only proteins, such as BIM, while in mode 2 (the indirect model), MCL-1 would bind to and directly sequester BAX and/or BAK to limit their activity. Mode 2 is suggested to be a more efficient mechanism of inhibition of apoptosis since it involves direct activity against that of the effector proteins and

would require upregulation of BH3-only proteins (and hence high stress conditions) to displace effector proteins from pro-survival proteins[219]. Furthermore, it has also been suggested that retrotranslocation of BAX/BAK, from the mitochondria to the cytosol, by BCL-2 family members might be considered a 'mode 0' of apoptosis regulation[41].

Further complicating the activation models of apoptosis, recent work has shown that the proposed subgroups of BH3-only proteins (i.e. sensitisers versus activators) are also not as simple as previously thought, as some BH3-only proteins which were thought to function exclusively as sensitiser BH3-only proteins (NOXA) have been shown to be capable of functioning like activators of BAX/BAK. Thus, an 'interconnected hierarchical model' of apoptosis was suggested[220].

There is no doubt that future work to unravel this hierarchy will reveal even more complex behaviour of the BCL-2 protein family. Altogether, studies seem to point towards a bi-modal, complex activation of apoptosis, where both major subsets of pro-apoptotic proteins (effectors and BH3-only proteins) are inhibited by the pro-survival proteins and this inhibition must be un-done in a step-wise manner. Activator BH3-only proteins may convert BAX/BAK to their active forms (direct activation) and subsequently, block the activity of anti-apoptotic proteins, thus relieving their inhibition of BAX/BAK insertion in the mitochondrial membrane. This suggests that the majority of the BCL-2 family interactions are highly dynamic and intricate, with multiple levels of control in place to regulate MOMP.

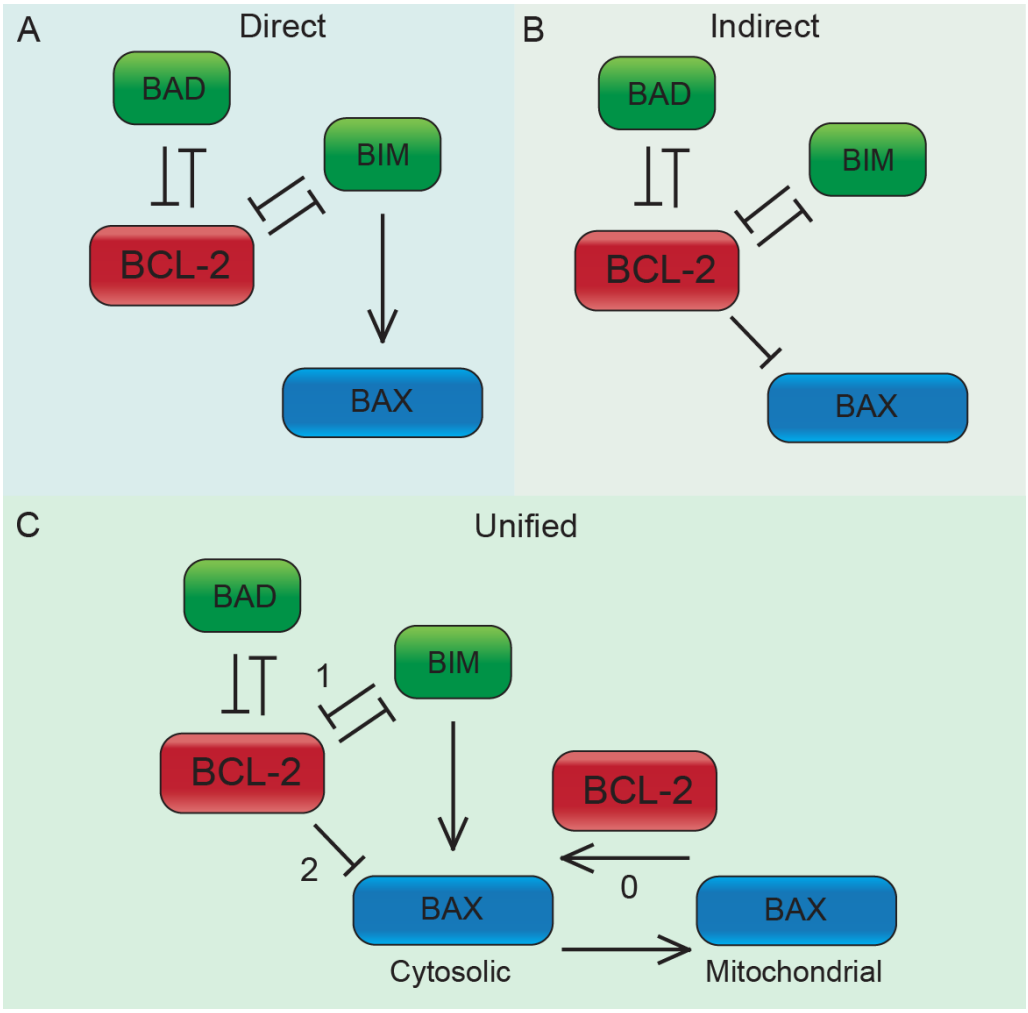


Figure 1.4 The proposed activation models of apoptosis. Multiple models of activation of the effector BCL-2 family proteins have been proposed. The direct activation model (A) suggests that 'activator' BH3-only proteins, like BIM, bind to and directly activate BAX/BAK following relief of their inhibition by pro-survival proteins thanks to 'sensitiser' BH3-only proteins. The indirect model (B) suggests pro-survival proteins, like BCL-2, bind to and inhibit effector proteins. Effector proteins are then indirectly activated by 'activator' BH3-only proteins, which block the activity of pro-survival proteins. Extensive studies support the notion of both of these types of activation occurring in the unified model (C) (with mode 1 being direct and mode 2 being indirect activation) to allow extremely precise control of effector protein activation. Even further complexities exist in the form of retrotranslocation of BAX/BAK from the mitochondria to the cytosol by pro-survival proteins (mode 0) and the hierarchical model of apoptosis, where some 'sensitiser' BH3-only protein can function like activator BH3-only proteins.

1.12 Targeting the BCL-2 family – a key therapeutic approach in cancer

Until recently, most chemotherapeutic approaches exploited the fact that cancer cells are genetically unstable and divide very quickly. Agents such as cisplatin are able to rapidly incorporate into the DNA of cells that are highly proliferative, leading to massive DNA damage as the cells try to replicate. Excessive DNA damage and inability to repair it subsequently triggers cell death[221]. While these approaches are often some of the first-line approaches to cancer therapy and are consistently used in the clinic, the focus of translational cancer medicine has shifted towards targeted approaches which exploit more specific aspects of individual cancers.

Key targets in many cancers include hyperactive proteins such as kinases and many compounds have recently been developed against such proteins, such as the landmark compound vemurafenib – a inhibitor that targets the BRAF V600E mutation in melanoma[222]. This mutation of a valine residue to a glutamic acid residue leads to hyperactivation of BRAF kinase and its downstream targets in the MEK/ERK signalling pathway, promoting oncogenicity and this is a key oncogenic driver in more than 50% of melanomas[223–225]. Vemurafenib initially demonstrated remarkable results following use in late-stage melanoma, with complete regression of tumours at a remarkable rate in some patients. However, unfortunately, in most cases, some tumour cells remained and acquired mutations which made them resistant to further vemurafenib treatment, as other signalling pathways were co-opted in order to promote oncogenic signalling (reviewed in [226]). While targeted therapy of proteins like kinases can be initially very effective, they are also highly susceptible to resistance mechanisms, especially when the targeted proteins exist at a nexus of signalling pathways that might allow tumour cells to circumvent the inhibition of the protein they are addicted to.

Recent therapeutic strategies that target the BCL-2 family of proteins have demonstrated much promise in overcoming the issue of acquired resistance. The proteins that are targeted act downstream of most major signalling pathways therefore acquired resistance is much more difficult to achieve, as there are many fewer pathways to be exploited by cancer cells. Thus, much emphasis has been placed on ways to induce apoptosis by altering the behaviour of the BCL-2 protein family. Designing compounds to directly inhibit the BCL-2 family has proven challenging however, as to inhibit their activity requires disruption of protein-protein interactions.

Successful targeting of the BCL-2 family members has now been achieved by several strategies, including by stabilising the pro-apoptotic BCL-2 family members to promote apoptosis, by reducing the expression levels of the anti-apoptotic members at the level of transcription or by directly disrupting the BCL-2 family interactions. Early compounds in this field include flavopiridol, a cyclin-dependent kinase (CDK) inhibitor which was described as potent inducer of apoptosis in CLL cells[227] and was later shown to be able to induce cell death in both CLL and multiple myeloma, thanks to indirect transcriptional downregulation of MCL-1 and IAPs[228,229]. Although initial Phase I/II studies suggested that continuous flavopiridol infusion had no effect on relapsed CLL patients[230], adjusting the dose and timings of administration in Phase II showed extremely promising results, with high response rates in CLL[231]. However, the non-specific nature and potential toxicity of the compound limited its use in the clinic.

The pro-apoptotic BCL-2 family can also be targeted indirectly – although to induce apoptosis, their expression must be elevated rather than downregulated. One mechanism to do this is by using histone deacetylase inhibitors (HDACis), which can relieve the epigenetic silencing of BH3-only proteins such as NOXA and BIM. Such

an indirect approach can also potentially increase the expression of pro-survival proteins (namely MCL-1) in CLL, however. While HDAC inhibitors could induce some apoptosis despite the increase in both pro- and anti-apoptotic proteins, inhibition of MCL-1 using CDK inhibitors could potentiate the HDACi-induced apoptosis[232,233]. Some studies have also shown that inhibition of the proteasome can also induce apoptosis by stabilising BH3-only proteins. One example of an effective proteasome inhibitor is Bortezomib, which has been shown to stabilise NOXA levels, thereby abrogating MCL-1 pro-survival activity[123]. Bortezomib has been used with success in treatment of multiple myeloma[234] and mantle cell lymphoma[235,236] but has struggled to perform in other malignancies. One issue of using such broad action inhibitors such as HDACis or proteasome inhibitors is their inherent non-specificity in modulating expression levels of numerous short-lived proteins that may impinge on the apoptotic pathways. This means that it is possible that levels of tumour-promoting or pro-survival proteins can be elevated using such approaches, thus negating the effect of the increase of pro-apoptotic BH3-only proteins. Nonetheless, these types of inhibitors have proven useful in some cancers and may be of use in combination therapy in the future. It is clear, however, that directly targeting the anti-apoptotic proteins themselves is of great importance.

1.13 Inhibitors of the BCL-2 protein family - BH3 mimetics

Early BH3 mimetics functioned as pan-BCL-2 antagonists

Designing compounds to directly inhibit the BCL-2 family has proven challenging as to inhibit their activity requires disruption of protein-protein interactions between the anti- and pro-apoptotic BCL-2 family members. However, such compounds have recently been developed in the form of BH3 mimetics - small molecule inhibitors that mimic the pro-apoptotic BH3-only members in an attempt to compete with the pro-

apoptotic members to bind and inhibit their anti-apoptotic counterparts. To be defined as a BH3 mimetic, a compound must satisfy two minimum requirements: it should be able to (1) induce apoptosis in a BAK/BAX-dependent manner (2) bind to the pro-survival BCL-2 family members with nanomolar affinity[237].

Early work described the activity of several putative BH3 mimetics – natural or synthetic compounds which seemed to have BH3 mimetic-like behaviour, able to inhibit the activity of one or more of the pro-survival BCL-2 family members *in vitro*. Key examples include obatoclax[238] and derivatives of gossypol (apogossypol, sabutoclax[239,240]) – which exhibited pan-BH3 mimetic-like properties in binding to the key pro-survival proteins and displacing effector proteins to induce apoptosis, albeit at micromolar concentrations. Obatoclax, however, could induce apoptosis in BAX/BAK null cells, suggesting it was not a true BH3 mimetic. Meanwhile, gossypol (and its derivatives), natural compounds derived from cottonseeds, had been known to have anti-neoplastic activity for more than 50 years[241], although were only designated as a putative BH3 mimetic 40 years later[242]. Despite the multiple steps of re-engineering of gossypol to other compounds like apogossypol, TW37 and sabutoclax, these compounds represented poor therapeutic potential, as they had weak binding affinities for the anti-apoptotic proteins[243,244]. Moreover, many of these compounds could also induce BAX/BAK-independent apoptosis, indicating likely off-target toxicities[245–247].

While many of these compounds were BH3 mimetic-like, none could be considered true BH3 mimetics and most demonstrated both off-target effects in addition to a lack of specificity within the BCL-2 family itself. With the knowledge that different cancers can rely on different BCL-2 family proteins for survival, specific targeting of

one or more members of the anti-apoptotic BCL-2 with true BH3 mimetics was pursued.

Development of synthetic, BH3-like peptides

Since it was remarked that native BH3-only proteins are direct antagonists of the pro-survival proteins, Walensky *et al*. set about designing synthetic peptides based off the structure of the endogenous BH3-only proteins[248]. Stable, stapled peptides based on the BH3 motifs of multiple BH3-only proteins, including BIM, BAD and BID were generated by stabilising peptides with hydrocarbon groups and were named SAHBs – stabilised alpha-helix of BCL-2 domains. The ability of these peptides to induce apoptosis was assessed by measuring *in vitro* cytochrome *c* release or phosphatidylserine (PS) externalisation in cancer cells, with the peptides demonstrating a BAK-dependent increase in both measurements[211,248]. These studies provided the basis for the development and identification of compounds with similar properties, able to bind to the hydrophobic groove of anti-apoptotic proteins and induce BAX/BAK-dependent apoptosis.

1.14 The advent of *bonafide* BH3 mimetics

Development of ABT-737

ABT-737 was developed following an NMR-based screen searching for small molecules that could specifically bind to the BH3 binding groove of BCL-X_L. Promising hits from the screen were subjected to extensive structural modifications to yield the BAD-like BH3 mimetic, ABT-737. It was designed based on how BAD bound to BCL-X_L, and hence called a BAD-mimetic. True to its name, ABT-737 could bind to and inhibit BCL-2, BCL-X_L and BCL-w but not MCL-1, just like BAD. The binding affinity of ABT-737 to the aforementioned anti-apoptotic proteins is

extremely low at ≤1 nM and it was shown to be able to induce apoptosis as a single agent in both haematological and solid cancer cell lines and could induce significant cytochrome *c* release in isolated mitochondria at concentrations as low as 10 nM. ABT-737 also showed low nanomolar activity in follicular lymphoma and CLL patient samples as well as xenograft models of small cell lung cancer, where it reduced tumour volume and greatly improved survival of mice[249].

Development of ABT-263

Despite its remarkable ability to induce apoptosis in cell lines and mouse models, the therapeutic potential of ABT-737 was limited by its poor oral bioavailability[246]. To first overcome this, a second-generation inhibitor, ABT-263 (Navitoclax) was developed through re-engineering of ABT-737. ABT-263 displayed similar binding affinities for BCL-2, BCL-X_L and BCL-w but did not bind the other pro-survival proteins as expected. The bioavailability of ABT-263 was assessed by oral gavage in several species and was shown to be ~20%, which increased to ~50% by incorporation into a lipid formula[246]. Like ABT-737, ABT-263 was also able to induce cytochrome *c* release as well as apoptosis in solid and haematological cancer cell lines and regression of tumours in xenograft mouse models, all at clinically relevant doses[246]. This suggested that ABT-263 could have great therapeutic potential for treating various tumours in the clinic. The specificity of ABT-263 was also demonstrated by the fact that it could not induce apoptosis in BAX/BAK null cells, indicating that ABT-263 functions through the apoptotic pathway and can be considered a *bonafide* BH3 mimetic[246].

ABT-199 – the first BCL-2 specific and highly effective BH3 mimetic

Due to extremely promising pre-clinical outcomes, ABT-263 was taken forward to multiple Phase I and II clinical trials in both lymphoid and solid (small cell lung cancer) malignancies. While ABT-263 resulted in extensive apoptosis and increased survival of patients, this was marred by its dose-limiting thrombocytopenia[250–253]. Studies confirmed that the thrombocytopenia observed was, in fact, an on-target toxicity, as ABT-263 inhibited multiple members of the pro-survival BCL-2 family, including BCL-$X_{L,}$ which is required for survival of platelet cells[254].

To circumvent the unwanted toxicity of ABT-263, ABT-199 (Venetoclax), a BCL-2-specific inhibitor, was developed for use in CLL, where BCL-2 is a major contributing factor to disease progression[255,256]. ABT-199 showed a remarkable sub-nanomolar affinity for BCL-2 and induced significant apoptosis in several BCL-2-dependent malignancies including non-Hodgkin's lymphoma cell lines. *In vivo* xenograft studies also demonstrated that ABT-199 can work as a monotherapy or in combination with other chemotherapeutic agents to promote tumour regression and increase survival, all without causing thrombocytopenia[256]. Furthermore, an initial study of ABT-199 in patients suffering from refractory CLL demonstrated that ABT-199 can induce a rapid reduction (within 24 h of administration) in tumour burden in patients while sparing platelets[135,256]. ABT-199 has since been FDA-approved to treat refractory CLL with the 17p deletion[257] and is being explored for the treatment of other BCL-2-driven malignancies, both as a single agent and in combination with other therapies, including non-Hodgkin's lymphoma[258], acute myeloid leukaemia[259] and multiple myeloma[260,261].

Development of BCL-X_L specific inhibitors

With the successful development of a specific and highly potent BCL-2 inhibitor complete, it set the basis for development of specific inhibitors of the other pro-survival BCL-2 family members, including BCL-X_L and MCL-1. Several inhibitors have been developed that specifically target BCL-X_L, the first being WEHI-539, identified in a high-throughput screen of ~100,000 compounds. The compound was shown to have sub-nanomolar affinity for BCL-X_L and was able to induce apoptosis in MCL-1-null fibroblasts but not BAK-null MEFs, suggesting it acts through the apoptotic pathway as expected. As another marker of on-target activity, the effect of WEHI-539 was assessed on platelets derived from mice which should require BCL-X_L for survival. WEHI-539 was able to induce apoptosis in these cells, suggesting that it does indeed hit BCL-X_L[262].

While WEHI-539 was an extremely important leap in the development of BH3 mimetics, its chemical structure (with potentially toxic hydrazone groups) limited its use in the clinic[262–264]. Thus, further work was carried out in search of more BCL-X_L inhibitors, such as A-1155463[263] and A-1331852[265]. Both of these compounds were able to induce a BCL-X_L specific apoptosis in the nanomolar range, with A-1331852 being up to 50-fold more potent than A-1155463 while also being orally bioavailable. These inhibitors are extremely important steps forward in targeting the BCL-2 family but also useful tools for dissecting the functional interplay between the pro- and anti-apoptotic proteins and have provided a basis for the development of further therapeutic tools.

In search of a potent, MCL-1-specific BH3 mimetic

The development of BH3 mimetics targeting BCL-2, BCL-X_L and BCL-w proved to be relatively successful, however development of a specific MCL-1 inhibitor has proven much more difficult. This is thought to be linked to the fact that the MCL-1 hydrophobic binding groove possesses a very rigid conformation compared to the other anti-apoptotic BCL-2 family members. Furthermore, MCL-1 has a very high affinity for its endogenous BH3-only protein binding partners[266]. However, the MCL-1-specific BH3 mimetic field has developed at a very rapid pace in the past few years, beginning with the development of A-1210477[267]. This compound was developed as the lead MCL-1 inhibitor in a series of compounds derived from an indole-2-carboxylyic acid core following a structure-guided inhibitor design. The inhibitor displaced the endogenous ligands from the hydrophobic binding groove of MCL-1 and also induced the typical hallmarks of apoptosis in MCL-1-dependent cell lines[267]. While A-1210477 was shown to have a high binding affinity for MCL-1 (0.45 nM), the *in vivo* working concentration was in the micromolar range[267] which restricted its use to preclinical studies. However, A-1210477 demonstrated the classic features of a *bona fide* MCL-1 inhibitor and was an important step in the development of MCL-1 inhibitors[267].

Since the development of A-1210477, multiple other compounds which are purportedly better inhibitors of MCL-1 have been demonstrated. AMG-176 is a novel MCL-1 inhibitor which has been shown to have picomolar binding ability *in vitro* and was able to rapidly induce apoptosis in various haematological cancer cell lines as well as tumour xenograft models and patient samples. Furthermore, AMG-176 could synergise with other BH3 mimetics to induce apoptosis in AML models, suggesting that it may have great therapeutic potential[268]. Indeed, a clinical trial is currently

recruiting patients to test AMG-176 as an MCL-1 inhibitor in relapsed or refractory multiple myeloma and acute myeloid leukaemia (ClinicalTrials.gov Identifier: NCT02675452), further highlighting its potential.

Likewise, S63845 is another promising MCL-1 inhibitor, developed following an NMR-based fragment screen and structure-guided discovery. S63845 binds the BH3 binding groove of MCL-1, partly due to its carboxylate moiety strongly binding a well-known anchor point for anti-apoptotic BCL-2 family inhibitors at Arg263 (also seen in multiple other MCL-1 inhibitors[267]). S63845 could induce rapid apoptosis at nanomolar concentrations in several cell lines and also demonstrated potential for *in vivo* use with no apparent toxicity in mice[269]. Subsequent studies have confirmed the potency of S63845 and further explored its potential use in synergy with other chemotherapeutic agents in various cancers including AML[270], T-cell acute leukaemia[271] and breast cancer[202].

Thus, despite the challenges facing development of a specific MCL-1 inhibitor, recent advances have been greatly successful and multiple highly potent and specific MCL-1 inhibitors are now in the process of being developed for therapeutic use, while also providing excellent tools for the study of the biology of MCL-1.

1.15 Challenges facing BH3 mimetic therapy

Immense progress has been made in the BH3 mimetic field recently, however the use of these compounds in a clinical setting still faces a wide array of challenges. BCL-2 can now be effectively targeted using ABT-199 in a clinical setting, often achieving excellent results in specific subsets of cancers. Meanwhile, the targeting of BCL-X_L, while achievable, faces its own challenges in the form of on-target toxicity, as previously described. Moreover, now that effective inhibitors of these proteins have

been developed, possible resistance mechanisms are being highlighted in the literature. Indeed, it has been demonstrated that tumours may acquire resistance to ABT-199 through expression of MCL-1 or BCL-X_L[272,273], while other studies have shown that ABT-199 resistance may arise through downregulation of BCL-2 and/or BIM[274] or even acquired mutations in BCL-2 itself[275].

Inhibition of MCL-1, either as a first-line therapy or to overcome resistance to other BH3 mimetics, provides unique challenges too. The multi-faceted nature of MCL-1 along with its extensive healthy tissue expression means that targeting MCL-1 in human cancer may have undesirable side-effects and therefore must be carefully explored and controlled prior to potential MCL-1 inhibitors entering the clinic. Indeed, recent animal studies show that loss of MCL-1 (via genetic knockout) results in cardiotoxicity as a result of altered mitochondrial function – indicating the importance of MCL-1 in regulation of mitochondrial dynamics and bioenergetics[276,277]. Hence, further knowledge regarding how MCL-1 functions in both normal and cancerous cells is required in order to efficiently target MCL-1 in tumour cells while causing minimal harm to healthy cells. Until recently, the possibility of specifically targeting MCL-1 in cancer remained difficult, however following the development of inhibitors specific to individual BCL-2 family members, several novel MCL-1 inhibitors which work at clinically acceptable concentrations have now been established. While these inhibitors must be trialled extensively before use in patients, the advent of highly specific and highly potent BH3 mimetics which have enormous potential therapeutic value is a great step forward for the programmed cell death field.

1.16 Aims

Following the recent development of purportedly highly potent and specific MCL-1 inhibitors, this thesis aims to explore these novel compounds in order to confirm their ability to induce apoptosis alone and in combination with other BH3 mimetics, which could potentially represent highly effective chemotherapeutic approaches in the future. The first specific MCL-1 inhibitor, A-1210477, works in the micromolar range to induce apoptosis, however our group has multiple MCL-1 inhibitors, both published and unpublished, which are proposed to work in the nanomolar range, representing a huge potential improvement in MCL-1-targeting. Since MCL-1 is one of the most overexpressed genes in cancer and represents a key therapeutic target, validating the efficacy of these new inhibitors and exploring their mechanism is of great importance as it improves the scope of targeting MCL-1 in cancer.

The mechanism of action of MCL-1 and other BCL-2 family inhibitors will also be explored in order to identify the role of BH3-only proteins in apoptosis induction, which has recently been identified as a controversial point in the field. It is now known that the BH3-only proteins may be dispensable for apoptosis induction when both MCL-1 and BCL-X_L are neutralised, however it is not known if highly specific BH3 mimetics, which are now available for all major pro-survival proteins, can induce apoptosis in the absence of the BH3-only proteins. This is important to explore as this goes against the proposed mechanism of action of BH3 mimetics and raises important questions as to how they function.

Furthermore, this thesis will explore novel MCL-1-interacting proteins in order to attempt to explain BH3-only protein-independent apoptosis. The notion that undiscovered, BH3-only-like proteins exist, which could be binding to MCL-1 and

mediating apoptosis in the absence of the known BH3-only proteins is one critical hypothesis to explain the previously observed BH3-only protein-independent apoptosis. MCL-1 is known to be an unusually complicated member of the BCL-2 family, with multiple binding partners and functions lying outside of apoptosis, thus, this work will not only address the possibility of novel BH3-only-like proteins, but may also identify new functions or pathways that MCL-1 is involved in. This is a key angle of MCL-1 biology research as it is a complex protein whose diverse functions remain to be fully understood. This could mean that there may be on-target but unexpected effects of targeting MCL-1 in cancer.

With all of this taken into account, the main aims of this study are (1) to assess the specificity and potency of novel MCL-1 inhibitors as well as their mechanism of action, (2) to determine the requirements of the known BH3-only proteins in BH3 mimetic-induced apoptosis and (3) to identify novel MCL-1-interacting proteins which could mediate apoptosis in the absence of the 8 major BH3-only proteins.

Chapter 2

Materials and Methods

Contents

2.1 Cell culture

Cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) or American Type Culture Collection (ATCC, Manassas, VA, USA) unless otherwise specified in Table 2.1, and were cultured in appropriate cell culture media, as indicated in Table 2.1. Cell culture media was purchased from Life Technologies Inc (Paisley, UK) and supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine (Life Technologies). Cells were grown at 37°C, 5% CO_2 and were passaged every 48 h or according to their growth rate in order to maintain approximately 80% confluence. For passaging of adherent cell lines, media was aspirated from cell culture dishes, cells washed once with warmed PBS (ThermoFisher Scientific, MA, USA) and then incubated with 0.05% trypsin-EDTA (Gibco, ThermoFisher Scientific) at 37°C for 5 min. Fresh media was then added to the cells to inhibit the activity of trypsin and cell suspension divided into plates with new media accordingly. Suspension cell lines had media replaced every 48 h.

2.2 Cell lines

Table 2.1 – Cell lines used in this study, their specified media, BCL-2 family protein survival dependencies and additional notes including sources.

Media	Cell line and tumour origin	Reported survival dependency	Notes
Roswell Park Memorial Institute (RPMI) 1640	H1299, H23 (non-small cell lung carcinoma, adherent)	BCL-X_L and MCL-1, MCL-1	Purchased from ATCC.
	PC-3 (prostate cancer, adherent)	BCL-X_L and MCL-1	Gift from Y. Ke (University of Liverpool).
	MV-4-11, OCI-AML3, HL-60, U937, THP-1, MOLM-13 (acute myeloid leukaemia, suspension)	BCL-2 and MCL-1	Purchased from ATCC.
	U-2946 (diffuse large B-cell lymphoma, suspension)	MCL-1	Purchased from DSMZ.
	Jurkat (acute T-cell leukaemia, suspension)	MCL-1	Wild-type, caspase-9 null, caspase-8 null and FADD null Jurkat cells were gifts from J. Blenis (Weill Cornell Medicine, USA).
	K562, KCL-22 (chronic myeloid leukaemia, suspension)	BCL-X_L	Gift from R. Clark (University of Liverpool).
	MAVER-1 (mantle cell lymphoma, suspension)	BCL-2	Gift from J. Slupsky (University of Liverpool).
	H929 (multiple myeloma., suspension)	MCL-1	Purchased from ATCC. Additionally supplemented with 0.02% 2-mercaptoethanol.
	Chronic lymphocytic leukaemia (CLL) patient cells, suspension	BCL-2	Freshly isolated from CLL patient blood samples after patient consent and ethical approval.
Dulbecco's modified Eagle Medium (DMEM)	HELA (cervical carcinoma, adherent)	BCL-X_L and MCL-1	Purchased from ATCC.
	SUIT-2 (pancreatic cancer, adherent)	BCL-X_L and MCL-1	Gift from A. Mielgo (University of Liverpool).
	MDA-MB-231 (triple negative breast cancer, adherent)	BCL-X_L and MCL-1	Gift from P. Meier (Institute of Cancer Research, UK).

McCoy's 5A Modified	HCT116, HT-29 (colon carcinoma, adherent)	BCL-X_L and MCL-1	HCT116 WT, DKO and 8KO cells were from our collaborator X. Luo (University of Nebraska). HCT116 BAX/BAK KO cells were from R. J. Youle (National Institute of Health, USA).
			HT-29 cells were a gift from J. Parsons (University of Liverpool).
DMEM/F-12	A549 (non-small cell lung carcinoma, adherent)	BCL-X_L and MCL-1	Purchased from ATCC.

2.3 Cell line authentication

Genomic DNA from all cell lines was isolated using the DNeasy kit (Qiagen, Cambridge, UK) and the samples were subjected to short tandem repeat (STR) profiling to ensure authenticity using GenePrint® 10 (Promega, Madison, WI, USA) at the University of Liverpool Cell Line Authentication Facility. Percentage matches of cell lines to multiple cell databases are indicated in Table 2.2.

Table 2.2 – Cell lines were authenticated using STR profiling and matched against multiple databases. Cells were required to have at least an 80% match to online databases. *MDA-MB-231 cells were originally thought to be HCC-1187 cells, however STR profiling revealed that they matched 83% to MDA-MB-231 versus 34% to HCC-1187.

Cell line	% Match	Databases
OCI-AML3	100%	DSMZ and ExPASy
HL-60	100%	DSMZ, PHEngland and ExPASy
MOLM-13	100%	DSMZ, ExPASy
MV-4-11	100%	DSMZ, ATCC
THP-1	100%	DSMZ, ATCC, PHEngland
U-937	100%	DSMZ, ATCC, PHEngland
K562	83%	DSMZ
U-2946	100%	DSMZ
H929	79%	DSMZ, ATCC
MAVER-1	94%	DSMZ
KCL-22	100%	DSMZ
H1299	100%	DSMZ/ATCC
A549	86%	DSMZ, ATCC, ExPASy
PC-3	92%	ATCC
SUIT-2	100%	DSMZ
HCT116 WT	89%	DSMZ, ATCC
HCT116 8KO	89%	DSMZ, ATCC
HT-29	100%	DSMZ, ATCC
HELA	100%	PHEngland, ExPASy
MDA-MB-231	34%*	DSMZ
H1299	100%	DSMZ, ATCC

2.4 Reagents and inhibitors

All reagents, chemicals and buffers, unless otherwise specified, were from Sigma-Aldrich (now Merck) Darmstadt, Germany)). Inhibitors used throughout the study are indicated in Table 2.3. Large-scale pre-prepared subcellular fractions (extranuclear, nuclear, 'S100', mitochondria) for immunoprecipitation and mass spectrometry analysis were purchased from Ipracell (Ipratech, Belgium) and lysed according to the immunoprecipitation protocol outlined.

Table 2.3 – Inhibitors used, their targets and sources. Selleck represents Selleck Chemicals Co (Houston, TX, USA), Abbvie represents Abbvie (Chicago, IL, USA), Active Biochem represents Active Biochem (New Jersey, USA).

Inhibitor	Target	Source
ABT-199	BCL-2	Selleck
A-1331852	BCL-X_L	Abbvie
A-1210477	MCL-1	Abbvie
S-30	MCL-1	Collaborator gift
AMG-176	MCL-1	Amgen
S63845	MCL-1	Selleck
MG-132	Proteasome	Active Biochem
Z-VAD-FMK	Caspases	Selleck

2.5 Antibodies

Table 2.4 – Antibodies used in different protocols, divided into functional subsets. Clone numbers and epitopes are indicated where available. The applications where each antibody was used is also indicated. 'AA' represents amino acids. 'SC' represents Santa Cruz Biotechnology (CA, USA), 'CST' represents Cell Signalling Technologies (MA, USA), 'Abcam' represents Abcam (Cambridge, UK), 'BD' represents BD BioSciences (CA, USA), 'Milli'. represents Millipore/Merck (Darmstadt, Germany), 'Enzo' represents Enzo Biochem Inc (NY, USA), 'Calbio'. represents Calbiochem Research Biochemicals (now Merck) and 'Upstate' represents Upstate USA Inc (NY, USA).

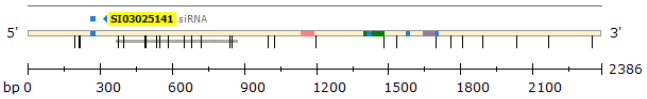
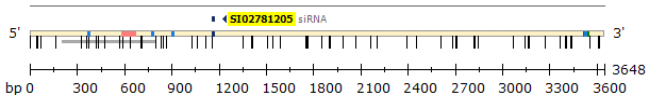
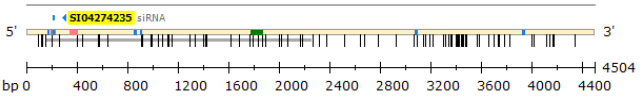
Antibody	Epitope	Application(s)	Source	Catalogue #
BCL-2 family				
BCL-2 (100)	AA 41-54	Immunoblotting	SC	sc-509
BCL-2 (6C8)	Recombinant protein	Immunoprecipitation	BD	51-1513GR
BCL-X_L	Around Asp61	Immunoblotting	CST	2762
BCL-X_L (E-18]	AA 1-100	Immunoprecipitation	Abcam	ab32370
MCL-1 (S-19)	AA 89-139	Immunoblotting	SC	sc-819
MCL-1 (Y-37)	AA 100-200	Immunoprecipitation	Abcam	ab32087
PUMA	C terminal	Immunoblotting	CST	4976
BIM (C34C35)	Around Pro25	Immunoblotting	CST	2933
BIK	N-terminal	Immunoblotting	SC	sc-1710
BAD	Around Ser112	Immunoblotting	CST	9292
BMF	Around Gly81	Immunoblotting	CST	5889
HRK	AA 30-50	Immunoblotting	SC	sc-6971
NOXA (114C307)	Recombinant protein	Immunoblotting	Milli.	OP180
BID	Cleavage site	Immunoblotting	CST	2002
BAK (AB-1)	Active Site	Activation assay	Calbio.	AM-03
BAK	Internal	Immunoblotting	SC	sc-832

BAX (C3)	AA 55-178	Immunoblotting & Immunocytochemistry	BD	610983
BAX (6A7)	Active site	Activation assay	Enzo	ALX-804-224-C100
BAX (N-terminal)	N-terminal	Immunoprecipitation	Upstate	06-499
Apoptosis				
Cytochrome *c*	Around residue 62	Immunocytochemistry	BD	556432
Caspase-8 (1C12)	C-terminal	Immunoblotting	CST	9746
Caspase-9	Asp315	Immunoblotting	CST	9502
FADD	Ser194	Immunoblotting	CST	2782
Misc.				
Tubulin (6-11B-1)	Around Lys40	Immunoblotting	Abcam	ab24610
GAPDH (FL-335)	AA 1-335	Immunoblotting	SC	sc-25778
HSP60 (C24)	Recombinant protein	Immunocytochemistry	BD	611562
BAP31	C-terminal	Immunoblotting	Abcam	ab37120
DRP-1	AA 601-722	Immunoblotting	BD	611113
GM130	AA 869-982	Immunoblotting	BD	610822
Vimentin (V9)	Whole vimentin (porcine eye lens)	Immunoblotting	SC	6260
ATPB (3D5)	Whole mitochondria (human heart)	Immunoblotting	Abcam	ab14730
NDUFA9 (20C11)	Whole purified complex I (bovine)	Immunoblotting	SC	sc-58392
PARP-1 (F-2)	AA 764-1014	Immunoblotting	SC	sc-8007

2.6 RNA interference

All siRNAs (small interfering RNA oligoduplexes) were purchased from Qiagen (Cambridge, UK). Knockdowns were performed in Opti-MEM Reduced Serum Media (Life Technologies), using Interferin transfection reagent (Polyplus, Illkirch, France). Cells were seeded at their required densities to be confluent following a 72 h incubation with a 10 nM final concentration of siRNAs.

Table 2.5 – siRNA catalogue numbers and their target regions of the indicated proteins. The targeted region of the proteins is indicated by the highlighted region, with the grey bar below the transcript indicating the coding sequence of the gene. The BCL-X_L siRNA targets the 5' untranslated region (UTR) while the MCL-1 and HRK siRNAs target the 3' UTR. The DRP-1 siRNA targets the coding region.

Protein targeted	siRNA catalogue number	Sequence Targeted
BCL-X_L	SI03025141	TTGGCTTTGGATCTTAGAAGA 
MCL-1	SI02781205	CCCGCCGAATTCATTAATTTA 
DRP-1	S104274235	CACGGTGGGCGCCGACATCAT 
Non-targeting control	1027310	

2.7 SDS-PAGE (Immunoblotting)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using in-house gels made with lower gel buffer (1.5 M Tris-HCL, 0.4% SDS, pH 8.8), upper gel buffer (Tris-HCL 0.5 M, 0.4% SDS, pH 6.8) Protogel acrylamide (30%) (National Diagnostics, Atlanta GA, USA), 10% APS and TEMED.

Cells intended for immunoblotting were collected, pelleted by centrifugation and lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) containing 20 µM MG-132 and a protease inhibitor tablet (Roche, Basel, Switzerland). Cells were incubated on ice for 15 min in RIPA lysis buffer then subjected to sonication using a Bandelin SONOPULS ultrasonic homogeniser (Bandelin, Berlin, Germany). Protein concentration was then determined in a colorimetric (Bradford) assay using the BioRad Protein Assay Dye Reagent (BioRad, CA, USA). Optical density at 600 nm (OD600) of samples was measured in a spectrophotometer (Eppendorf, Hamburg, Germany) and compared to a known standard of bovine serum albumin (BSA) to establish protein concentrations.

10-50 µg of protein lysate was then combined with 4x NuPAGE LDS sample buffer (Life Technologies) and heated for 5 min at 70°C (ThermoFisher Scientific). Samples, alongside the SeeBlue™ Plus2 Pre-stained Protein Standard (ThermoFisher Scientific) were then subjected to SDS-PAGE in electrode buffer (25 mM Tris-HCL, 192 mM glycine, 10% SDS) at 130 V for 90 min then transferred to Amersham ProTran nitrocellulose membranes (VWR, Radnor, PA, USA) in transfer buffer (25 mM Tris-HCL, 192 mM glycine, 20% methanol) at 100 V for 90 min. Membranes were subsequently blocked in non-fat 5% milk in TBS-T (Tris-buffered saline with

0.1% Tween, 20 mM Tris-HCL, 150 mM NaCl, 0.1% Tween-20) for 30 min then incubated with the indicated primary antibodies, diluted 1:1000 in TBS-T, overnight at 4°C. Membranes were then rinsed with TBS-T and incubated with either anti-mouse or anti-rabbit IgG, HRP-linked secondary antibodies (Cell Signalling Technology) for 1 h, then washed three times in TBS-T before visualisation of protein bands with ECL reagents (GE Healthcare, IL, USA) either using X-ray film (GE Healthcare) or the ChemiDoc Imaging system (BioRad).

2.8 Immunoprecipitation

For immunoprecipitation (IP) experiments, Protein G Dynabeads (30 µl/sample) (ThermoFisher Scientific) were washed three times with PBS-T (PBS with 0.05% Tween-20) before being resuspended in PBS-T with 5 µg of antibodies against the protein of interest. The antibody-protein mix was rotated for 4 h at 4°C and beads subsequently washed with 0.2 M triethanolamine (TEA). The antibody-bead conjugates were then cross-linked using 5.4 mg/ml dimethylpimelidate (DMP) at room temperature for 30 min. The cross-linking reaction was then neutralised with 50 mM Tris-HCL pH 7.5 at room temperature for 15 min, with agitation. Bead-antibody complexes were then washed three times in PBS-T and resuspended in 500 µg of cell lysate.

Cell lysates for immunoprecipitation were prepared using 1% CHAPS lysis buffer (150 mM KCl, 50 mM HEPES (pH 7.4) 1% CHAPS) containing 20 µM MG-132 and a protease inhibitor tablet. Cells were incubated with 1% CHAPS lysis buffer for 45 minutes on ice and centrifuged at 14,000 RPM for 10 min at 4°C to remove cell debris. Bead-antibody complexes were incubated with the lysates overnight at 4°C with rotation and then flow-through (immunodepleted lysates collected post-immunoprecipitation) collected and the beads washed three times with PBS-T. Protein

was eluted from beads using 2x NuPAGE LDS sample buffer (Life Technologies) by incubation at 55°C for 10 min with shaking and samples subsequently loaded onto gels alongside an input (whole cell lysate from the same lysates used for the immunoprecipitation) and in some cases, a bead control (beads with no antibody bound, incubated with lysates) and a flow-through. Immunoblotting for immunoprecipitation experiments was carried out according to the immunoblotting protocol described. Immunoprecipitation for mass spectrometry analysis was performed in the same way but with minor changes. Lysates were incubated for 30 min at 4°C with 30 µl protein G Dynabeads to pre-clear and reduce non-specific binding of protein to beads. Furthermore, pre-elution, the beads were washed with low-salt PBS and water and protein was eluted using 1% Rapigest (Waters, UK) into lo-bind tubes (Eppendorf).

2.9 Coomassie staining of protein gels

Gels for Coomassie staining were subjected to electrophoresis in the same way as done for standard SDS-PAGE. Upon completion of electrophoresis, the gels were fixed using a mix of 45% methanol and 10% glacial acetic acid for 1 h at room temperature. Gels were then washed and incubated with EZBlue Gel Staining Reagent overnight at room temperature, washed 3 times with water for 5 min and images acquired using the BioRad ChemiDoc imaging system.

2.10 Fast protein liquid chromatography

Fast protein liquid chromatography (FPLC), used to fractionate cell lysates to analyse BCL-2 family protein complexes, was performed using an FPLC ÄKTA system with Superose 6 and Superdex 200 columns (GE Healthcare). Columns were washed prior to use with water then equilibrated with running buffer (50 mM Tris, 150 mM KCL,

1 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF, pH 8). Columns were also subjected regular runs with protein standards to determine fraction sizes. Cells intended for gel filtration were lysed in 1% CHAPS on ice for 45 min and then centrifuged at 14,000 RPM at 4°C for 10 min and the supernatant collected as protein lysate. Lysates were filtered to remove any remaining debris and subjected to Bradford assays as described previously to determine protein concentration. 500 µl protein lysate (minimum 20 mg/ml) was then loaded on to the columns using a 500 µl loop and collected in 50 x 500 µl fractions. Collected fractions were either mixed directly with 4x LDS sample buffer or saved as pure lysate for further experiments. The UNICORN Control System software (GE Healthcare) was used to control the columns. Column peaks were used to determine which fractions would be loaded on gels, typically beginning with fraction 16 of Superose 6 and ending with fraction 35 of Superdex 200.

2.11 Flow cytometry – apoptosis

Cells were seeded at a density of 0.1 million cells/well and exposed to the required drug treatment for the indicated time. Following incubation at 37°C, cells were collected – cells from haematological malignancies were directly transferred to tubes while adherent cells were washed once with PBS, trypsinised and collected in their media before being centrifuged to pellet the cells. For Annexin-V-FITC and propidium iodide (PI) labelling, cells were resuspended in 500 µl of 1x Annexin Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4 with Annexin-V (1:20,000, made in-house) and 5 µl PI added just before analysis. To assess the extent of labelling, cells were passed through the Attune NxT flow cytometer (ThermoFisher) using red and blue lasers for PI and Annexin V staining respectively. 10,000 events were captured per sample and at least three replicates were analysed for each experiment.

2.12 Flow cytometry – BAX/BAK activation

To measure BAX and BAK activation, cells were exposed to the indicated treatments, collected and fixed with 2% paraformaldehyde (PFA) at room temperature for 10 min. Fixed cells were then washed with PBS and re-suspended in permeabilisation buffer (0.1% saponin, 0.5% BSA in PBS) for 10 min. Primary antibodies (mouse anti-BAX 6A7 or mouse anti-BAK AB-1) were then added to the cells at a volume of 1:100 in permeabilisation buffer and cells incubated for 1 h at 4°C. Goat-anti-mouse IgG-AlexaFluor-488 conjugated secondary antibody was then mixed with the cells and incubated for 1 h at 4°C. Activated BAX or BAK was then detected using flow cytometry using a blue (BL1) laser (530/30 nm emission filter) as the excitation source for the AlexaFluor-488 tag of the secondary antibodies.

2.13 Flow cytometry – BH3 profiling

Cells were collected in TEB buffer (Derived from Trehalose Experimental Buffer, 135 mM trehalose, 50 mM KCl, 20 µM EDTA, 20 mM EGTA, 0.1% BSA, 5 mM succinate, 10 mM HEPES-KOH pH 7.5) and permeabilised with digitonin (0.002%) and incubated at room temperature with the indicated peptides for 90 min. Tetramethylrhodamine ethyl ester (TMRE), a positively charged dye that accumulates in active mitochondria as a result of membrane potential[278] was then added (200 nM final) to each tube and the cells then incubated a further 30 min at room temperature and loss of mitochondrial membrane potential (ψ_m) assessed using flow cytometry (blue (BL2) laser, 574/26 emission filter). FCCP (Carbonyl cyanide-4-phenylhydrazone), a mitochondrial un-coupler was used as a positive control for depolarisation[279].

Peptides used in BH3-profiling were MS-1 (RPEIWMTQGLRRLGDEINAYYAR) and BAD (LWAAQRYGRELRRMSDEFEGSFKGL) from New England Peptide (Gardner, MA, USA).

2.14 Immunocytochemistry

Cells were seeded at 0.1 million cells/well on sterile coverslips and left to grow for the required time, up to 70% confluence. Coverslips were removed from media and cells fixed in 4% (w/v) paraformaldehyde for 10 min then permeabilised in 0.5% (v/v) Triton X-100 in PBS for 10 min, both at room temperature. Coverslips were then incubated with primary antibody diluted 1:300 in 3% BSA for 1 h followed by a 1 h incubation with an AlexaFluor fluorescently-conjugated secondary antibody (Life Technologies) diluted in 1:2000 in 3% BSA. Coverslips were then washed briefly three times in water and mounted on glass slides using Polymount (Polysciences, PA, USA) and imaged using a 3i Marianas spinning disk confocal microscope, fitted with a Plan-Apochromat × 63/1.4 NA Oil Objective, M27 and a Hamamatsu ORCA-Flash4.0 v2 sCMOS Camera (Intelligent Imaging Innovations, GmbH, Gottingen, Germany).

2.15 Molecular biology

mRNA extraction

To assess mRNA levels of BCL-2 family members in HCT116 cells, the three cell lines used (WT, DKO and 8KO) were subjected to RNA extraction using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) according to the kit protocol. Briefly, 1 x 10^7 cells were harvested per cell line and lysed in RLT buffer. 1 volume of 70% ethanol was then added to the lysate and mixed before being transferred to a RNeasy Mini spin column in a collection tube and the tubes centrifuged together for 15 sec at 8000 x *g*.

The flow-through was discarded and Buffer RW1 was added to the spin column and centrifuged again. The flow-through was discarded and RPE buffer was added and the tubes spun again. This step was then repeated before the column was placed in a 1.5 ml collection tube and 30 µl RNase-free water added to the column. The tubes were spun at 8000 x g for 1 min and the RNA yield quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific).

Generation of cDNA

The mRNA from the RNA extraction above was then used to generate cDNA from the three cell lines using reverse transcription. To generate cDNA, 2 µg RNA was mixed with 1 µl 10 mM dNTPs, 50 µg Oligo dT and nuclease-free water and incubated at 65°C for 5 min then the mixture combined with 2 µl 10x reverse transcriptase buffer, 50 mM 2 µl $MgCl_2$, 1 µl DTT, 1 µl RNase out (recombinant RNase inhibitor), 1 µl SuperScript II RT polymerase (200 units/µl) and nuclease-free water. The mix was then subjected to PCR with the following conditions: 50 ^oC for 100 min, 85 ^oC for 5 min. All reverse transcription materials were purchased from Invitrogen (CA, USA).

qPCR

qPCR was performed using gene-specific QuantiTect Primer Assay primers (Qiagen) for BCL-2 (QT00025011), BIK (QT00070777) and HRK (QT00203588) (Qiagen) using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Estonia). The qPCR reaction was run on Stratagene Mx3005P (Thermo Fisher) under the following cycling conditions: 1 cycle: 95 ^oC for 15 min. 45 cycles: 95 ^oC for 15 sec, 60 ^oC for 20 sec, 72 ^oC for 30 sec, 78 ^oC for 11 sec. 1 cycle: 95 ^oC for 1 min, 55 ^oC for 30 sec and 97 ^oC continuously. Relative mRNA expression levels of proteins of interest were calculated using the following formula[280]:

$$= 2^{-(\Delta CT_{\text{gene of interest}} \Delta CT_{\text{gapdh}})}.$$

2.16 Mass spectrometry and analysis

Three individual replicates of eluates from immunoprecipitation of MCL-1 in each Ipracell subcellular fraction, plus bead controls (beads and lysates with no antibody) were submitted for mass spectrometry analysis. Mass spectrometry and statistical analysis was performed by The Centre for Proteome Research at The University of Liverpool by Deb Simpson.

Protein lists were returned for each subcellular fraction with peptide counts, calculated *p* values based on the three replicates, *q* values to adjust for false discovery rate (FDR), maximum fold change in the bead control versus immunoprecipitation and confidence scores. Proteins were selected for pathway analysis if they were detected > 2-fold higher in the immunoprecipitation versus bead control, > 2 spectral counts (i.e. more than 2 peptides of an individual protein detected) and < 10% frequency in a database of 400+ mass spectrometry experiments (the CRAPome). Subsequently, only proteins with fold change of > 5 in the immunoprecipitation versus bead control and a *q* value of < 0.02 were selected to further remove non-significant proteins. Where protein lists were still high in number following this triage, pathway analysis was performed using the Reactome and proteins associated with apoptosis highlighted.

2.17 Statistics

For time-course studies, a two-way ANOVA was performed. All other studies were analysed for statistical significance with one-way ANOVA and the asterisks depicted correspond to the following *p* values: * for $p \leq 0.05$, ** for $p \leq 0.005$ and *** for $p \leq 0.001$.

Chapter 3

MCL-1 inhibitors and the BCL-2 protein family

Contents

3.1 Background

BH3 mimetics, the novel group of pharmacological compounds, were designed to function like BH3-only proteins - binding to the hydrophobic groove of the pro-survival proteins and displacing them from their pro-apoptotic BH3-only protein partners. Subsequently, the native pro-apoptotic BCL-2 family members are proposed to be free to induce apoptosis. In order to be classified as a *bona fide* BH3 mimetic, a compound must comply with two key criteria: bind to target anti-apoptotic proteins with a high affinity and induce a BAX- and/or BAK-dependent apoptosis[245]. As described earlier, the first clinically viable BH3 mimetic was ABT-263 (Navitoclax), an orally available form of ABT-737, which inhibits BCL-2, BCL-X_L and BCL-w and potently induces apoptosis in several solid and haematological cancers[246]. Navitoclax was identified as a very strong chemotherapeutic candidate for chronic lymphocytic leukaemia (CLL) as many of these malignancies demonstrate dependence on BCL-2 for survival[12,281,282]. Although initial results of trials of ABT-263 in CLL were encouraging, studies were halted by a dose-limiting thrombocytopenia – a result of ABT-263's ability to target BCL-X_L in addition to BCL-2. The inhibition of BCL-X_L resulted in the death of platelets as they are extremely dependent on the anti-apoptotic BCL-X_L for survival[254]. While this was an on-target effect of ABT-263, it meant that the use of Navitoclax in patients was limited. Thus, ABT-263 was re-engineered and was soon followed by ABT-199 (Venetoclax), a BCL-2-specific inhibitor, which could induce cytotoxicity at a nanomolar range while sparing platelets[256]. ABT-199 has proven to be greatly successful in the clinic and is now FDA-approved for use in patients with CLL refractory to at least one prior treatment. However, potential resistance mechanisms to ABT-199 have already been reported in some diseases[273–275]. One of the major resistance factors is likely upregulation of MCL-1 – an anti-

apoptotic protein which has remained largely untargeted by specific BH3 mimetics, until recently. MCL-1 is a key survival protein in many cancers and is associated with resistance to various chemotherapeutic approaches. As a result, there has been an intense effort to identify specific MCL-1 inhibitors that have a high potency.

While indirect methods for inhibiting MCL-1 have been established, only recently has a *bona fide* MCL-1-specific inhibitor which works at a clinically relevant concentration been demonstrated, in the form of S63845[269]. Efforts to develop MCL-1 specific inhibitors which work at low nanomolar concentrations have proven difficult and it is thought that this, in part, is due to the properties of the BH3 binding groove of MCL-1 which has been shown to be very rigid and furthermore, tightly binds the native BH3 only proteins, making them hard to displace[266]. In addition to S63845, multiple other MCL-1 specific compounds have been or are in development and many groups and pharmaceutical companies are all vying to develop the best MCL-1 inhibitors for future use in large number of cancers.

Here, a panel of four of the most recently described MCL-1 inhibitors (A-1210477, AMG-162, S-30 and S63845) were assessed to determine whether novel MCL-1 inhibitors have improved the potential of targeting MCL-1 in cancer. While A-1210477 is known to work in the micromolar range, it has not been compared to newer inhibitors such as S-30 and AMG-162. The ability of these inhibitors to induce the intrinsic pathway of apoptosis was assessed using cancer cell lines that lack distinct members of the apoptotic pathway, while the specificity of the inhibitors to exclusively target MCL-1 was assessed using a panel of cell lines that depend on BCL-2, BCL-X_L or MCL-1 for survival. The initial report on S63845 suggested high specifity to MCL-1, however, observations made here could imply otherwise. Choosing S63845 as the major focus of the subsequent studies, the potential for combining S63845 with other

BH3 mimetics was explored in a range of cancer types, while its ability to displace BH3-only members from MCL-1 was correlated with its ability to induce apoptosis in order to further characterise the mechanisms underlying MCL-1 inhibition and apoptosis induction in cancer.

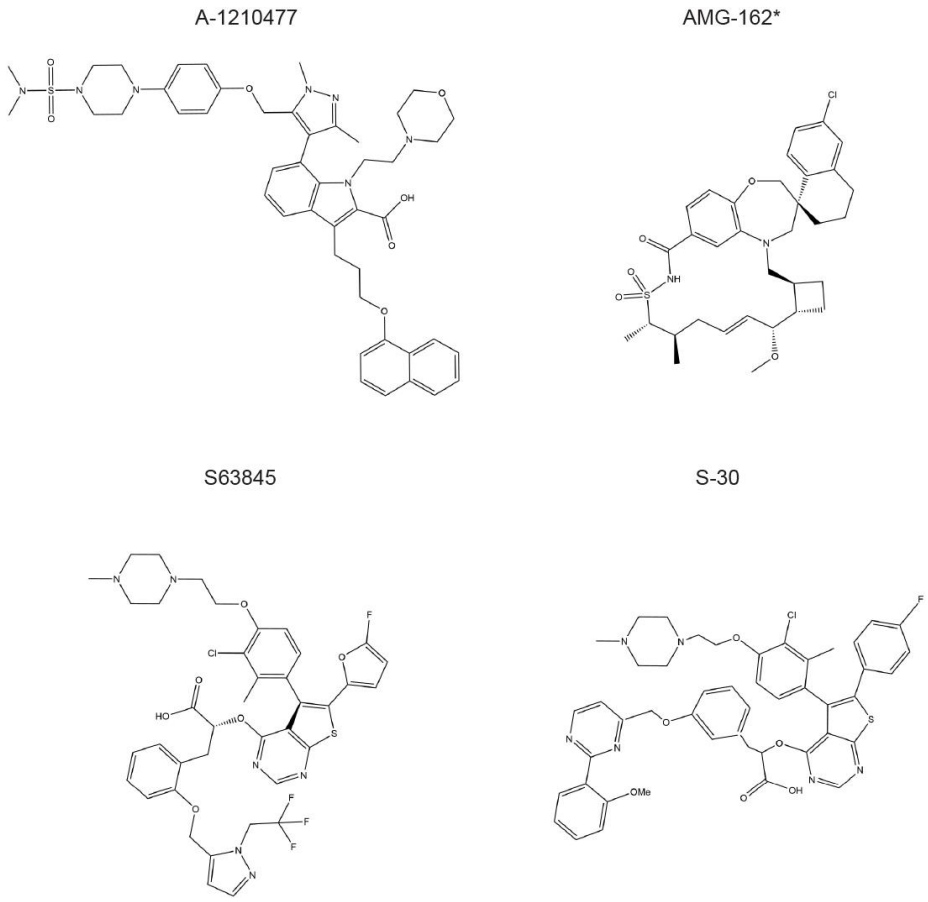


Figure 3.1. The chemical structures of four key MCL-1 inhibitors. The chemical structures of the four MCl-1 inhibitors A-1210477, AMG-162, S-30 and S63845, demonstrating their structural differences and similarities. *The chemical structure of AMG-162 is unavailable therefore a close analogue (AMG-176) has been utilised.

3.2 MCL-1 inhibitors induce rapid apoptosis in MCL-1-dependent cell lines.

In order to test the potential of the selected MCL-1 inhibitors to induce apoptosis in a MCL-1-specific manner, two cell lines known to be dependent on MCL-1 for survival, H929[267] and U-2946[283], were exposed to a concentration range of A-1210477, S63845, AMG-162 or S-30 for 4 h. Apoptosis was assessed using FITC-labelled Annexin V (which binds to externalised phosphatidylserine during apoptosis[284]) labelling in flow cytometry (Fig. 3.2). A-1210477 only induced significant apoptosis in both cell lines at 10 µM, in line with previous findings that it is effective in the micromolar range[267]. AMG-162 started to induce apoptosis at lower concentrations than A-1210477, however significant apoptosis could only be observed at ~1 µM, suggesting AMG-162 has an IC_{50} of ~500 nM. Meanwhile, both S63845 and S-30 had an IC_{50} of ~100 nM in both cell lines, suggesting that they are both equally potent and very effective MCL-1 inhibitors. While all four inhibitors were able to induce apoptosis in both MCL-1 dependent cell lines, it was evident that S63845 and S-30 are significantly better inhibitors than A-1210477 and AMG-162, both able to induce apoptosis at nanomolar concentrations (Fig 3.2).

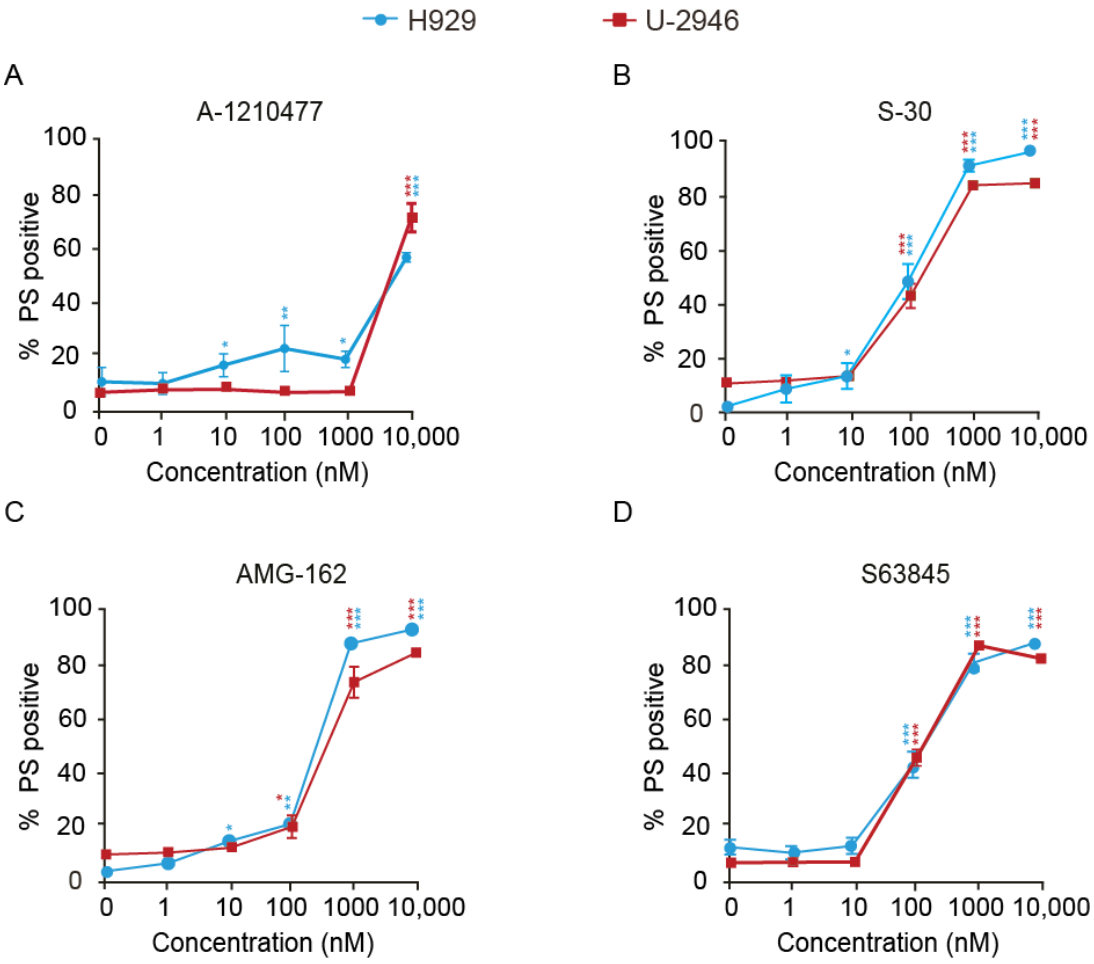


Figure 3.2. MCL-1 inhibitors can induce rapid apoptosis in MCL-1-addicted cell lines. H929 and U-2946 cells were exposed to a concentration range of either (A) A-1210477, (B) S-30, (C) AMG-162 or (D) S63845 for 4 h and apoptosis was assessed by phosphatidylserine (PS) externalisation using Annexin V labelling in flow cytometry. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

3.3 MCL-1 inhibitors bind to and stabilise MCL-1.

It is known that the binding of MCL-1 inhibitors to MCL-1 causes its stabilisation – an unexpected effect of compounds designed to ablate the activity of MCL-1[267,269]. While it is not entirely understood how MCL-1 is stabilised by its own inhibitors, it is possibly linked to the MCL-1-NOXA interaction, which is disrupted following BH3 mimetic exposure. NOXA is thought to continually promote the translocation, phosphorylation and degradation of MCL-1, thus it has been proposed that when BH3 mimetics disrupt the MCL-1-NOXA interaction, MCL-1 starts to stabilise[126,285]. In order to verify that the four drugs tested here were indeed binding to and stabilising MCL-1, HELA cells were exposed to the indicated compounds at their approximate IC_{50} measures at various timepoints up to 24 h and MCL-1 protein levels observed using immunoblotting (Fig. 3.3). At early timepoints in all conditions, of the three known bands of MCL-1 (a ~40 kDa isoform associated with anti-apoptotic activity[175], a ~38 kDa middle band and a ~36 kDa lower band, which has been associated with the inner mitochondrial membrane functions of MCL-1[175] and perhaps, its nuclear functions[177]) only the 40kDa isoform could be clearly seen (Fig 3.3). However, following exposure to the MCL-1 inhibitors, stabilisation of MCL-1 started to become obvious as early as 2 h and MCL-1 continued to stabilise from this point, with much higher levels of the long isoform of MCL-1 being detectable at 24 h in all treatments. Surprisingly, despite being one of the less potent MCL-1 inhibitors at inducing apoptosis, AMG-162 seemed to stabilise MCL-1 better than S-30 and S63845, as stabilisation occurred at earlier timepoints in the AMG-162-treated cells. This could be a result of 10-fold difference in concentration used, however. Regardless of this, all four BH3 mimetics clearly stabilised MCL-1, indicating that they all bind to MCL-1 and therefore this is likely the mechanism by which they induce apoptosis.

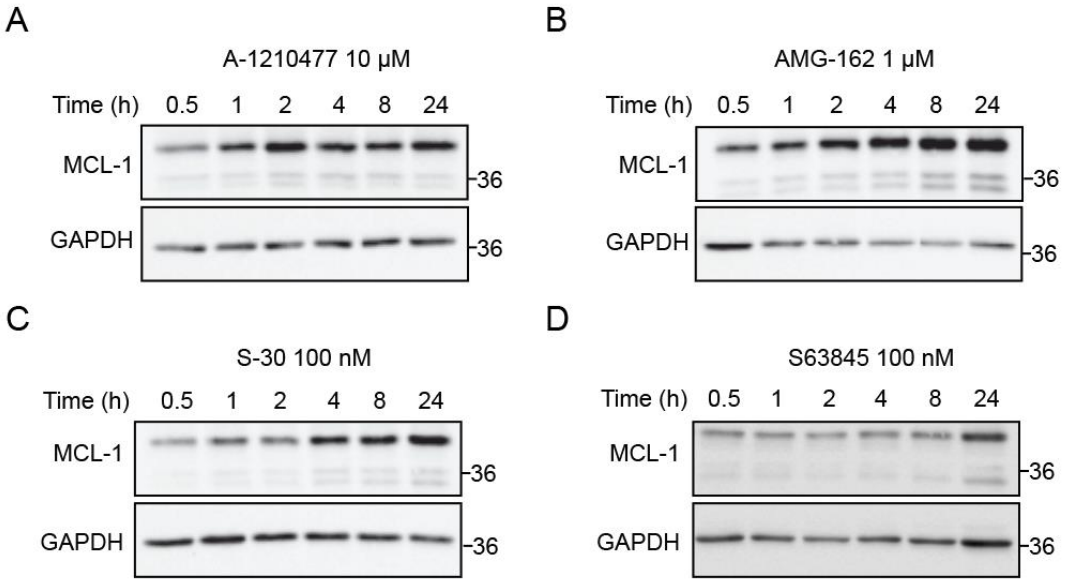


Figure 3.3. MCL-1 inhibitors continually stabilise MCL-1 over a 24 h period. HELA cells were exposed to either (A) A-1210477, (B) AMG-162, (C) S-30 or (D) S63845 at their respective approximate IC_{50} values (as determined in Fig. 3.2) at the indicated time points. Cells were collected and lysed in RIPA buffer for immunoblotting for MCL-1 and GAPDH as a loading control. Approximate molecular weights of bands are indicated.

3.4 S63845 does not inhibit BCL-X_L

Of the four MCL-1 inhibitors tested, S63845 and S-30 were clearly the most potent, able to induce significant apoptosis within 4 h at low nanomolar concentrations. Since these two inhibitors displayed similar activity, further experiments were focused on S63845 (along with A-1210477 as a control in some cases), as these inhibitors have been published[267,269] and were readily available.

S63845 was designed to specifically target MCL-1 and, as expected, it can induce apoptosis in MCL-1-dependent cell lines. However, given its therapeutic potential, it

was important to confirm that S63845 exclusively targets MCL-1 and not the other anti-apoptotic BCL-2 family members which share a high degree of homology with each other. It must be noted that previous BH3 mimetics, such as Navitoclax, targeted multiple members of the BCL-2 family, thus resulting in on-target thrombocytopenia, therefore new BH3 mimetics should be specific to individual proteins to avoid effects like this in the future. To assess the specificity of S63845 to induce apoptosis in a MCL-1- but not BCL-X_L- dependent manner, K562 and KCL-22 cells (two BCL-X_L-dependent chronic myeloid leukaemia cell lines[149,286,287]) were exposed to increasing concentrations of S63845, alongside A-1210477 and the BCL-X_L inhibitor A-1331852 (as a positive control) and apoptosis assessed by PS externalisation in flow cytometry (Fig. 3.4). As expected, S63845 (as well as A-1210477) failed to induce apoptosis in both K562 and KCL-22 cells, with only a small increase (~20%) in cell death observed for S63845 at high concentrations (10 μM). However, extensive apoptosis was observed in both cell lines following exposure to A-1331852, with an IC_{50} of ~100 nM but apoptosis becoming evident at concentrations as low as 10 nM. This data suggested that S63845 does not target BCL-X_L to induce apoptosis, supporting MCL-1 specific activity.

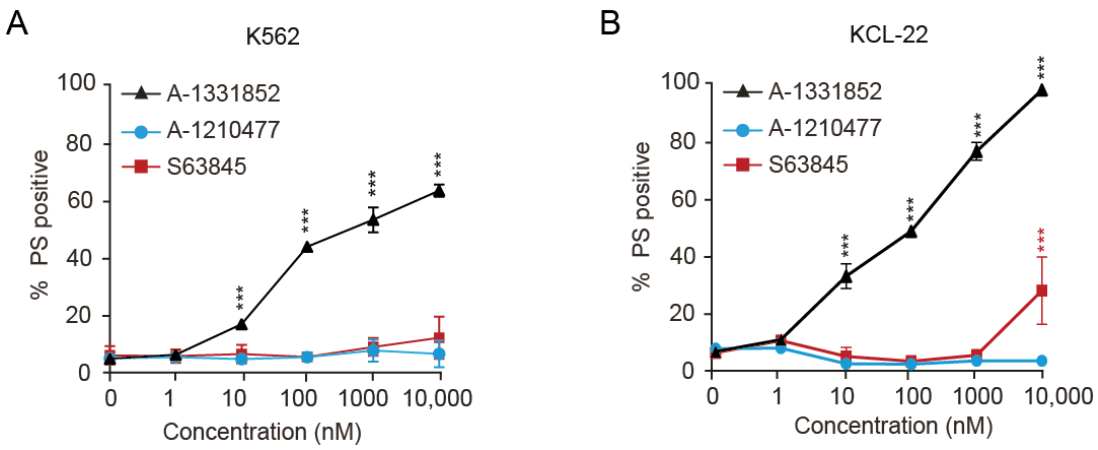


Figure 3.4. S63845 does not have any inhibitory activity against BCL-X_L. The BCL-X_L-addicted cell lines K562 and KCL-22 were exposed to A-1331852, A-1210477 or S63845 for 24 h and apoptosis was assessed by PS externalisation using Annexin V labelling in flow cytometry. S63845 and A-1210477 failed to induce apoptosis in these cells, while A-1331852 induced significant apoptosis at 100 nM. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

3.5 S63845 induces apoptosis in BCL-2 dependent cells

Further to the previous experiment, to ensure that S63845 also did not have inhibitory activity against BCL-2, S63845 was tested in MAVER-1, a BCL-2-dependent cell line derived from a mantle cell lymphoma malignancy[288]. As expected, exposure of MAVER-1 cells to the BCL-2 inhibitor ABT-199 resulted in extensive apoptosis, with an IC_{50} of ~100 nM but apoptosis being detectable at as low as 10 nM (Fig. 3.5A). Surprisingly however, S63845 appeared to induce apoptosis in the MAVER-1 cell line, albeit at higher concentrations than ABT-199. At 100 nM S63845, ~20% cell death was detected, increasing to 30% and 40% at 1 µM and 10 µM respectively. While it was clear that S63845 could not induce apoptosis to the same extent at 100

nM as ABT-199, S63845 appeared to have some unexpected inhibitory activity against BCL-2. A-1210477 could also induce low levels of apoptosis in MAVER-1 cells, although this was limited to ~20% and was only detectable at 10 μM. To confirm this observation, the same experiment was performed in chronic lymphocytic leukaemia (CLL) patient cells, which are known to be highly BCL-2 dependent[251,255,256]. In line with the previous data, exposure of these cells to even 1 nM ABT-199 induced 60% cell death, rapidly rising to 100% cell death with increasing concentrations (Fig. 3.5B). A-1210477 did not induce significant apoptosis in the CLL patient cells until 10 μM, where ~70% of cells were apoptotic. Remarkably however, S63845 could induce more than 60% apoptosis in the CLL patient cells at 100 nM, reaching a complete cell death like ABT-199 at higher concentrations. This data suggests that S63845 can potentially inhibit BCL-2 in an unexpected, off-target manner. The original report on S63845 demonstrating its capacity as an MCL-1 inhibitor did explore the *in vitro* binding of S63845 to other BCL-2 family members in addition to assessing cell death in various cell lines with different dependencies, but they did not report any binding or inhibitory capacity of S63845 against BCL-2[269]. While higher concentrations of S63845 were required to inhibit BCL-2 in MAVER-1 cell lines, only 100 nM was required to induce > 60% death in CLL patient cells, which are known to be exquisitely BCL-2 dependent. This suggests that the use of S63845 in the clinic must be carefully explored, since off-target effects can be observed even at the nanomolar range in patient cells and hitting BCL-2 could have adverse effects on healthy cells which require it for survival[134].

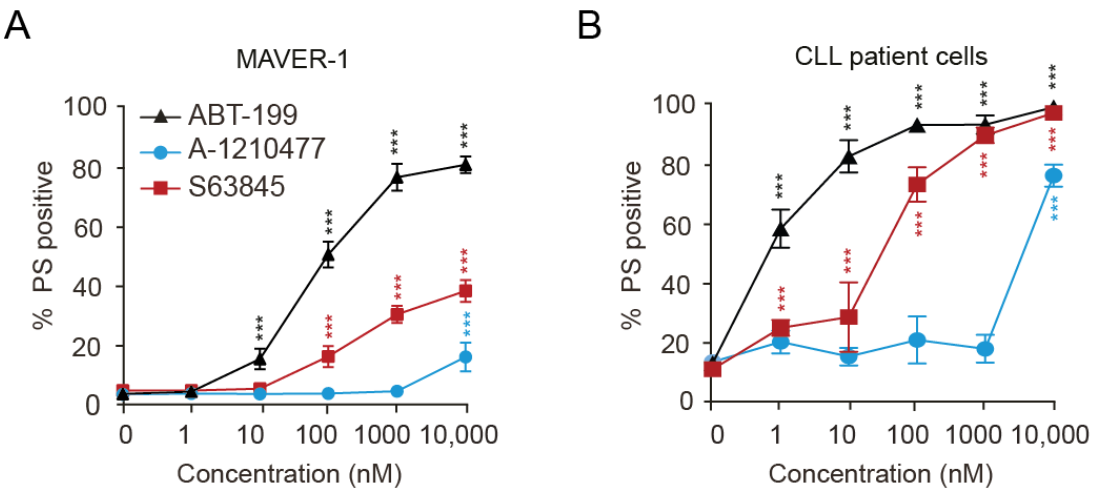


Figure 3.5. BCL-2-dependent cells undergo apoptosis following exposure to S63845. (A) The BCL-2-addicted cell line MAVER-1 and (B) CLL patient cells were exposed to a concentration range of either ABT-199, A-1210477 or S63845 for 8 h and apoptosis was assessed by PS externalisation using Annexin V labelling in flow cytometry. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

3.6 S63845 induces the intrinsic and not extrinsic apoptotic pathway.

The remarkable result of S63845 inducing apoptosis in BCL-2 addicted cell lines was very surprising and warranted exploration of whether S63845 was only inducing the intrinsic apoptotic pathway and not other cell death pathways, as expected. In order to test this, Jurkat cell lines deficient in key components of the intrinsic or extrinsic pathway were used: wild-type (WT), caspase-9 deficient (the apical initiator caspase of the intrinsic pathway which acts as part of the apoptosome[47]), FADD deficient (the adaptor protein which links the extrinsic pathway TNF receptor superfamily to the caspases to form the DISC[54,55]) and caspase-8 deficient (one of the key caspases in the DISC[54,289]). These cells were exposed to a concentration range of S63845 for 24 h and cell death assessed by PS externalisation in flow cytometry (Fig. 3.6). The WT cells

started to undergo apoptosis at low concentrations of S63845, as low as 100 nM, reaching 80% death at 10 µM. Both the caspase-8- and FADD-deficient cells also underwent similar levels of apoptosis at the same concentrations, while the caspase-9-deficient cells did not undergo any apoptosis, even at 10 µM S63845. This data confirmed that S63845 induces the intrinsic apoptotic pathway, as cells can still undergo apoptosis even when key components of the extrinsic pathway are missing while no apoptosis occurs when caspase-9, the apical caspase of the intrinsic pathway, is missing.

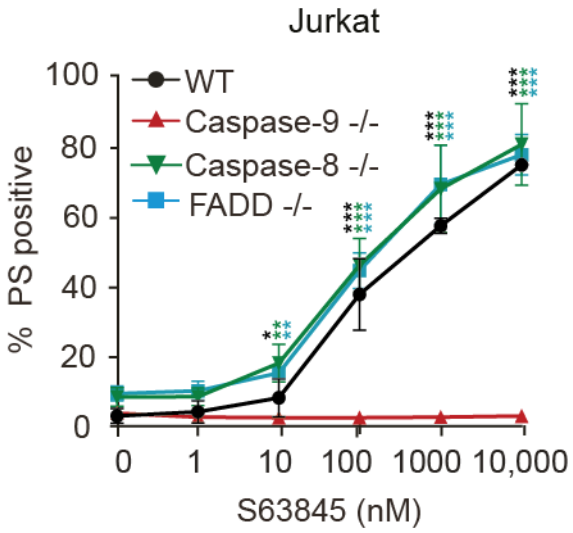


Figure 3.6. S63845 induces cell death via the intrinsic and not extrinsic apoptotic pathway. Wild-type (WT) Jurkat T cell leukaemia cells and cells deficient in key arms of the intrinsic and extrinsic apoptotic pathways were exposed to a concentration range of S63845 for 24 h and apoptosis was assessed by PS externalisation using Annexin V labelling in flow cytometry. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

3.7 S63845 is more potent than ABT-199 in inducing apoptosis in AML cell lines

Since S63845 appeared to induce the intrinsic apoptotic pathway in both an MCL-1- and BCL-2 dependent manner, it is possible that it might be of use in certain cell lines which have a BCL-2 and MCL-1 dependence. Recent reports indicate that cells derived from acute myeloid leukaemia (AML) patients can be effectively targeted with a BCL-2 and/or MCL-1 inhibitor, although other studies suggest that these cells depend primarily on BCL-2 for survival[272,290,291]. Despite this, MCL-1 inhibitors are currently entering clinical trials to treat AML and multiple myeloma patients (Clinical trials - NCT02979366; NCT02675452; NCT02992483). Since S63845 inhibits MCL-1, and (perhaps) to a lesser extent BCL-2, we investigated whether S63845 could induce apoptosis alone or in combination with other BH3 mimetics in a panel of AML cell lines. Six AML cell lines were exposed to a concentration range of either S63845 or ABT-199 alone or, a combination of the two, with a fixed concentration of ABT-199 at 100 nM and a concentration range of S63845 and then assessed for apoptosis by PS externalisation in flow cytometry (Fig. 3.7). Of the six cell lines tested, MV-4-11 cells (Fig. 3.7A) exhibited the highest sensitivity to both S63845 and ABT-199 individually, with IC_{50s} of ~20 nM and ~50-100 nM) respectively. The combination of S63845 and ABT-199 induced almost complete death at just 10 nM S63845, demonstrating the exquisite sensitivity of these cells to BH3 mimetics. THP-1 cells were much more resistant to ABT-199 (Fig. 3.7B), requiring ~1 µM to induce even 20% death, however they were considerably more sensitive to S63845, which induced ~75% apoptosis at 100 nM. The combination of S63845 and ABT-199 however, like in MV-4-11 cells, induced high levels of apoptosis (~90%) even at 10 nM. Meanwhile, OCI-AML3 cells were completely resistant to ABT-199 at all concentrations and only

underwent apoptosis following S63845 exposure at higher concentrations (Fig. 3.7C), with an IC_{50} of ~500 nM and extensive death induced by 1 µM S63845. S63845 and ABT-199 combination treatment was considerably more effective than the single inhibitors at inducing apoptosis however, with ~20% apoptosis occurring at 10 nM of the combination therapies and ~70% at 30 nM. Some of the AML cell lines tested were considerably more resistant to all treatments. U937 cells were completely resistant to ABT-199 and only underwent ~40% apoptosis at 1 µM S63845 (Fig. 3.7D), while the combination treatment of ABT-199 and S63845 did not vastly increase cell death compared to S63845 alone. Similarly, MOLM-13 cells were resistant to ABT-199 and relatively insensitive to S63845 (Fig. 3.7E) – 1 µM S63845 only induced ~30% apoptosis, while the combination treatment could induce significant apoptosis with 1 µM S63845 and 100 nM ABT-199. Finally, HL-60 cells were largely resistant to both BH3 mimetics individually but 1 µM S63845 could induce ~30% apoptosis (Fig. 3.7F). While the HL-60 cells were somewhat sensitive to the combination treatment, they were clearly less sensitive than many of the other cell lines tested as even at ~500 nM S63845, only 40% cell death could be seen in combination with ABT-199.

Overall, it would therefore seem that, while AML cells can differ quite drastically in their sensitivity to BH3 mimetics (which could perhaps be a result of their dependency on other pro-survival BCL-2 family proteins) S63845 is a better inducer of apoptosis alone than ABT-199 alone in these cells. Whether this can be attributed to the fact that S63845 might have some off-target BCL-2 inhibitory activity in combination with MCL-1 remains unclear, as the AML cells tested might tend to rely on MCL-1 over BCL-2 for survival. It is clear however that S63845 might be of therapeutic use in some cases of AML.

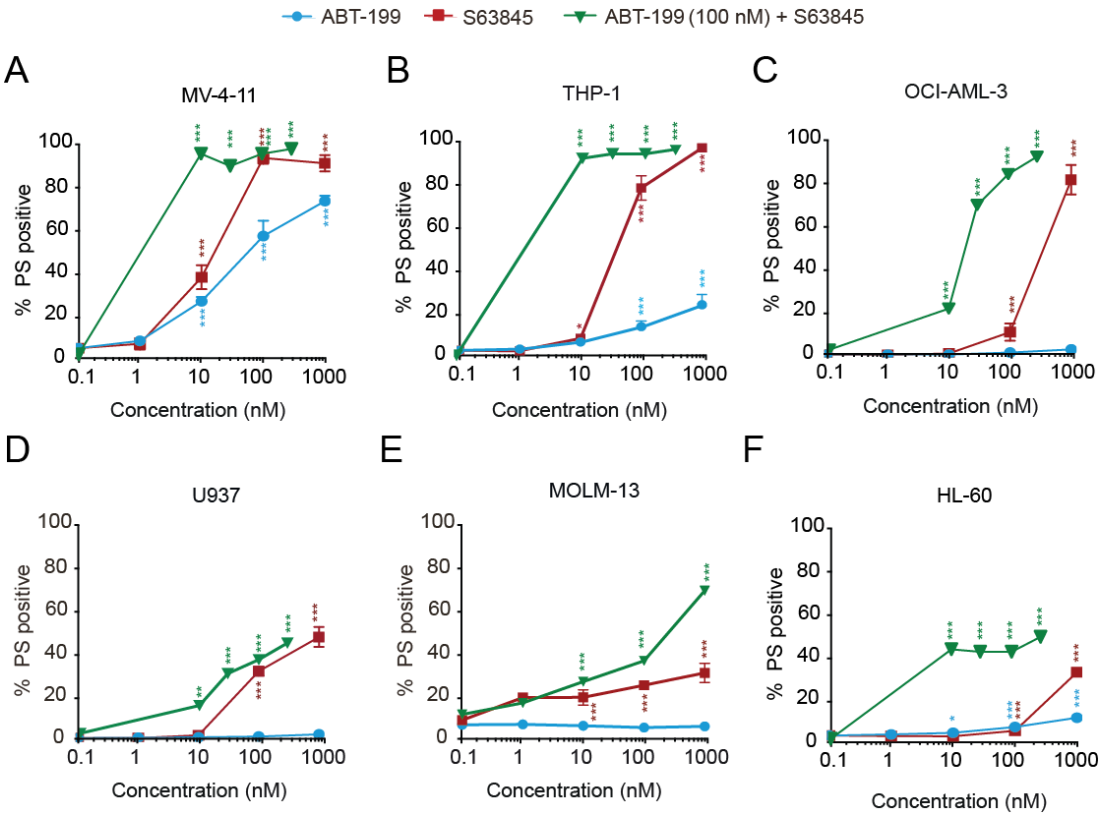


Figure 3.7. S63845 can be combined with ABT-199 to induce apoptosis in AML cell lines. AML cell lines were exposed to a concentration range of either S63845 or ABT-199 alone or concentration range of S63845 with ABT-199 at a constant concentration of 100 nM for 24 h and assessed for PS externalisation using Annexin V labelling in flow cytometry. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

3.8 Resistance to ABT-199 and/or S63845 in some AML cell lines could be attributed to BCL-X_L.

As previously mentioned, some of the AML cell lines were considerably more resistant to inhibition of BCL-2 and MCL-1 than others. In order to test whether any of the resistant AML cell lines had some dependency on BCL-X_L and hence explain why they were not susceptible to the combination of S63845 and ABT-199, U-937, MOLM-13 and HL-60 cells were exposed to A-1331852 (a BCL-X_L inhibitor) alone

or in combination with the other previously tested BH3 mimetics and apoptosis detected by PS externalisation in flow cytometry (Fig. 3.8). In both U-937 (Fig. 3.8A) and HL-60 cells (Fig. 3.8C), where the combination of S63845 and ABT-199 could only induce ~50% apoptosis even at higher concentrations, A-1331852 failed to induce significantly more apoptosis in combination with ABT-199 and S63845, suggesting these cells do not depend on BCL-X_L for survival and instead, one of the more obscure anti-apoptotic BCL-2 family members could be promoting survival. Meanwhile, in the MOLM-13 cells, 100 nM of A-1331852 alone, unlike S63845 and ABT-199, could induce significant apoptosis (~60% at 100 nM) and in combination with either ABT-199 or S63845 could induce up to 80% apoptosis, suggesting a key role for BCL-X_L in survival in this cell line (Fig. 3.8B).

Overall, it was evident from this data that many AML cell lines are indeed dependent on MCL-1 and/or BCL-2 and that S63845 is better at inducing apoptosis in these cell lines than ABT-199, perhaps in part because S63845 can potentially inhibit BCL-2 in addition to MCL-1. It is also possible that many of these cells are more dependent upon MCL-1 than BCL-2 for survival and thus, hitting them with an MCL-1 inhibitor can induce more apoptosis than a BCL-2 inhibitor. With this in mind, further studies were undertaken on the AML cell lines to explore the dynamics of the BCL-2 family interactions with and without BH3 mimetics, in order to determine if the sensitivity to certain BH3 mimetics is a result of disruption of certain interactions between BH3-only proteins and their anti-apoptotic partners, as would be expected in the proposed mechanism of BH3 mimetic action.

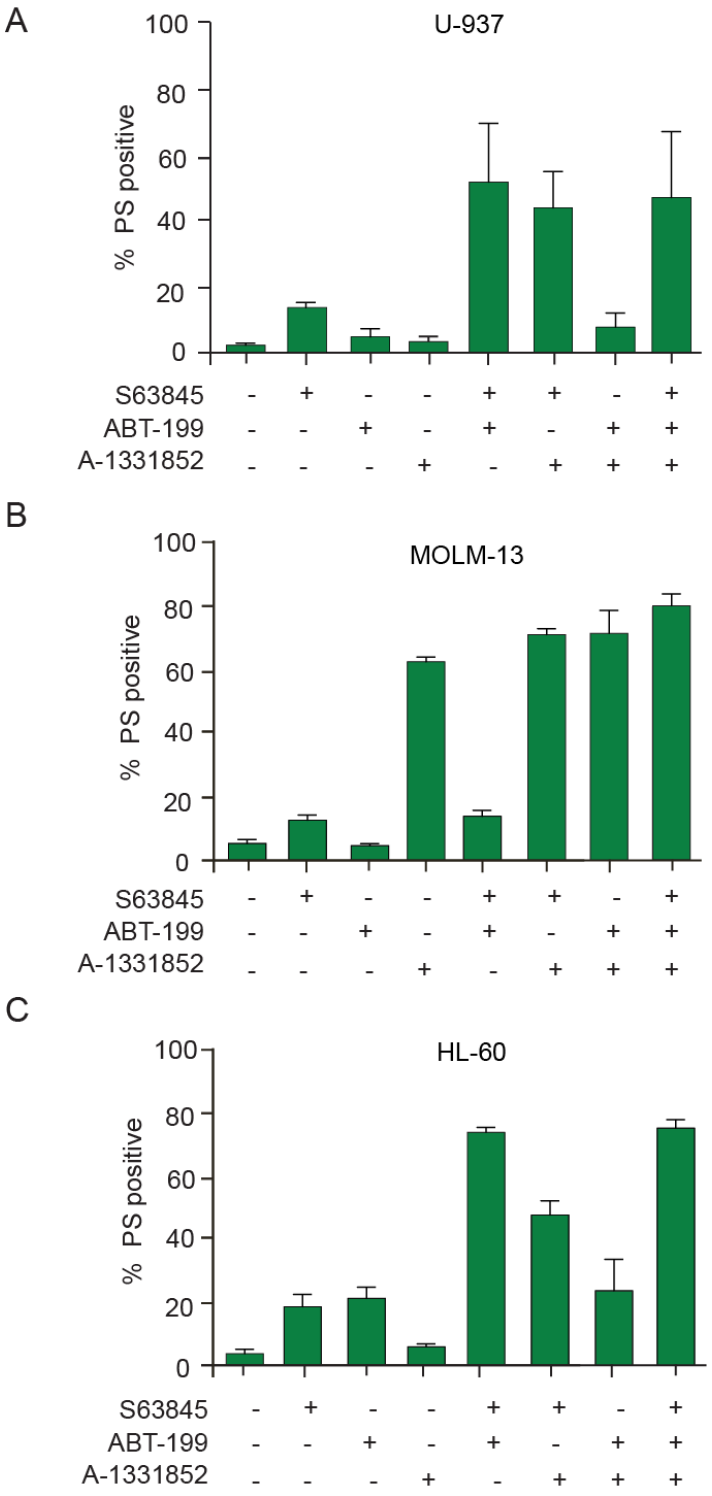


Figure 3.8 AML cell lines resistant to BCL-2/MCL-1 inhibition exhibit varying sensitivity to inhibition of BCL-X_L. The indicated cell lines were exposed to the different BH3 mimetics (all at 100 nM) for 24 h and apoptosis assessed by PS externalisation. Error bars = Mean ± SEM.

3.9 BCL-2 appears to be the major anti-apoptotic protein in MV-4-11 cells despite a marked sensitivity to S63845.

Mechanistically, BH3 mimetic-induced apoptosis is usually attributed to the displacement of BH3-only proteins from their anti-apoptotic partners, leading to BAX/BAK activation. Since the different AML cell lines tested exhibited varied responses to BH3 mimetic-induced apoptosis, it could be suggested that there is varied binding and/or displacement of the different BH3-only proteins to their known survival counterparts which determines the sensitivity of a cell to a certain BH3 mimetic. For example, a cell which is largely dependent upon MCL-1 would be expected to have large amounts of BIM, NOXA and PUMA bound to MCL-1, which would get displaced to initiate apoptosis upon addition of S63845 or another appropriate BH3 mimetic. To explore the BCL-2 family interactions in AML cells during apoptosis, one extremely sensitive (MV-4-11) (Fig. 3.9) one moderately sensitive (OCI-AML-3) (Fig. 3.10) and one resistant (HL-60) (Fig. 3.11) cell line were chosen as representative cells of each level of sensitivity and subjected to BCL-2 family immunoprecipitation experiments to detect the interactions of the key survival proteins with the major BH3-only proteins. Drug treatments were performed at different concentrations and time-points between cell lines to better represent the IC_{50} measures of the sensitive versus resistant cells. Cells were collected for lysis after ~50% apoptosis was achieved (measured by PS externalisation, data not shown).

In MV-4-11 cells, which are highly sensitive to both ABT-199 and S63845, the promiscuous activator BH3-only protein BIM was bound preferentially to BCL-2, with traces also bound to MCL-1 (Fig. 3.9). The activator BH3-only protein BID could not be seen bound to either MCL-1 or BCL-2, while PUMA, another activator BH3-

only protein which has promiscuous binding activity, was found bound to BCL-2 exclusively. NOXA, a sensitiser BH3-only protein, could be seen bound to MCL-1 in traces and not BCL-2 (as would be expected), while BAD, another sensitiser BH3-only protein, could not be seen bound to BCL-2 despite being present in the input at reasonable levels. Following exposure of the MV-4-11 cells to ABT-199, only a very small amount of the BIM bound to BCL-2 was displaced, with most appearing to remain bound. PUMA was also not significantly displaced from BCL-2 following ABT-199 treatment. The small amount of BIM bound to MCL-1 was readily displaced from MCL-1 following exposure to S63845 and, similarly, the traces of NOXA bound to MCL-1 underwent dissociation from MCL-1 too. Taken together, the results of this experiment were surprising – MV-4-11 cells were very sensitive to both ABT-199 and S63845 yet, significant levels of the BH3-only proteins, particularly BIM and PUMA, were preferentially bound to BCL-2 over MCL-1. Moreover, neither BIM nor PUMA were displaced from BCL-2 following ABT-199 treatment. How ABT-199 can induce such significant death at low concentrations in these cells is therefore somewhat of a mystery, as BIM, the only BH3-only protein bound to BCL-2, was not significantly displaced from BCL-2 following ABT-199 treatment. Further adding to this, S63845 could induce extensive apoptosis even when most BIM was sequestered by BCL-2 and only a very low level was released from MCL-1 following S63845 exposure. Thus, there appear to be discrepancies in the binding/release of BH3-only proteins to their pro-survival partners and apoptosis induction following BH3 mimetic treatment. It was therefore of great interest to explore whether this observation was specific to MV-4-11 cells or if it was perhaps a more general feature of AML and possibly other cell lines.

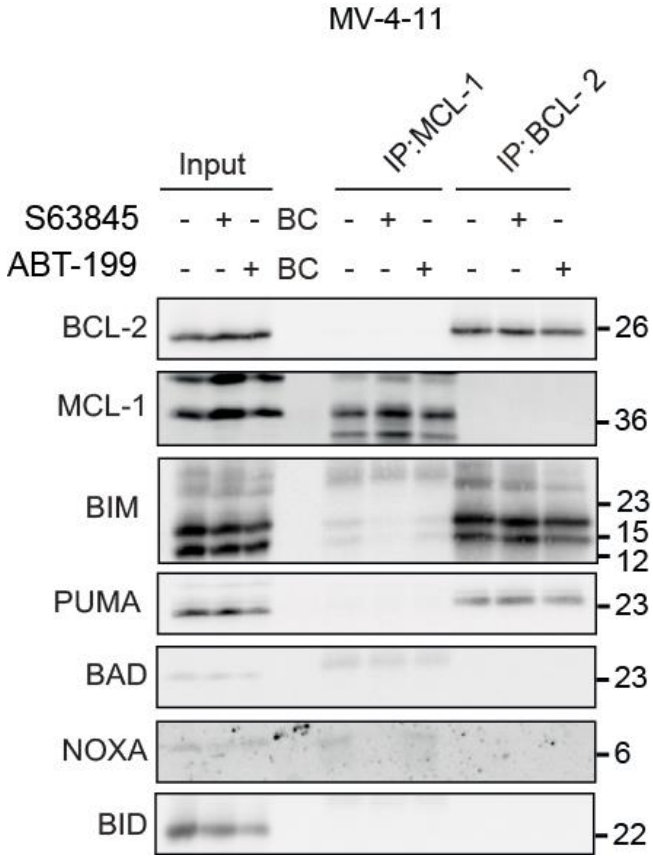


Figure 3.9. BCL-2 family protein interactions and displacements following BH3 mimetic exposure in MV-4-11 cells. Immunoprecipitation of MCL-1 and BCL-2 was carried out in MV-4-11 cells following exposure to S63845 (30 nM) or ABT-199 (50 nM) for 6 h. Collected cells were lysed and incubated with bead-antibody conjugates with antibodies against MCL-1 and BCL-2. The eluted complexes were immunoblotted for the indicated proteins. The input represents total cell lysate, IP indicates immunoprecipitation and BC indicates bead control. Approximate molecular weights of proteins are indicated alongside the blots.

3.10 BCL-2 appears to be the major anti-apoptotic protein in OCI-AML3 cells despite a marked sensitivity to S63845.

To further examine the notion of BCL-2 family interactions not reflecting sensitivity to BH3 mimetics, the same experiment was performed in the moderately sensitive OCI-AML3 cell line (Fig 3.10). In this cell line, binding patterns of BH3-only proteins to pro-survival proteins were similar to those seen for MV-4-11. BIM was largely bound to BCL-2 and a smaller amount bound to MCL-1, while PUMA was preferentially bound to BCL-2 and traces of NOXA were bound to MCL-1. Neither BAD nor BID appeared to bind either anti-apoptotic protein. In contrast to the MV-4-11 cells, exposure to ABT-199 resulted in a slightly more obvious displacement of BIM and PUMA from BCL-2, despite the cells being largely resistant to ABT-199 treatment. Furthermore, BIM and NOXA bound to MCL-1 were readily displaced with S63845, supporting the fact that these cells are sensitive to S63845. The observation that MV-4-11 and OCI-AML3 cells share a very similar binding pattern of BCL-2 family interactions yet displayed significantly different sensitivities to S63845 and ABT-199 is quite remarkable and unexpected. Strikingly, ABT-199 was capable of inducing displacement of BIM and PUMA from their major binding partner (BCL-2) in OCI-AML3 cells which are markedly less sensitive than MV-4-11 cells, where BIM is displaced less from BCL-2. This further suggested that the interactions between the pro- and anti-apoptotic BCL-2 protein family members may be more complicated than thought in terms of dictating sensitivity to BH3 mimetics.

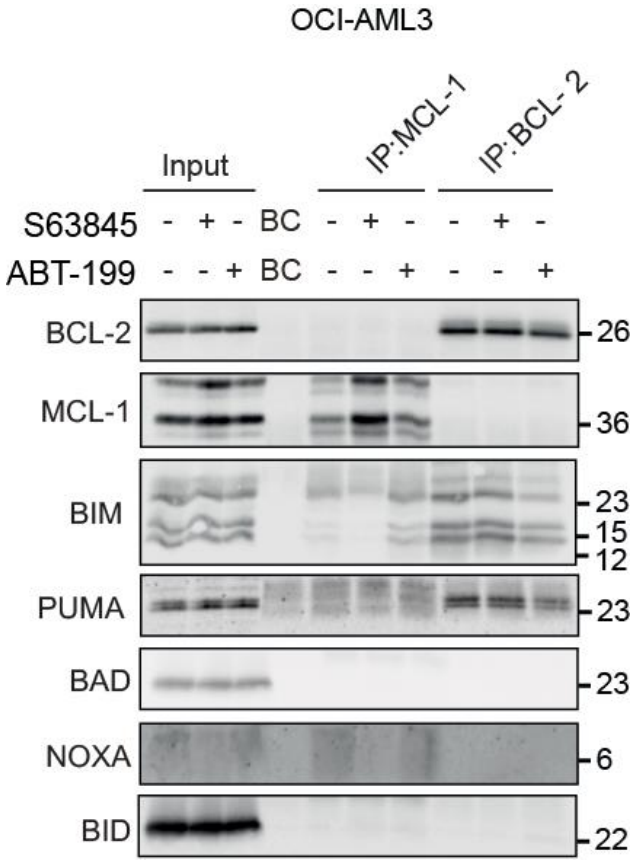


Figure 3.10. BCL-2 family protein interactions and displacements following BH3 mimetic exposure in OCI-AML3 cells. Immunoprecipitation of MCL-1 and BCL-2 was carried out in OCI-AML3 cells following exposure to S63845 (30 nM) or ABT-199 (50 nM) for 8 h. Collected cells were lysed and incubated with bead-antibody conjugates with antibodies against MCL-1 and BCL-2. The eluted complexes were immunoblotted for the indicated proteins. The input represents total cell lysate, IP indicates immunoprecipitation and BC indicates bead control. Approximate molecular weights of proteins are indicated alongside the blots.

3.11 BCL-2 appears to be the major anti-apoptotic protein in HL-60 cells despite a marked resistance to both ABT-199 and S63845 treatment.

Both cell lines tested previously for BCL-2 family interaction profiles were very or somewhat sensitive to at least one or a combination of BH3 mimetics. The resistant HL-60 cell line, however, did not undergo extensive apoptosis with S63845 or ABT-199 alone, while the combination could only achieve ~40% apoptosis. Furthermore, these cells did not depend on BCL-$X_{L,}$ the other major anti-apoptotic protein, for survival, as shown in Figure 3.8C. It was therefore of interest to detect where BH3-only proteins were being sequestered and what could cause displacement in these cells, in order to better understand the concept of BCL-2 family interacting proteins reflecting sensitivity to BH3 mimetics. In the HL-60 cells, binding patterns were remarkably similar to those seen for MV-4-11 and OCI-AML3 cells. BIM and PUMA bound almost exclusively to BCL-2, while BID and BAD were not bound to either MCL-1 or BCL-2 (Fig 3.11). Moreover, of all the BH3-only members, only traces of NOXA appeared to interact with MCL-1. The lack of BH3-only proteins, apart from traces of NOXA, being bound to MCL-1 is compatible with HL-60 cells being very resistant to S63845 treatment. The large amounts of BIM bound to BCL-2 were only slightly displaced following ABT-199 treatment which is, again, compatible with the cells being resistant to ABT-199. Meanwhile, PUMA was partially displaced by treatment of HL-60 cells with ABT-199, which could suggest that ABT-199 could therefore induce apoptosis through PUMA alone, but with the cells being very resistant to BH3 mimetics, this is evidently not the case. This was a remarkable observation as the HL-60 and MV-4-11 cells, despite looking very similar in BH3-only protein binding preferences, demonstrated vastly different sensitivities to BH3 mimetics. Taken together, these results suggest that interactions between the pro- and

anti-apoptotic BCL-2 family members may not necessarily reflect the relative sensitivities of different AML cell lines to undergo BH3 mimetic-mediated apoptosis and may suggest involvement of other survival factors, including perhaps other anti-apoptotic proteins but also a more complex level of control of the cell fate decision-making process.

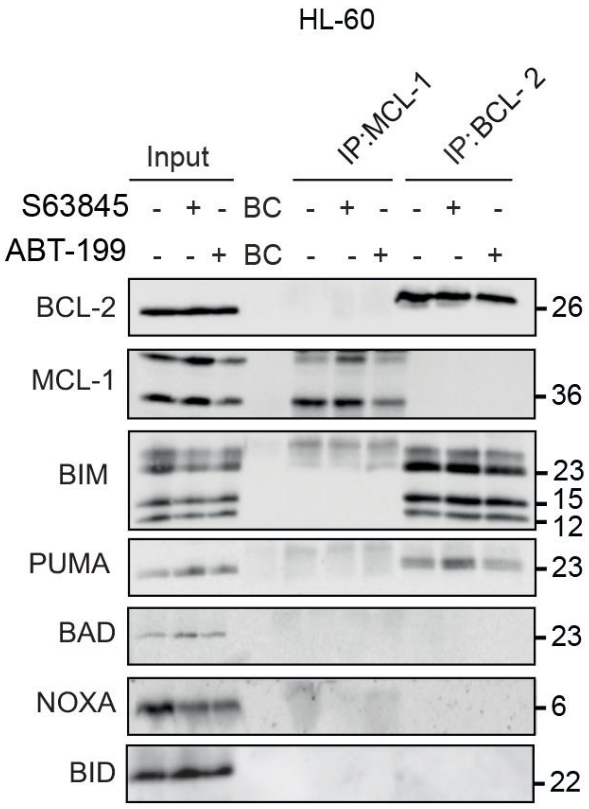


Figure 3.11. BCL-2 family protein interactions and displacements following BH3 mimetic exposure in HL-60 cells. Immunoprecipitation of MCL-1 and BCL-2 was carried out in HL-60 cells following exposure to S63845 (30 nM) or ABT-199 (50 nM) for 8 h. Collected cells were lysed and incubated with bead-antibody conjugates with antibodies against MCL-1 and BCL-2. The eluted complexes were immunoblotted for the indicated proteins. The input represents total cell lysates, IP indicates immunoprecipitation and BC indicates bead control. Approximate molecular weights of proteins are indicated alongside the blots.

3.12 S63845 can combine with A-1331852 to induce apoptosis in solid tumour cell lines.

To further explore the phenomenon of BCL-2 family interactions not reflecting BH3 mimetic sensitivity (while also examining the possibility of using S63845 in combination with other BH3 mimetics), several solid tumour cell lines, which typically depend on both BCL-X_L and MCL-1 for survival[199,245,292–295], were exposed to S63845 and A-1331852 (at a fixed concentration of 100 nM) for 4 h and apoptosis assessed by PS externalisation in flow cytometry. Six cell lines derived from various kinds of solid tumour malignancies including H1299 and A549 (non-small cell lung carcinoma (NSCLC)), HCT116 and HT-29 (colorectal cancer), SUIT-2 (pancreatic cancer) and PC-3 (prostate cancer) were used.

H1299 cells did not undergo apoptosis following exposure to either S63845 or A-1331852 alone at any concentration but with the combination treatment, began to undergo apoptosis at 100 nM, with an IC_{50} of ~100 nM. H1299 cells were completely apoptotic following exposure to 10 µM S63845 and 100 nM A-1331852 (Fig. 3.12A). Meanwhile, the second NSCLC cell line, A549, was somewhat more sensitive to the single compounds. S63845 began to induce apoptosis alone at higher concentrations of 10 µM, while A-1331852 induced just less than 20% apoptosis at 1 µM and ~30% at 10 µM (Fig. 3.12B). These cells were significantly more sensitive to the combination treatment – just 10 nM S63845 combined with 100 nM A-1331852 could induce ~30% apoptosis, while 100 nM of both S63845 and A-1331852 induced almost 90% cell death.

Like A549 cells, HCT116 cells were somewhat sensitive to the single BH3 mimetics – S63845 induced ~30% apoptosis at 10 µM while A-1331852 could induce ~20%

death at 100 nM and ~30% death at 1 µM (Fig. 3.12C). Again, however, the combination of the two inhibitors was the most effective at inducing death - 100 nM of each inhibitor induced ~60% cell death and almost 100% of cells were apoptotic following exposure to 10 µM S63845 and 100 nM A-1331852 together. The second colon carcinoma cell line tested, HT-29, presented a very different death profile. Both single drugs could induce ~40% death but only at 10 µM, while 100 nM of the drugs combined also induced ~40% apoptosis (Fig. 3.12D). Even at higher concentrations of S63845 however, only 60% apoptosis could be achieved, suggesting there may be another factor promoting survival here – perhaps another pro-survival BCL-2 family protein such as BCL-w. Indeed, less well explored pro-survival proteins have been implicated in survival of colorectal cancer cells[164], supporting this concept.

The pancreatic cell line, SUIT-2, was similar to H1299 cells in that they were largely insensitive to single BH3 mimetics but very sensitive to the combination treatment. Only at 10 µM could either S63845 or A-1331852 alone induce apoptosis and even then, this was limited to ~20%. The combination of the two inhibitors, however, induced ~40% death at 100 nM and almost complete death at 1 µM S63845 combined with 100 nM A-1331852 (Fig. 3.12E). Finally, the prostate cancer cell line tested, PC-3, demonstrated a similar apoptotic profile to SUIT-2 cells, although were overall less sensitive to BH3 mimetics. S63845 did not induce apoptosis until 10 µM, where it induced ~20% death, while A-1331852 induced 20% apoptosis at 1 µM and ~30% at 10 µM alone (Fig 3.12F). Surprisingly, unlike the other cells, the combination of 100 nM of each inhibitor did not induce significant apoptosis, while increasing the concentration of S63845 10-fold to 1 µM alongside 100 nM A-1331852, resulted in massive cell death, approaching 90%. These cells appeared to have an IC_{50} of ~500 nM S63845 when combined with 100 nM A-1331852.

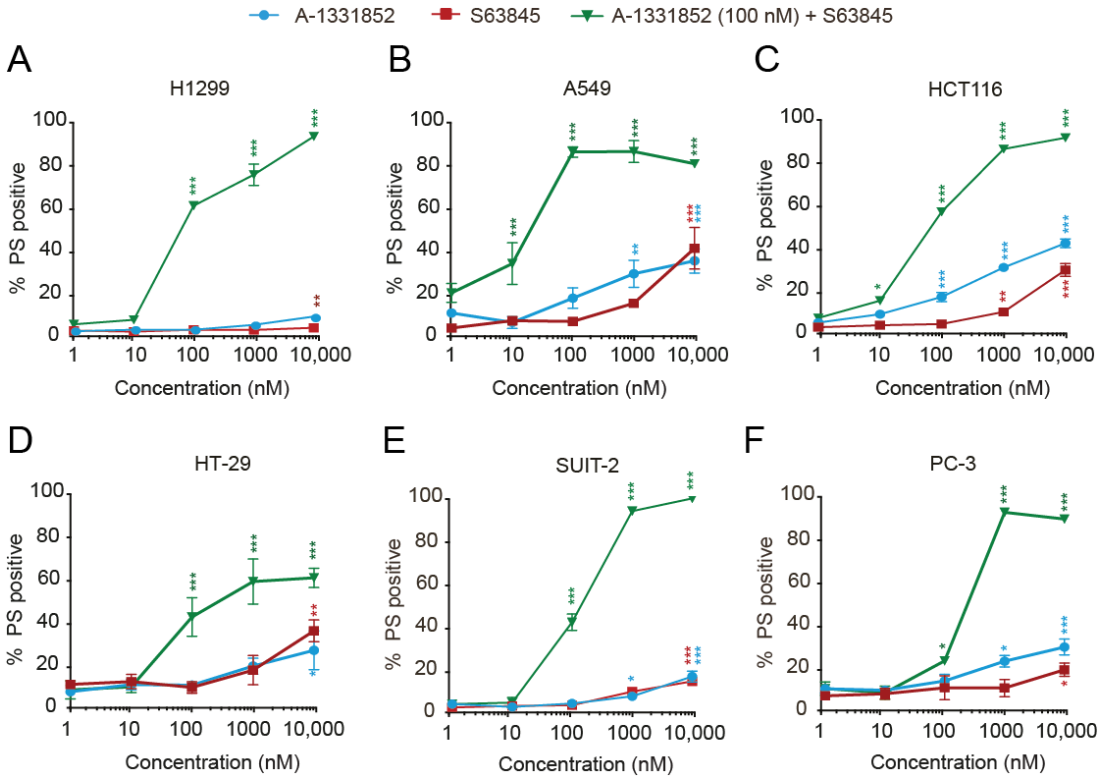


Figure 3.12. S63845 can be combined with A-1331852 to induce apoptosis in cell lines derived from various solid tumours. Solid tumour cell lines derived from different types of cancers were treated with a concentration range of S63845 and a constant concentration of 100 nM of A-1331852 for 4 h and assessed for PS externalisation using Annexin V labelling in flow cytometry. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

Overall, this data demonstrated that while some cells derived from solid tumour cells can be moderately sensitive to single BH3 mimetics, they mostly require inhibition of both MCL-1 and BCL-X_L in order for significant death to be induced. Since BCL-X_L and MCL-1 have different BH3-only protein binding partners, the interactions and displacement events with each of the BH3 mimetics was next studied using immunoprecipitation, as done for the AML cell lines.

3.13 H1299 cells demonstrate a proto-typical BCL-2 family protein interaction profile.

To corroborate earlier findings in AML cell lines and assess interactions between the different BH3-only proteins with their key pro-survival counterparts, namely BCL-X_L and MCL-1, immunoprecipitation studies were repeated in three of the solid tumour cell lines. All the cell lines tested demonstrated sensitivity to the combination of BCL-X_L and MCL-1 inhibition, while a couple of cell lines also showed low levels of sensitivity to the single BH3 mimetics. Thus, H1299 and SUIT-2 cells, which were sensitive to the combination BH3 mimetic treatment, and HCT116 cells, which were slightly sensitive to A-1331852 inhibition at 100 nM and combination treatment, were chosen as the model cell lines. Since these cells demonstrated very similar sensitivities to the combination treatment, it was thought that their BCL-2 family binding profiles would be similar, however this proved to not be the case.

In H1299 cells, BIM appeared to preferentially bind MCL-1, while, BID could not be seen bound to MCL-1 or BCL-X_L, suggesting it does not play a major role in the intrinsic apoptotic pathway in these cells (Fig. 3.13). Meanwhile PUMA, which can also bind both MCL-1 and BCL-X_L, preferentially bound to BCL-X_L, though a small amount of PUMA was also bound to MCL-1. NOXA exhibited the expected selective

binding to MCL-1, while BAD demonstrated specific binding to BCL-X_L. Following exposure of H1299 cells to S63845, BIM was clearly displaced from MCL-1. Interestingly, while little BIM appeared to bind BCL-X_L under control conditions, in the S63845 treatment, where BIM was displaced from MCL-1, small amounts of BIM could be seen bound to BCL-X_L instead, suggesting that when displaced from its anti-apoptotic protein of choice, BIM is capable of binding the other key anti-apoptotic protein, in this case BCL-X_L. This could, in part, explain why no death was seen with the single BH3 mimetics, since BIM is not actually freed upon S63845 or A-1331852 treatment and is instead sequestered by BCL-X_L, which will prevent its pro-apoptotic activity. In the case of PUMA (which could bind both MCL-1 and BCL-X_L), exposure of the cells to A-1331852 resulted in dramatic displacement of PUMA from BCL-X_L, while S63845 also caused moderate displacement of PUMA from MCL-1. Unlike BIM however, the counter anti-apoptotic protein did not appear to sequester PUMA when displaced from the other. Both BAD and NOXA underwent displacement from their respective anti-apoptotic partners following A-1331852 and S63845 treatment respectively. H1299 cells therefore represent a very typical model of BCL-2 family interactions, particularly in terms of displacement with BH3 mimetics. In the H1299 cells, displacement of BIM, PUMA and NOXA from MCL-1 can drive apoptosis but only when BCL-X_L is also neutralised, as BCL-X_L can sequester any free BIM released from MCL-1 with S63845 treatment, while also sequestering BAD.

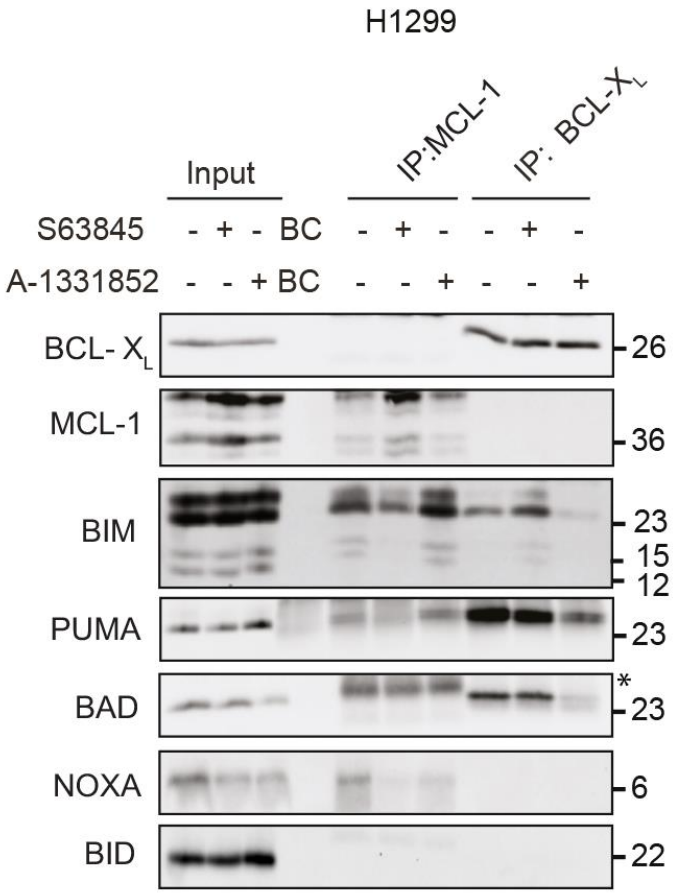


Figure 3.13. BCL-2 family protein interactions and displacements following BH3 mimetic exposure in H1299 cells. Immunoprecipitation of MCL-1 and BCL-X_L was carried out in H1299 cells following exposure to S63845 (100 nM) or A-1331852 (100 nM) for 2 h. Collected cells were lysed and incubated with bead-antibody conjugates with antibodies against MCL-1 and BCL-X_L. The eluted complexes were immunoblotted for the indicated proteins. The input represents total cell lysates, IP indicates immunoprecipitation, and BC indicates bead control. Approximate molecular weights of proteins are indicated alongside the blots.

3.14 SUIT-2 cells do not display the exact same binding pattern as H1299 cells, despite having the same BH3 mimetic sensitivity.

SUIT-2 cells were not particularly sensitive to single BH3 mimetics alone but underwent significant apoptosis with a combination of the BCL-X_L and MCL-1 inhibitors at nanomolar concentrations (Fig. 3.12E). In SUIT-2 cells, BIM was bound to both MCL-1 and BCL-X_L almost equally while PUMA was surprisingly not bound to either MCL-1 or BCL-X_L (Fig. 3.14). Furthermore, unlike in H1299 cells where no BID could be seen bound to either anti-apoptotic protein, a small amount of BID could be seen bound to BCL-X_L in SUIT-2 cells. NOXA was bound exclusively to MCL-1 and BAD was bound exclusively to BCL-X_L, as would be expected. Following exposure to BH3 mimetics, BIM was displaced to differing extents– a slight displacement from MCL-1 following S63845 treatment was observed, while a complete displacement from BCL-X_L could be seen (Fig. 3.14) with A-1331852 treatment. Moreover, the complete displacement of BIM from BCL-X_L with A-1331852 treatment was also followed by increased binding of BIM to MCL-1, similar to what was seen with BIM in H1299 cells but in reverse. There was also a possible slight increase in binding of BIM to BCL-X_L following its displacement from MCL-1 after exposure of the cells to S63845.

Both BAD and NOXA were also significantly displaced from their binding partners following A-1331852 and S63845 treatment respectively. Overall, H1299 and SUIT-2 cells exhibited significant differences in their BCL-2 family interaction profile, despite similar expression levels of the different proteins and responses to the combination of A-1331852 and S63845. This further re-enforced the idea of BCL-2 family interactions not exclusively dictating sensitivity to BH3 mimetics.

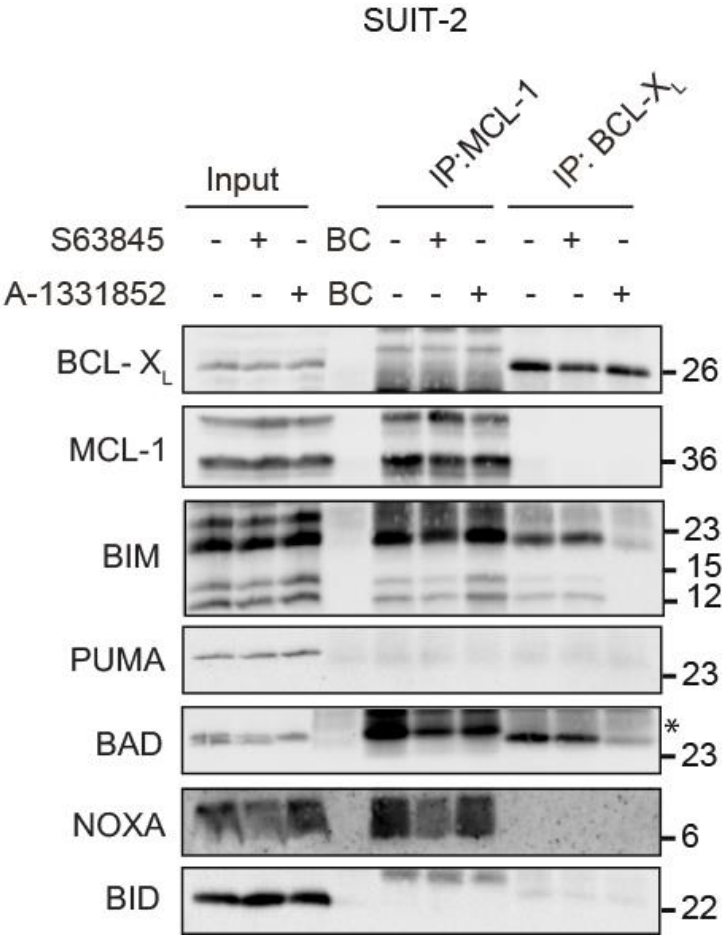


Figure 3.14. BCL-2 family protein interactions and displacements following BH3 mimetic exposure in SUIT-2 cells. Immunoprecipitation of MCL-1 and BCL-X_L was carried out in SUIT-2 cells following exposure to S63845 (100 nM) or A-1331852 (100 nM) for 2 h. Collected cells were lysed and incubated with bead-antibody conjugates with antibodies against MCL-1 and BCL-X_L. The eluted complexes were immunoblotted for the indicated proteins. The input represents total cell lysates, IP indicates immunoprecipitation and BC indicates bead control. Approximate molecular weights of proteins are indicated alongside the blots.

3.15 Most BH3-only proteins bind to BCL-X_L in HCT116 cells even though both MCL-1 and BCL-X_L must be neutralised for extensive apoptosis.

The inconsistencies in binding/displacement patterns versus sensitivity to BH3 mimetics were even more evident in HCT116 cells, which were moderately sensitive to inhibition of BCL-X_L but required dual inhibition of BCL-X_L and MCL-1 for extensive apoptosis. In these cells, BIM was exclusively bound to BCL-X_L, with very little binding to MCL-1 (Fig. 3.15). A band was present in the BIM blots in the MCL-1 immunoprecipitation lane, however it is at a molecular weight which is not the same as any of the other bands in the input or BCL-X_L immunoprecipitation, suggesting this band may not be BIM. Furthermore, BID, like SUIT-2 cells was bound to BCL-X_L but in very small amounts compared to the expression in the input. For the BH3-only proteins which have specific binding partners, binding was very similar to the other cells as expected. NOXA was only bound to MCL-1, while BAD was bound to BCL-X_L. The effects of BH3 mimetics on these interactions were very interesting – both BIM and PUMA were almost completely displaced from BCL-X_L following A-1331852 treatment but were not in turn sequestered by MCL-1. This is in line with HCT116 cells being somewhat sensitive to A-1331852 treatment alone, however the lack of sequestration of free BIM/PUMA by MCL-1 (which is not bound to any other BH3-only protein apart from NOXA) is surprising, since MCL-1 must be neutralised alongside BCL-X_L for full apoptosis. The traces of BID bound to BCL-X_L were also displaced following exposure of cells to A-1331852, while both BAD and NOXA were displaced from their respective binding partners with the indicated treatments.

Collectively the data in solid tumour cell lines shows that while the BH3-only proteins which are specific to certain anti-apoptotic proteins do not vary in binding and are

always displaced with the appropriate BH3 mimetics, the more promiscuous members of the BH3-only protein family, i.e. PUMA, BIM and BID vary greatly in binding to the different anti-apoptotic proteins, even in cells which demonstrate virtually the same sensitivity to BH3 mimetics. For example, BIM binds MCL-1 in the H1299s, both MCL-1 and BCL-X_L in SUIT-2 cells and exclusively BCL-X_L in in HCT-116 cells, yet all three cell lines require both inhibitors to induce significant apoptosis. Moreover, PUMA also showed discrepancies between the cell lines – binding MCL-1 and BCL-X_L to differing extents in H1299s, neither anti-apoptotic protein in SUIT-2 cells and only BCL-X_L in HCT-116. It could be suggested that the similarities in binding/displacement of the specific BH3-only proteins (i.e. NOXA/BAD) is what is dictating the sensitivity of the three cell lines to BH3 mimetics, explaining why they share the same sensitivity. However, the many discrepancies identified suggest that something much more complex is happening. For example, BIM and PUMA and displaced to dramatically different extents in the different cell lines, despite them sharing the same sensitivity to BH3 mimetics, while MCL-1 can sequester freed BH3-only proteins in some cell lines but not others. These results suggest that the preferential sequestration of different BH3-only proteins to distinct anti-apoptotic counterparts does not solely dictate dependency of a cell line on an individual anti-apoptotic protein and therefore could suggest that there is more at play than just the BH3-only proteins in determining the sensitivity of a cell to BH3 mimetics.

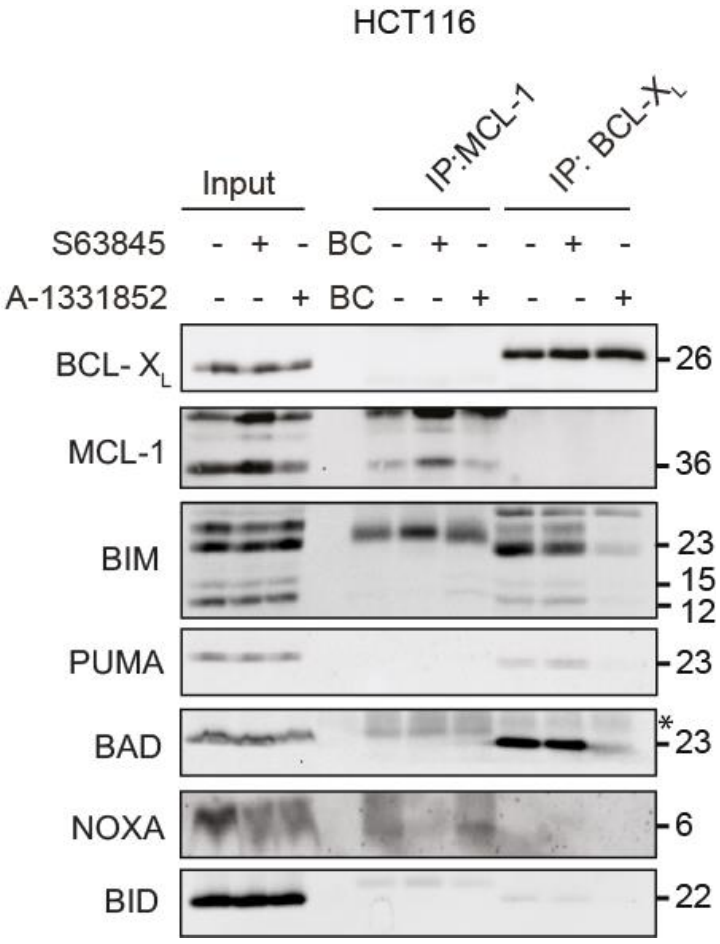


Figure 3.15. BCL-2 family protein interactions and displacements following BH3 mimetic exposure in HCT116 cells. Immunoprecipitation of MCL-1 and BCL-X_L was carried out in HCT116 cells following exposure to S63845 (100 nM) or A-1331852 (100 nM) for 2 h. Collected cells were lysed and incubated with bead-antibody conjugates with antibodies against MCL-1 and BCL-X_L. The eluted complexes were immunoblotted for the indicated proteins. The input represents total cell lysates, IP indicates immunoprecipitation and BC indicates bead control. Approximate molecular weights of proteins are indicated alongside the blots.

3.16 Discussion

Targeting the BCL-2 family in cancer has shown promising results for therapy despite the fact that, up until recently, inhibition of MCL-1 has been difficult. Novel inhibitors are, however, showing great promise. Here, three novel MCL-1 inhibitors were explored alongside A-1210477, the best previous inhibitor, to assess their ability to induce MCL-1-specific apoptosis and combine with other BH3 mimetics in a variety of cell lines derived from both solid and haematological malignancies. All four of the inhibitors tested were able to stabilise MCL-1 continually up to 24 h and induce MCL-1-specific apoptosis in MCL-1-addicted cell lines, though with very different IC_{50} values. While A-1210477 could only induce apoptosis at 10 µM, the three novel MCL-1 inhibitors could induce apoptosis at lower concentrations, with S63845 and S-30 both having approximate IC_{50} values of an impressive 100 nM. This corroborates previous findings that A-1210477 requires micromolar concentrations to induce apoptosis[267] while S63845 is considerably better at inducing MCL-1-dependent apoptosis, though we show here that S63845 can induce apoptosis at a 100-fold less concentration than A-1210477 while the original report on S63845 suggests the difference is actually 1000-fold less[269]. This could perhaps be a result of the differences in approaches used to examine apoptosis – here cells were incubated for 4 h with S63845 and apoptosis assessed by PS externalisation, while in the original report, cells were incubated with the compound for 48 h and apoptosis assessed by MTT assays. Regardless of this, S63845 is clearly a very potent MCL-1 inhibitor with great promise for future use in targeting MCL-1 in a therapeutic context. Moreover, it will be greatly useful as a tool (alongside other highly specific BH3 mimetics) to dissect the BCL-2 family pathways, allowing a better understanding of apoptosis and its regulation.

With S63845 appearing to be a very potent inhibitor of MCL-1, it was chosen as the focus of the study. Remarkably, it was observed that S63845 could induce apoptosis in BCL-2-addicted cell lines, suggesting it had some activity against BCL-2. While Kotschy *et al*. reported no binding of S63845 to the other anti-apoptotic proteins[269], it is clear from the data here that S63845 may possess some ability to induce apoptosis in BCL-2-addicted cell lines. It could be suggested that both MAVER-1 and CLL patient cells have some MCL-1 dependence and therefore, when exposed to S63845, undergo apoptosis. In the case of CLL patient cells, this certainly could be the case, as patient cells are typically very sensitive to exposure to drugs and thus, a small amount of MCL-1 inhibition could release enough BH3-only proteins to activate BAX and/or BAK and commit the cells to apoptosis. It could also be suggested however that S63845 is inducing a non-specific, non-apoptotic cell death in these cells. This is unlikely to be the case however, as S63845 could not induce apoptosis in Jurkat cells lacking caspase-9, which is critical to the intrinsic apoptotic pathway. Clearly there is more to S63845 activity in BCL-2 addicted cells than meets the eye and further work is necessary to understand how S63845 causes apoptosis in these cells before it is used in therapy. With the possibility of S63845 having inhibitory activity against MCL-1 and BCL-2, it was hypothesised that it might be of use in AML malignancies since they are thought to be largely BCL-2- and/or MCL-1-addicted[272,290,291,296]. To test this, a panel of AML cell lines was exposed to S63845 and/or ABT-199 to test whether S63845 alone could induce significantly more apoptosis than ABT-199 alone and whether the two drugs combined could induce complete apoptosis. The six cell lines tested demonstrated starkly different sensitivities to S63845 and/or ABT-199, with some being very sensitive to the single BH3 mimetics alone and some being completely resistant to

single BH3 mimetic exposure. S63845 largely demonstrated a better capacity to induce apoptosis in most of the cell lines tested and when combined with ABT-199, high levels of apoptosis could be induced in some of the more sensitive cell lines, although some of the AML cell lines demonstrated resistance to even a combination of S63845 and ABT-199, suggesting the involvement of other anti-apoptotic proteins in their survival. Previous reports demonstrate that MCL-1 is a key player in resistance to ABT-199 monotherapy in AML[272,290,291] and that targeting MCL-1 can overcome this resistance, which is in line with the data here showing that dual inhibition of both MCL-1 and BCL-2 can potently induce apoptosis in some AML cell lines. The resistance to both MCL-1 and ABT-199 inhibition seen here also suggested that BCL-X_L might play a role in ABT-199 resistance which was shown here and which has also been described previously by Wood *et al.*[272], where MCL-1 and BCL-X_L were shown to be upregulated following BCL-2 inhibition and furthermore, inhibition of BCL-X_L or MCL-1 could overcome ABT-199 resistance in multiple AML cells[272]. Only one cell line, MOLM-13, was sensitive to BCL-X_L inhibition however, suggesting that further additional factors are at play. Regardless of the difference is sensitivities, it is evident that BH3 mimetics will be a useful in treating AML malignancies, especially when tools such as BH3 profiling[297] can be used on patient samples to determine tumour cell addictions to BCL-2 family proteins and potential resistance mechanisms.

It was also demonstrated here that S63845 can synergise with a BCL-X_L inhibitor, A-1331852, to induce apoptosis in a range of different cell lines derived from solid tumours which typically depend on both MCL-1 and BCL-X_L for survival[199,245,292,293,298]. While this combination of BH3 mimetics is clearly very effective in inducing cancer cell death, the therapeutic potential of this drug

combination might be limited since inhibition of both MCL-1 and BCL-X_L may have side-effects on normal cells. However, this work demonstrates that these cell lines depend exquisitely upon the two anti-apoptotic protein members which could be targeted alone or in combination with other chemotherapeutic agents to induce a cancer cell specific death.

Having shown that S63845 could synergise with both ABT-199 and A-1331852 to induce apoptosis in a range of cell lines, the apoptosis induced by these inhibitors was assessed further, by means of immunoprecipitation, to observe the interactions and possible displacements of BH3-only proteins from their respective anti-apoptotic partners. Remarkably, in both suspension and solid tumour cell lines, the sensitivity of a cell line to specific inhibitors of pro-survival BCL-2 family proteins did not always match the binding and displacement patterns of BCL-2 family members. For example, in the case of MV-4-11 which are extremely sensitive to both S63845 and ABT-199, BIM was bound to both MCL-1 and BCL-2 but only readily displaced from MCL-1 and not BCL-2 with the respective BH3 mimetics. Moreover, no other BH3-only protein was released from BCL-2 following exposure to ABT-199. It is therefore unclear, following the defined mechanisms of apoptosis initiation, how ABT-199 can induce apoptosis in these cell lines, if no native BH3-only proteins are released from BCL-2 to activate the effectors of apoptosis, BAX and BAK. Similar observations could be made in solid tumour cell lines, with a particularly interesting case for HCT116, which require inhibition of MCL-1 and BCL-X_L for extensive apoptosis induction, yet with the exception of NOXA, no BH3-only proteins are bound to MCL-1 and almost all BIM is sequestered by BCL-X_L.

It could be suggested that in these cell lines, minute amounts of BIM or another BH3-only protein could be released from BCL-2 following ABT-199 treatment, which is enough to induce apoptosis but is not detectable by immunoblotting. In support of this, in MV-4-11 cells, a small amount of BIM is seen bound to MCL-1 and is readily displaced when the cells are exposed to S63845. However, it is striking that more BIM is not released from BCL-2, when it is completely displaced in other cases following ABT-199 exposure. Moreover, the striking dependence of HCT116 cells on both MCL-1 and BCL-X_L goes against this.

In order to better understand the BCL-2 family interactions during apoptosis, further techniques could be used, such as proximity ligation or dissociation assays in shorter, immunofluorescent live-cell imaging. This would allow for observation of the protein-protein interactions during the onset of apoptosis and would also eliminate any issue of detergent-induced changes in interactions which have been previously implied by other studies[299,300]. Moreover, this would allow for better observations of the whole BCL-2 family subcellular pools, as lysis of cells with CHAPS for immunoprecipitation may have resulted in loss of some degree of proteins present in the insoluble fraction, since CHAPS may not allow for full recovery of such proteins. A live-cell imaging approach would also require use of Z-VAD-FMK to inhibit caspases and thereby prevent downstream apoptotic signalling, which would remove the possibility of any artefacts seen in the immunoprecipitation experiments where cells might already be undergoing extensive apoptosis. Shorter time-points could also prevent this, as here, cells were collected for immunoprecipitation when ~50% of cells were apoptotic in order to ensure full displacement of BH3-only proteins from pro-survival proteins, however

it is possible that displacement occurs much earlier before apoptosis takes place, thus live-cell imaging at shorter time-points would improve any observations made.

Taken together, this data seems to suggest that the interactions and dispensability of the BCL-2 family in BH3 mimetic-induced apoptosis is much more complicated than originally thought. Indeed, multiple recent reports has shown that some, if not all, of the BH3-only proteins may actually be dispensable for BAX/BAK activation and subsequent apoptosis following suppression of both MCL-1 and BCL-X_L[220,301,302]. To further explore the role of the BH3-only proteins in BH3 mimetic-induced apoptosis in cancer cell lines, a genetically modified cell line lacking the 8 key BH3-only proteins, thought to be essential to apoptosis, was utilised and will be discussed in the next chapter.

Chapter 4

The dispensability of the BH3-only proteins in apoptosis

Contents

4.1 Background

In the previous chapter, it was shown that the interactions between the different BCL-2 family proteins might not always necessarily reflect the sensitivity of cell lines to undergo apoptosis following exposure to specific BH3 mimetics. If this is the case, the importance of the BH3-only proteins in determining whether a cell will die following suppression of the pro-survival proteins is put in question. As described earlier, there are multiple competing theories which try to explain how the BCL-2 protein family interactions regulate apoptosis. The most well-accepted model of effector protein activation is the unified model, where anti-apoptotic BCL-2 proteins restrain the activity of both pro-apoptotic BH3-only proteins and the effector proteins (BAX and BAK). Upon receipt of an apoptotic stimulus, two main 'modes' of apoptosis can ensue – 'mode 1' where increased levels of BH3-only proteins bind to and activate the effector proteins (direct activation) and 'mode 2' where effector proteins are released from anti-apoptotic proteins as increased levels of BH3-only proteins displace the pro-survival/effector protein interactions (indirect activation). In reality, both of these processes are likely to occur, resulting in a bi-modal activation of the effector proteins, allowing for finer control over BAX and BAK activation. It has been made evident from all of the proposed models that BH3-only proteins, in one way or another, are considered essential to the activation of BAX and BAK. Recent work by our collaborators, however, suggests otherwise, however[303]. The lab of Professor Xu Luo at The University of Nebraska Medical Centre developed a panel of BCL-2 family knockout HCT116 cell lines using CRISPR-Cas9 technology which revealed some very surprising results concerning the BCL-2 family and their dispensability in apoptosis. HCT116 cells, as confirmed in this study as well as in our findings (Fig 3.12C), depend on both MCL-1 and BCL-X_L for survival. Neutralisation

of these two proteins by RNA interference or other approaches in these cells resulted in rapid induction of apoptosis in a BAX/BAK-dependent manner. Surprisingly, the 'activator' BH3-only proteins BIM, PUMA and BID were dispensable for activation of BAX/BAK and the ensuing apoptotic pathway[304]. This suggested that inhibition of MCL-1 and BCL-X_L alone was sufficient to activate BAX/BAK and induce apoptosis[304], perhaps with the help of the other sensitiser BH3-only proteins (for example NOXA, BIK, HRK). Indeed, some reports have suggested that the 'sensitisers' do not exclusively function to sensitise a cell to apoptosis induction by altering the activity of anti-apoptotic proteins and instead, may be able to activate apoptosis themselves[220]. To follow up on this, the team, led by Professor Luo, further developed their HCT116 BH3-only protein KO cells to an octa-knockout (8KO) cell line, apparently not expressing any of the eight key BH3-only proteins: BIM, BID, PUMA, NOXA, HRK, BMF, BIK and BAD. While these cells were resistant to apoptosis induced by general apoptotic stimuli, such as serum starvation, thapsigargin (an ER stress inducer[305]) and the death ligand TRAIL, the 8KO cells underwent extensive BAX/BAK-dependent apoptosis following neutralisation of MCL-1 and BCL-X_L, just like the wild-type parental cells, ruling out a requirement of all known BH3-only proteins in apoptosis induction. This is supported by previous studies which have also outlined the lack of an absolute requirement of certain BH3-only proteins, such as BIM, PUMA and BID, in activation of apoptosis[217,220], while other reports suggest that even some sensitiser BH3-only proteins can weakly activate the effector proteins[90,306].

Collectively, these studies suggest that a simple model of activation of BAX and/or BAK by BH3-only proteins is unlikely to exist and instead, the interactions and

activity of the BCL-2 family are much more complicated, with the notion of activator *versus* sensitizer BH3-only proteins being largely irrelevant.

Data from the previous chapter suggested that BH3-only protein interactions with the anti-apoptotic proteins do not exclusively determine sensitivity to BH3 mimetics, while previous studies imply that BH3-only proteins are not required for apoptosis when BCL-X_L and MCL-1 are neutralised. Here, it was therefore asked whether the BH3-only proteins were required to mediate apoptosis following exposure to highly potent and specific BH3 mimetics, which has not previously been studied using any of the knockout cells. We demonstrate that in the absence of the eight key BH3-only proteins, inhibitors of MCL-1 and BCL-X_L can still induce apoptosis, going against the central dogma of BH3 mimetic design and challenging the current understanding of how BCL-2 protein family interactions work.

4.2 HCT116 8KO cells do not express the eight key BH3-only proteins.

The HCT116 8KO cells, alongside parental wild-type (WT) and double knockout (DKO, lacking BAX and BAK, used as a negative control) were a gift from Professor Luo (University of Nebraska Medical Centre, Nebraska). In order to first verify the knockouts in each of the cell lines, the cells were subjected to immunoblotting using antibodies against each of the key BH3-only proteins. The cell lysates were also probed with antibodies against the anti-apoptotic BCL-2 family as well as tubulin, as a loading control (Fig. 4.1A). The HCT116 WT cells clearly demonstrated reasonable levels of most of the BCL-2 family proteins in the immunoblots including BAX, BAK, BIM (with all isoforms identified), BID, BAD, PUMA (identified by the lower, faint band), NOXA and BMF (lower band). HRK, BCL-2 and BIK were not detectable at the protein level in any of the HCT116 cells. As expected, BAX and BAK were not

present in the DKO cell lines, while the rest of the BCL-2 family were unaffected in the DKO cells. The 6 BH3-only proteins detectable by immunoblotting (BIM, BID, BAD, PUMA, NOXA, BMF) were not present in the 8KO cell lines. The anti-apoptotic proteins MCL-1 and BCL-X_L were detectable at similar levels across all three cell lines, suggesting that a lack of the BH3-only proteins does not influence levels of the anti-apoptotic proteins.

Since HRK, BIK and BCL-2 and were not detectable at the protein level in any of the HCT116 cells, qPCR was performed using cDNA generated from the HCT116 WT, DKO and 8KO cells to look at the levels of mRNA. While the relative expression of BCL-2 mRNA was similar among the three cell lines, expression of BIK and HRK mRNA relative to the housekeeping gene, GAPDH, seemed to exhibit differences in the three cell lines (Figure 4.1B). BIK mRNA levels in the HCT116 8KO cells was marginally lower compared to the WT and DKO cells (Fig. 4.1D), whereas HRK mRNA seemed to be expressed at higher levels in the 8KO cells than in the WT or DKO cells. This seemed to indicate that the cells had residual HRK, although it was undetectable at the protein level (Fig. 4.1A). This was in discrepancy with the data presented in the original article showcasing the HCT116 8KO cells demonstrated that HRK was indeed knocked out by assessing the HCT116 cells for frameshift mutations of the CRISPR target region[303]. However, it must be noted that (1) the mRNA expression levels of BCL-2, BIK and HRK were overall extremely low (< 0.001 %) compared to GAPDH and (2) the knockouts were induced by frameshift mutations, which may not necessarily reduce mRNA expression, but which will block protein expression. This, along with the fact that HRK was undetectable at the protein level, confirmed that the 8KO cells were in fact devoid of the 8 BH3-only members, as reported by Professor Luo.[303]

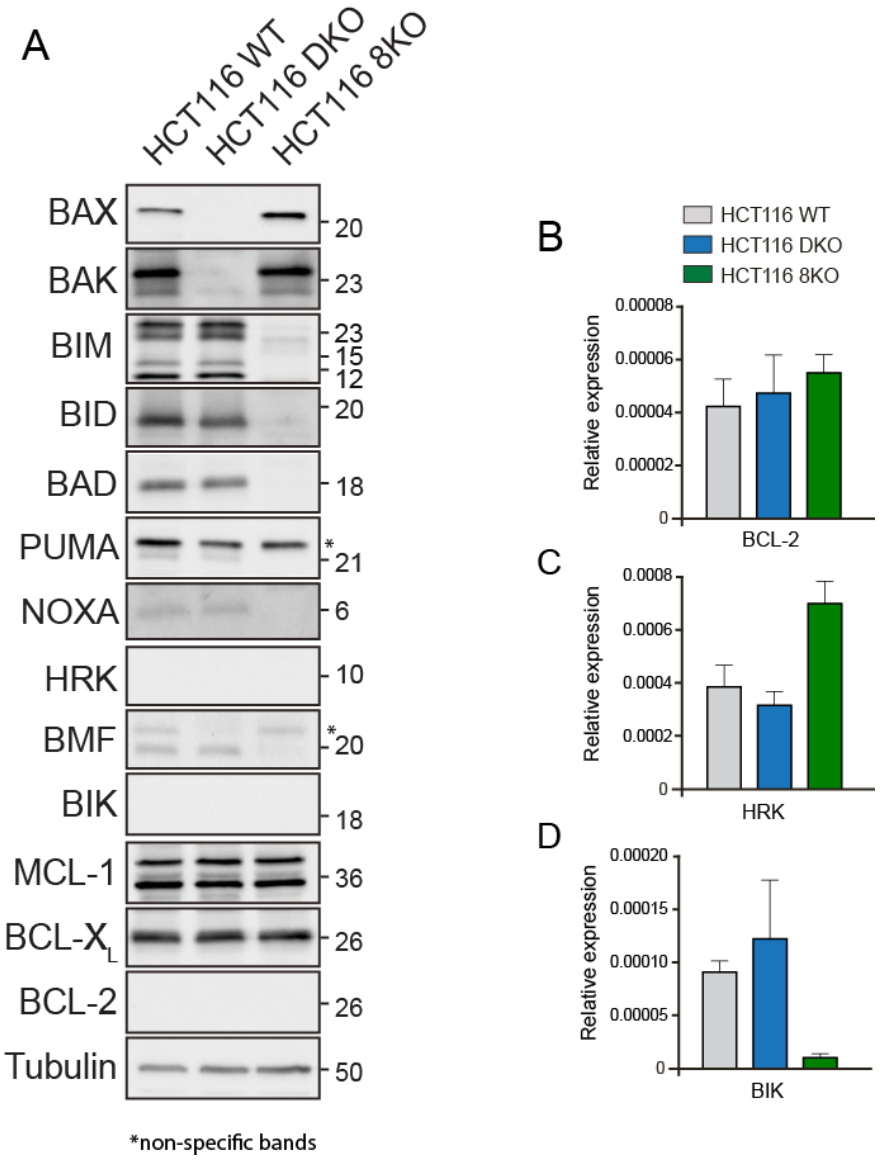


Figure 4.1. HCT116 8KO cells do not appear to express the eight key BH3-only proteins. (A) HCT116 WT, DKO and 8KO cells were lysed in RIPA buffer and subjected to immunoblotting to determine the levels of the BCL-2 family proteins and confirm the knockout of the indicated proteins. Tubulin was detected as a loading control. Approximate molecular weights of proteins are indicated alongside the blots. (B, C, D) For proteins which were not detectable by immunoblotting, qPCR was performed to determine the relative expression of the proteins compared to the house-keeping gene GAPDH.

4.3 Genetic suppression of MCL-1 and BCL-X_L can induce apoptosis in the absence of the known BH3-only proteins.

The original report showcasing the HCT116 8KO cells demonstrated that neutralisation of BCL-X_L and MCL-1 could induce BAX activation and apoptosis in the absence of BH3-only proteins. This goes against the central dogma of apoptosis where BH3-only proteins are released from pro-survival counterparts to activate BAX and hinted at other mechanisms which can induce BAX activation.

In order to validate this observation, the HCT116 WT, DKO and 8KO cells were subjected to a knockdown of either MCL-1 or BCL-X_L alone or a combination of BCL-X_L and MCL-1, using short-interfering RNAs (siRNAs). Downregulation of both BCL-X_L and MCL-1 together could induce significant apoptosis in both HCT116 WT and 8KO, with approximate values of 85% and 70% respectively, while the DKO cells did not undergo apoptosis, demonstrating on-target BAX and/or BAK dependent apoptosis (Fig. 4.2A). While knockdown of MCL-1 alone did not induce significant apoptosis in any of the three cell lines, knockdown of BCL-X_L could induce significant apoptosis (~60%) in the WT cells. Interestingly, apoptosis observed following BCL-X_L downregulation occurred in a BH3-dependent manner, as 8KO cells failed to undergo apoptosis and behaved similar to DKO cells, unlike the WT cells (Fig. 4.2A). This suggested that there may be some key differences between MCL-1 and BCL-X_L-mediated apoptosis in terms of the requirement of BH3-only proteins in HCT116 cells.

Knockdown of MCL-1 and BCL-X_L was validated using immunoblotting – cells were incubated with siRNAs as previously described, collected and lysed for immunoblotting. While BCL-X_L is almost completely knocked down in all three cell lines, only the top band of MCL-1 appeared to be knocked down. The top band of

MCL-1, as previously mentioned, is thought to be the isoform of MCL-1 which regulates apoptosis, however, therefore the loss of the upper band is sufficient to induce apoptosis in combination with knockdown of BCL-$X_{L.}$ It is interesting to note that when one anti-apoptotic BCL-2 member is silenced, there is slight but noticeable increase in expression of the partner anti-apoptotic protein in most cases, suggesting there may be some kind of compensatory mechanism in place to help cells survive (Fig. 4.2B, C, D).

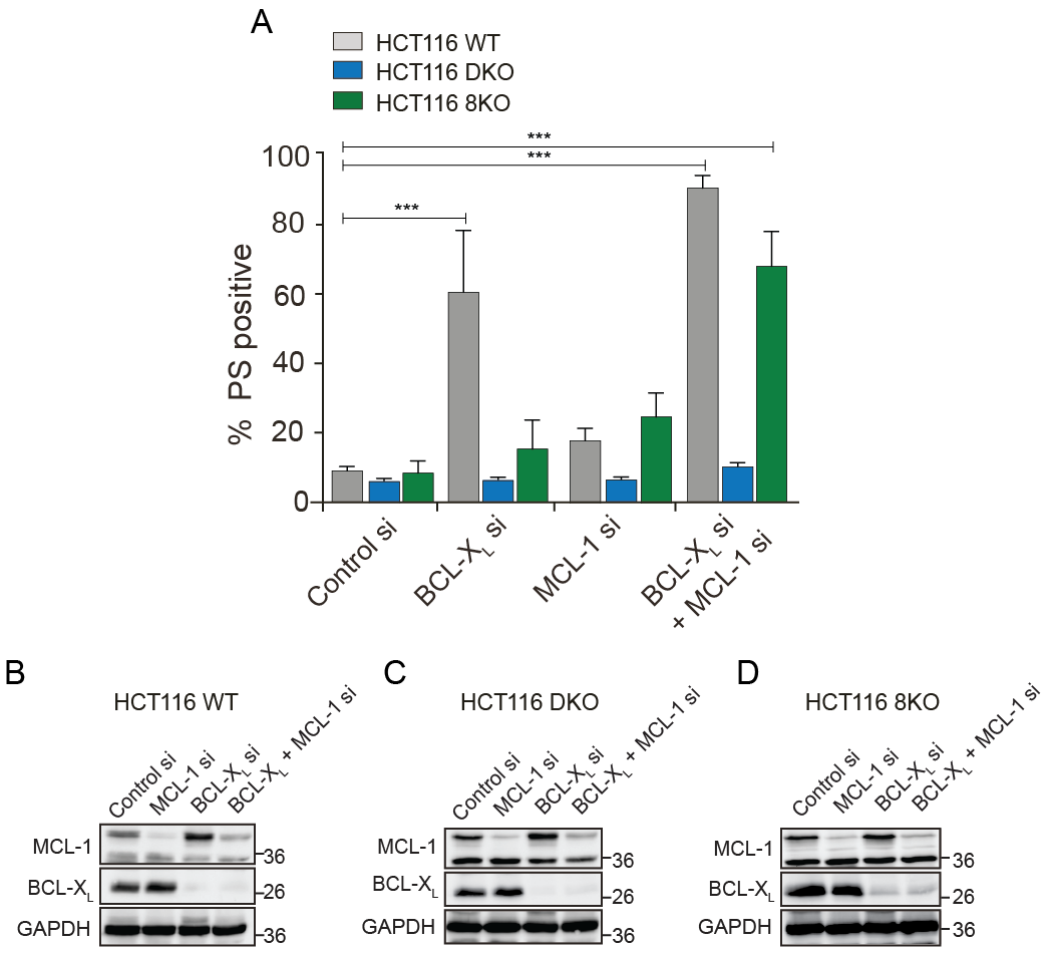


Figure 4.2. Knockdown of pro-survival proteins can induce apoptosis in a BAX- and BAK-dependent but BH3-only protein-independent manner. (A) HCT116 WT, DKO and 8KO cells were incubated with the indicated siRNAs for 72 hours and apoptosis was assessed by PS externalisation using Annexin V labelling in flow cytometry. (B, C, D) Knockdown efficiency of MCL-1 and BCL-X_L in the three different cell lines was assessed by immunoblotting with GAPDH as a loading control. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

4.4 HCT116 8KO cells are similarly primed for death as their WT counterparts.

In order to further explore the surprising ability of 8KO cells to undergo apoptosis to a similar extent to that of WT cells, HCT116 WT and 8KO cells were subjected to BH3 profiling. BH3 profiling is a novel technique developed by the Letai group which uses synthetic peptides to mimic the BH3 motif of pro-apoptotic BH3 proteins, such as BIM, BAD and NOXA, to determine the sensitivity of cells to undergo apoptosis following exposure to certain chemotherapeutic agents[297]. The extent of priming is quantified by assessing mitochondrial membrane potential following exposure to a single peptide or a mixture of peptides. This gives an indication of the extent of apoptosis that would be induced if the relative proteins were targeted using chemotherapy[24,307].

Here, the synthetic peptides BAD (which specifically binds BCL-X_L) and MS-1 (a peptide specific to MCL-1[308]) were used alone and in combination to determine the priming of HCT116 WT and 8KO cells for apoptosis induction following BCL-X_L and/or MCL-1 inhibition. FCCP (Carbonyl cyanide-4-phenylhydrazone), a mitochondrial un-coupler[279], was used as a positive control for depolarisation which, as shown in Figure 4.3, induced complete depolarisation of mitochondria in comparison to the DMSO control. Mitochondrial depolarisation was assessed using TMRE, a dye which accumulates in active mitochondria and which is lost upon depolarisation of the mitochondrial membrane[278]. Addition of 10 μM BAD peptide resulted in ~30% depolarisation of the WT cells (but not the 8KO cells), confirming that the WT cells were somewhat dependent on either BCL-2, BCL-X_L or BCL-w. This is in agreement with data in Fig. 4.2A, in which the WT cells appeared to exhibit some apoptosis following the genetic knockdown of BCL-X_L. As expected, the extent

of mitochondrial depolarisation following BAD peptide in the 8KO cells was significantly less, further confirming previous data (Fig. 4.2A). Addition of the MCL-1-specific MS-1 peptide also resulted in some mitochondrial depolarisation, however this was in fact higher in the 8KO cells compared to the WT, with ~25% depolarisation in the WT and ~35% in the 8KO cells. This data suggested that there may be a slight dependence on MCL-1 alone, although this was not detected when cells were previously exposed to S63845. A combination of the two peptides together induced extensive depolarisation of ~85% in both the WT and 8KO cells, as seen with the concurrent genetic inhibition of MCL-1 and BCL-X_L. Thus, the 8KO cells behave in a very similar way to the WT cells, suggesting they are equally as primed for death when both MCL-1 and BCL-X_L are inhibited but not when only BCL-X_L alone is inhibited.

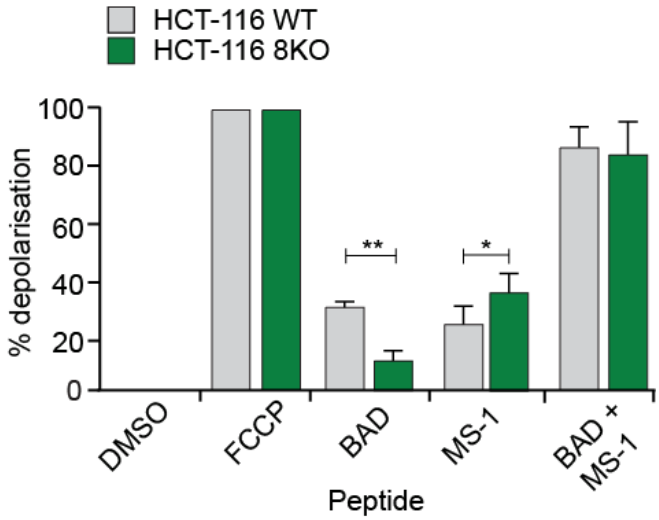


Figure 4.3. HCT116 WT and 8KO cells are equally primed for death following suppression of both MCL-1 and BCL-X_L but not BCL-X_L alone. The plasma membranes of HCT116 WT and 8KO cells were permeabilised and the indicated peptides or compound was incubated with the cells. TMRE was then combined with the mixture to assess mitochondrial depolarisation in flow cytometry. FCCP was used as a positive control. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$

4.5 Inhibition of BCL-X_L but not MCL-1 using BH3 mimetics can induce apoptosis in a BH3-dependent manner in HCT116 cells.

Since genetic inhibition of BCL-X_L but not MCL-1 could induce BH3-only protein-dependent cell death and since BH3 profiling data seemed to suggest that the BCL-X_L-specific BAD peptide could only induce significant depolarisation in the HCT116 WT cells, it was anticipated that direct pharmacological inhibition of BCL-X_L and MCL-1 would demonstrate similar differences. In order to test this, HCT116 WT and 8KO cells were incubated with either A-1331852 or S63845 (both at 100 nM) for 4 h and apoptosis assessed by PS externalisation in flow cytometry. Indeed, exposure of

cells to A-1331852, but not S63845, resulted in enhanced apoptosis in HCT116 WT cells, with ~30% of cells undergoing apoptosis following exposure to A-1331852 (Fig. 4.4). Remarkably, this apoptosis was not observed in the HCT116 8KO cells, in line with previous data presented here.

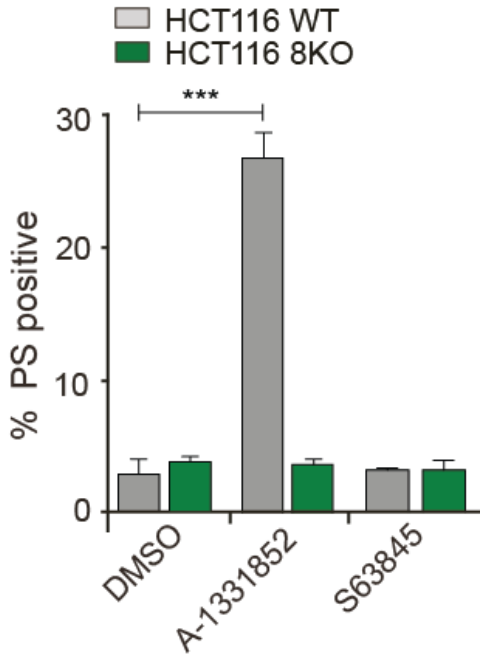


Figure 4.4. Pharmacological inhibition of BCL-X_L but not MCL-1 can induce apoptosis in a BH3-only protein-dependent manner. HCT116 WT and 8KO cells were exposed to 100 nM of A-1331852 or 100 nM S63845 for 4 h and apoptosis assessed by PS externalisation using Annexin V staining in flow cytometry. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$

4.6 BCL-X_L seems to be the major pro-survival protein in various cell lines.

Although most solid tumours depend on both BCL-X_L and MCL-1 for survival[199,245,292–294,309], it is clear from previous data that, surprisingly, inhibition of BCL-X_L alone can induce significant apoptosis in the colorectal carcinoma cell line, HCT116. In order to explore whether this trend extended to other solid tumour cell lines, a panel of five solid tumour cancer cell lines was exposed to either S63845 or A-1331852 (both at 100 nM) and apoptosis measured using PS externalisation at different timepoints. The five cell lines selected in addition to HCT116 WT cells were MDA-MB-231 (a breast cancer cell line), H1299, A549, PC-3 and SUIT-2. As expected, the HCT116 cells underwent ~50% apoptosis following 4 h exposure to A-1331852, while S63845 did not induce significant apoptosis compared to the DMSO control (Fig. 4.5A). Extension of treatment times to 24, 48 and 72 h did not see a significant increase in the extent of apoptosis however, with the extent of apoptosis still at ~50% even at 72 h. This suggests that inhibition of BCL-X_L alone is not enough to induce complete apoptosis in this cell line and that targeting of both BCL-X_L and MCL-1 was required to induce complete apoptosis (as seen in Fig 3.12C). The other cell lines tested demonstrated varied responses to inhibition of BCL-X_L over MCL-1. Treatment of the breast cancer cell line MDA-MB-231 with A-1331852 resulted in extensive apoptosis at 4 h (~60%), which increased moderately over time to ~80% at 72 h, suggesting these cells are very sensitive to inhibition of BCL-X_L (Fig. 4.5B). S63845 did not induce significant apoptosis at any time point in MDA-MB-231 cells. Meanwhile, in H1299 cells, neither S63845 nor A-1331852 alone could induce apoptosis at any timepoint, suggesting that inhibition of both MCL-1 and BCL-X_L is required to induce death in this cell line (Fig. 4.5C), as alluded to in the previous chapter for various cell lines (Fig. 3.12). The other non-small cell lung carcinoma cell

line, A549, did undergo some apoptosis however, with A-1331852 inducing ~35% apoptosis, which fluctuated until 72 h, supporting the idea suggested in the previous chapter that cells derived from the same type of cancer can exhibit varying dependence on pro-survival proteins (Fig. 4.5D). The two other cell lines tested, PC-3 and SUIT-2, mimicked A549 in undergoing moderate apoptosis following A-1331852 exposure, whereas S63845 did not induce more apoptosis than the DMSO control (Fig. 4.5E and F). The overall message from these experiments is that although inhibition of BCL-X_L can induce apoptosis in cells from various cancers, dual inhibition of MCL-1 and BCL-X_L (and possibly even other pro-survival proteins) is often required to induce complete apoptosis, as most cells from solid tumours do not exclusively depend on one pro-survival protein.

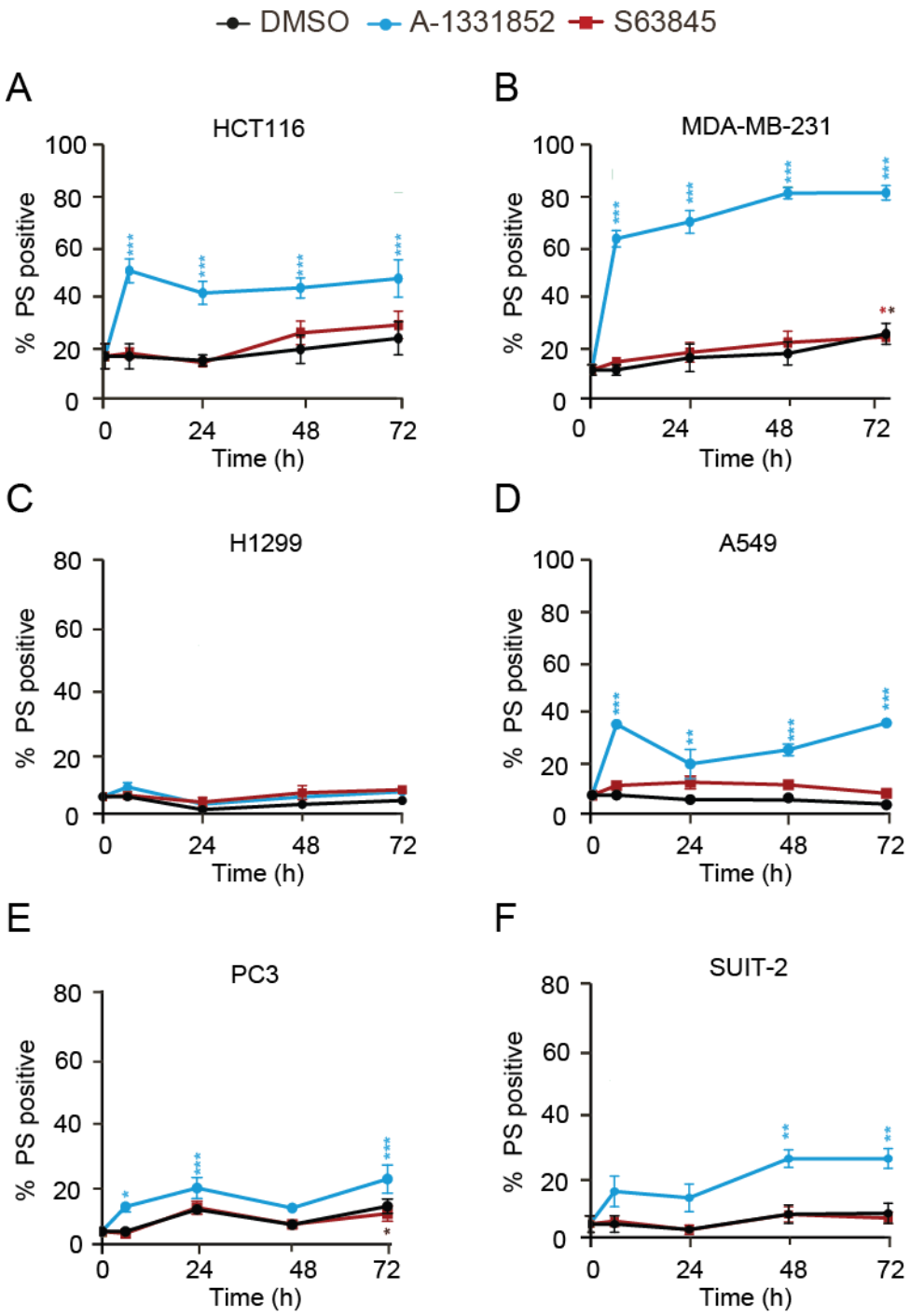


Figure 4.5. Pharmacological suppression of BCL-X_L but not MCL-1 is sufficient to induce apoptosis in some cell lines at extended timepoints. The indicated cell lines were exposed to either DMSO, A-1331852 (100 nM) or S63845 (100 nM) alone and collected at the indicated timepoints to be assessed for apoptosis by PS externalisation using Annexin-V labelling in flow cytometry. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

4.7 Dual inhibition of MCL-1 and BCL-X_L with highly specific BH3 mimetics can induce apoptosis even in the absence of BH3-only proteins.

It has been established in previous figures that inhibition of BCL-X_L alone can induce apoptosis in the HCT116 WT but not HCT116 8KO cells (which lack the 8 key BH3-only proteins). To test whether dual inhibition of BCL-X_L and MCL-1 would result in the same phenomenon, the HCT116 WT and 8KO cells were exposed to a combination of A-1331852 (100 nM) and a concentration range of S63845 for 4 h and apoptosis assessed using PS externalisation in flow cytometry. The parental WT control cells underwent significant apoptosis following a combination of inhibitors (both at 100 nM), with ~80% death achieved following exposure to 1 µM S63845 and 100 nM A-1331852 (Fig. 4.6). Remarkably, the HCT116 8KO cells also underwent a similar apoptosis, with slightly more cells dying at 100 nM of each drug than the WT but with approximately the same IC_{50} (around 100 nM). Thus, when both MCL-1 and BCL-X_L are inhibited using BH3 mimetics in the HCT116 8KO cells, there is no requirement for the BH3-only proteins – unlike when just BCL-X_L is inhibited. This data also extends the results of the original HCT116 8KO report[303] by demonstrating that cells can undergo apoptosis without the eight key BH3-only members even when using highly specific BH3 mimetics to inhibit MCL-1 and BCL-X_L. This goes against the accepted principle of how BH3 mimetics induce apoptosis and raises many questions about the BCL-2 family and BH3 mimetics in apoptosis, particularly regarding the mechanism of action of BH3 mimetics and how the effector proteins, BAX and BAK, behave in the HCT116 8KO cells.

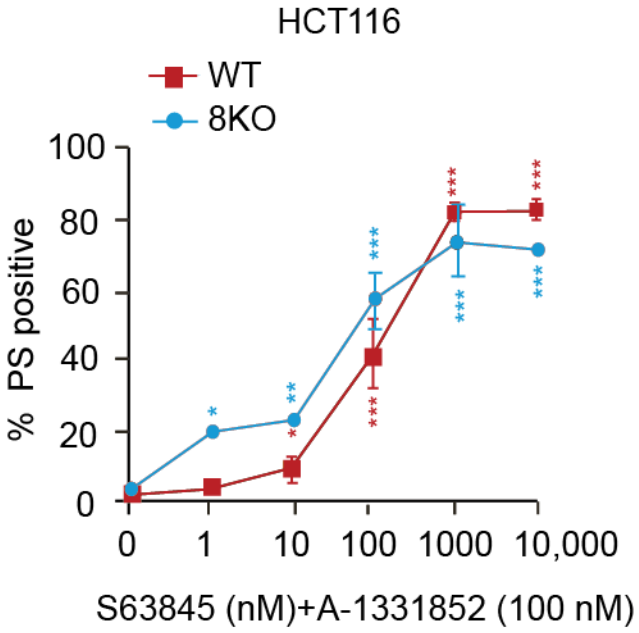


Figure 4.6. BH3 mimetics can induce apoptosis in a BH3-only protein-independent manner. HCT116 WT and 8KO cells were exposed to a concentration range of S63845 alongside a fixed concentration of A-1331852 (100 nM) for 4 h and the extent of cell death assessed by PS externalisation using Annexin V labelling in flow cytometry. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$

4.8 BH3 mimetic-induced apoptosis requires BAX but not BAK in HCT116 cells.

Since BH3-only proteins are not required for mediating BH3 mimetic-induced apoptosis in HCT116 cells, it is possible that BH3 mimetics could somehow directly activate BAX and/or BAK to induce apoptosis, perhaps by relieving their inhibition by pro-survival proteins. While previous data indicates that BH3 mimetics require the presence of the effector proteins to induce apoptosis, the exact requirement of BAX and/or BAK had not been established. Therefore, in order to determine the BAX/BAK requirement of the HCT116 cells in BH3 mimetic-induced death, four cell lines

(HCT116 WT, BAX KO, BAK KO and BAX/BAK DKO) were exposed to 100 nM A-1331852 with a concentration range of S63845 for 4 h and assessed for apoptosis by PS externalisation in flow cytometry (Fig. 4.7A). The WT cells underwent extensive apoptosis as expected, with an IC_{50} of ~100 nM of both S63845 and A-1331852. Meanwhile, the BAK KO cells also underwent apoptosis, with a similar IC_{50} to the WT cells, suggesting that BAK is not critical for apoptosis in HCT116 cells. However, BAX appeared to be essential for BH3 mimetic-induced apoptosis in HCT116 cells as neither the single BAX KO nor the BAX/BAK DKO cells underwent apoptosis following exposure to A-1331852 in combination with all concentrations of S63845. Knockout of the indicated proteins was confirmed by immunoblotting (Fig. 4.7B).

This data suggests that BAX is essential to BH3 mimetic induced apoptosis in HCT116 cells and BAK cannot compensate for a lack of BAX. This is in line with previous studies which suggest that HCT116 relies rely on BAX over BAK for apoptosis[310] and also suggests that even in the absence of BH3-only proteins, BAX is still an absolute requirement for HCT116 cells to undergo apoptosis.

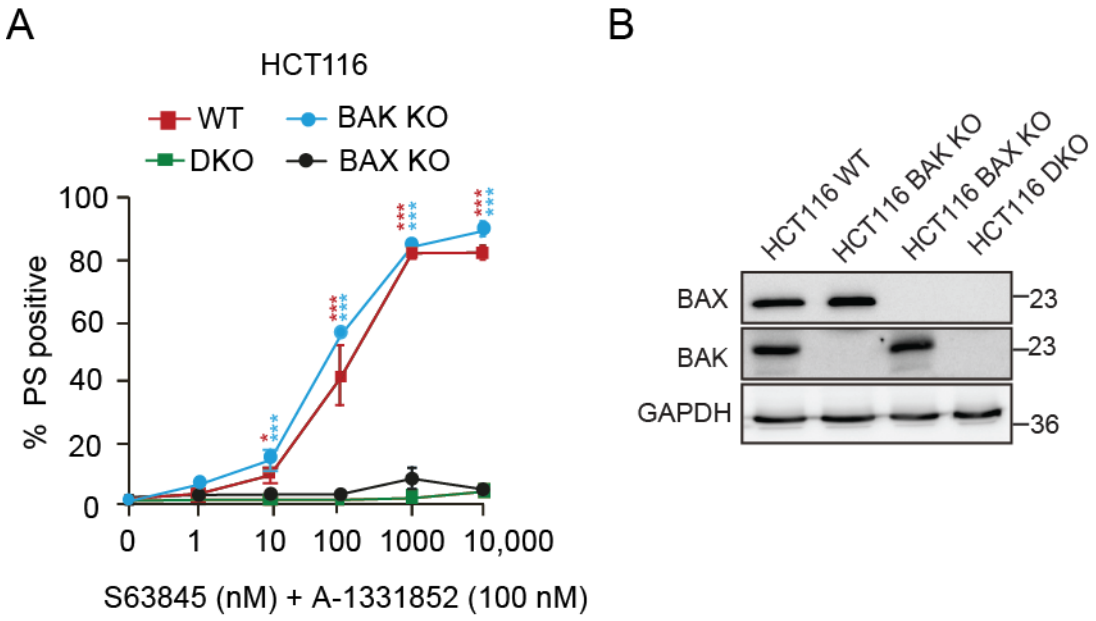


Figure 4.7. A combination of S63845 and A-1331852 induces BAX-dependent but BAK-independent death in HCT116 cells. (A) HCT116 WT, BAX KO, BAK KO and BAX/BAK DKO cells were exposed to a concentration range of S63845 alongside a fixed concentration of A-1331852 (100 nM) for 4 h and the extent of cell death assessed by PS externalisation using Annexin V in flow cytometry. (B) Western blot validation of the knockout cell lines with GAPDH as a loading control. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$

4.9 BAX appears to be already activated in both HCT116 WT and 8KO cells.

As described in the introduction, for a BAX-dependent apoptosis to occur, BAX must undergo a series of events, such as activation of monomeric BAX from its globular form where its key motifs are internalised, to an active form which then oligomerises to form a channel on the mitochondrial membrane, allowing the release of cytochrome *c*.

BAX was shown to be key to BH3 mimetic-induced apoptosis in HCT116 cells, therefore in order to assess whether BAX was differentially activated in HCT116 WT

versus 8KO cells, the level of activated BAX following BH3 mimetic exposure was detected using the BAX 6A7 antibody. This antibody specifically recognises residues in the α-1 helix of BAX which is only exposed following apoptotic stimuli[300,311]. The BAX 6A7 antibody was therefore used to label cells exposed to 100 nM S63845 and A-1331852 and fluorescence intensity detected in in flow cytometry. Surprisingly, in HCT116 cells, it appears that using this method to detect BAX activation is not ideal, as almost all BAX, with and without treatment, appeared to be activated in both WT and 8KO cells (Fig. 4.8A). A similar activation of BAK (detected by the anti-BAK AB-1 antibody) was observed in both WT and 8KO cells (Fig. 4.8B), suggesting that HCT116 cells were inherently posing some difficulty with respect to this particular assay. To confirm this idea, BAX/BAK activation assays were also carried out in H1299 cells. Although activated BAX was not detectable in H1299 (as these cells do not express BAX), activated BAK was clearly observed in H1299 following a combination of S63845 and A-1331852, while no activated BAK was detectable in the DMSO control, suggesting that the assay was working as anticipated, at least in the H1299 cells. It could perhaps be proposed that this constant activation of BAX is not an artefact of the experiment and therefore could explain why 8KO cells undergo apoptosis following neutralisation of the pro-survival proteins. However, this is unlikely to be the case, as neither the HCT116 WT or 8KO cells have high levels of basal death which would be expected with cells with such extensive basal BAX/BAK activation. Thus, it is likely that the high levels of activated BAX/BAK are merely an artefact of the experimental protocol used here.

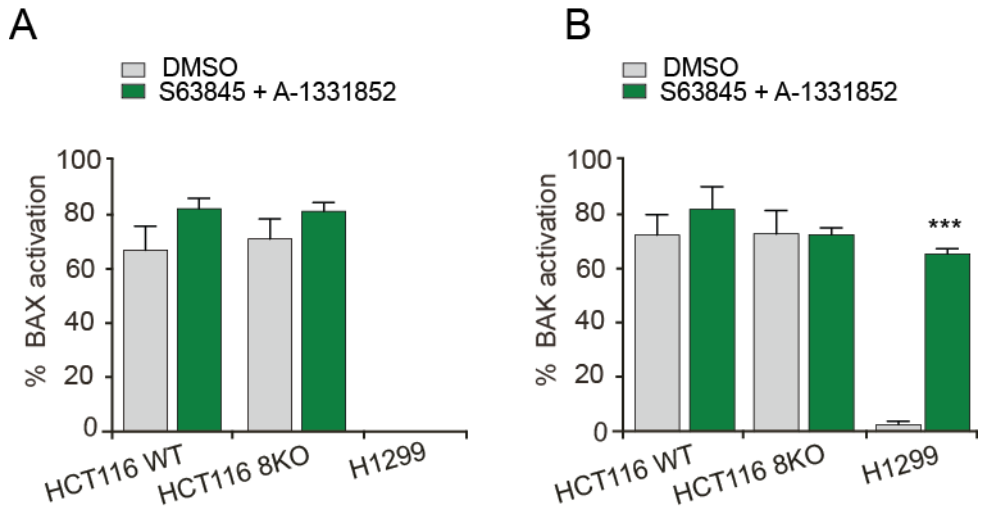


Figure 4.8. BAX and BAK activation are not detectable in HCT116 cells due to constant activation in all conditions. HCT116 WT and 8KO cells and H1299 cells (a positive control for the assay) were treated with a vehicle control or 100 nM S63845 and A-1331852 for 4 h, permeabilised and incubated with antibodies against active BAX (A) or BAK (B). While H1299 cells presented their typical BAK activation status, both HCT116 WT and 8KO cells presented constant BAX and BAK activation, regardless of treatment. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$

4.10 BAX translocates to the mitochondria even in the absence of BH3-only proteins.

Despite the limitations of BAX activation assays in the HCT116 WT and 8KO cells, BAX activity could be measured in other ways in order to assess whether it was undergoing the typical chain of events leading to cytochrome *c* release in the absence of the BH3-only proteins. One of these events is the translocation of BAX from the cytosol to the mitochondria. To ensure this was occurring in the same way in both the HCT116 WT and 8KO cells during BH3 mimetic-mediated apoptosis, the cells were pre-treated with 20 µM Z-VAD-FMK for 30 min. Z-VAD-FMK is a caspase inhibitor,

therefore prevents cells from dying by blocking events downstream of MOMP – this permits observation of BAX activity without the presence of apoptosis related artefacts. Cells were then treated with either DMSO or 100 nM of S63845, A-1331852 or a combination of the two for 2 h, before being fixed, permeabilised and incubated with an antibody against BAX. In the control (DMSO-treated) cells, BAX appeared in a speckled, dispersed pattern, all around the cell, typical of a cytosolic staining, in both HCT116 WT and 8KO cells (Fig. 4.9). However, upon treatment with the BH3 mimetics, this dispersed pattern was lost and instead, BAX could be seen at distinct foci, clustered together in large spots. While this pattern is not a typical mitochondrial pattern (hyper networked and organised), this was BAX translocating to the mitochondria, likely forming spots of high-molecular weight oligomers in preparation for cytochrome *c* release. It is not surprising that the resulting pattern is not typically mitochondrial, as the BAX oligomers are unlikely to be present as a homogenous population across the mitochondria and instead, are thought to be present at distinct recruitment sites involving multiple proteins, such as VDAC2[312] amongst others. Nevertheless, the translocation of BAX from the cytosol to the mitochondria was virtually indistinguishable between the WT and 8KO cells, with no mitochondrial BAX detected in the DMSO treated cells and BAX being mitochondrial in both WT and 8KO cells. It is interesting to note that, in support of previous findings, inhibition of BCL-X_L by A-1331852 alone could induce partial BAX translocation in the WT but not 8KO cells, suggesting that the differences in regulation of apoptosis by MCL-1 and BCL-X_L occur at the level of BCL-2 family interactions, prior to Bax translocation.

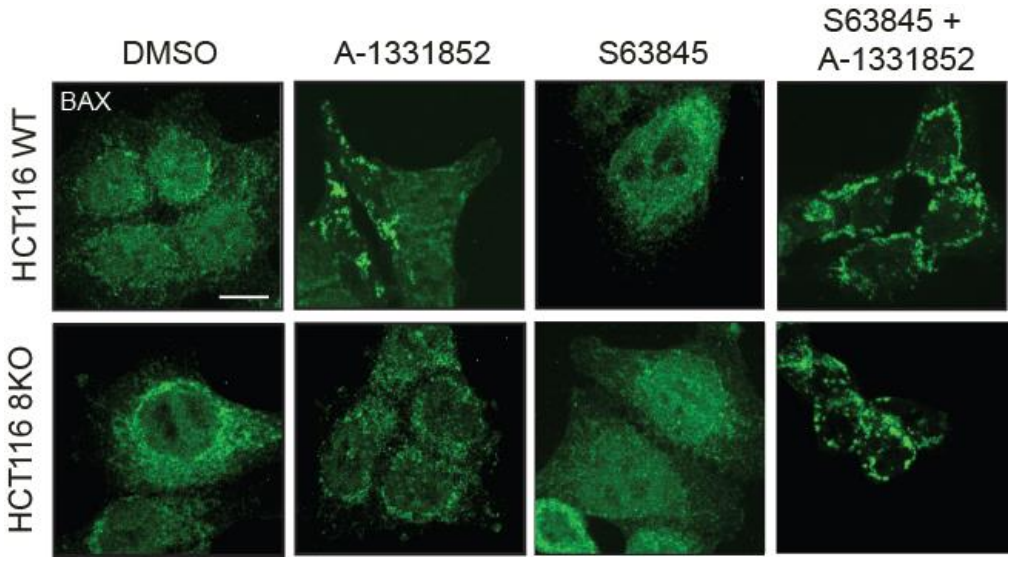


Figure 4.9. Pharmacological inhibition of MCL-1 and BCL-X_L can induce BH3-only protein-independent BAX translocation to the mitochondria. HCT116 WT and 8KO cells were pre-treated with 20 µM Z-VAD-FMK for 30 min then exposed to 100 nM of the indicated inhibitors for 2 h, fixed and stained for BAX to assess its localisation. Scale bar – 10 µm.

4.11 BAX forms high molecular weight oligomers following exposure to BH3 mimetics even in the absence of BH3-only proteins.

Since BAX translocates from the cytosol to the mitochondria in the same manner in both HCT116 WT and 8KO cells following BH3 mimetics, the ability of BAX to form high molecular weight oligomers without BH3-only proteins was next assessed. To examine this, both cell lines were pre-treated with Z-VAD-FMK followed by 100 nM S63845 and A-1331852 for two hours, as performed previously for analysis of BAX translocation, and then subjected to gel filtration chromatography over a Superose 6 column to separate protein complexes on the basis of their size.

The collected HCT116 WT and 8KO cell fractions were probed for BAX to assess whether oligomerisation could be observed following BH3 mimetic exposure. Since

BAX is considered to be cytosolic and typically monomeric[311] or possibly dimeric[313] it was anticipated to be present at low molecular weights (~20-50 kDa) in the control conditions. Indeed, in both HCT116 WT and 8KO, BAX could be seen in fractions which are proposed to be around 40-60 kDa in molecular weight, suggesting this pool of BAX exists at a low molecular weight - perhaps, in dimers (Fig. 4.10). Meanwhile, in the cells exposed to a combination of S63845 and A-1331852, BAX could be found largely at high molecular weights (~1500-2000 kDa) in both HCT116 WT and 8KO cells, suggesting it had oligomerised to high molecular weight complexes following exposure to BH3 mimetics to the same extent in both cell lines.

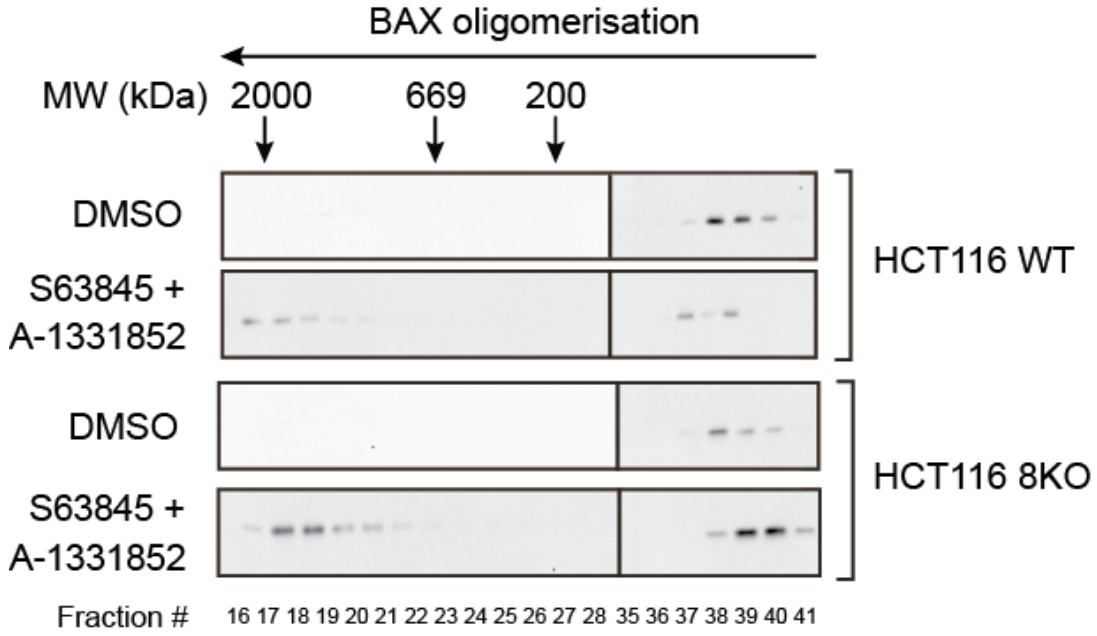


Figure 4.10. BAX undergoes oligomerisation following S63845 and A-1331852 treatment independently of the BH3-only proteins. HCT116 WT and 8KO cells were treated with 100 nM S63845 and A-1331852 for 2 h, lysed and proteins separated based on size using a Superose 6 column. High to low molecular weight fractions were then subjected to immunoblotting to detect the location of BAX. Vertical lines indicate separation of individual gels, with fractions ran in a non-continuous series.

4.12 Cytochrome *c* can be released by BH3 mimetics even in the absence of the known BH3-only proteins.

BAX translocates and forms oligomers in the same way in both WT and 8KO cells therefore, in theory, cytochrome *c* should also release in the same way, unless the BH3 mimetics are inducing cell death in the HCT116 8KO proteins in an unusual manner. To test this hypothesis, HCT116 WT and 8KO cells were exposed to S63845 and A-1331852 for 2 h following Z-VAD-FMK pre-treatment (as above) and probed for cytochrome *c*. In the control cells, cytochrome *c* was clearly localised within the mitochondria as expected. However, upon addition of S63845 and A-1331852, this networked, filamentous labelling is completely lost as the cytochrome *c* exits the mitochondria and becomes dispersed in the cytosol. This occurred in both the WT and 8KO cells in the same manner, suggesting that cytochrome *c* release can occur regardless of the presence of the BH3-only proteins following inhibition of MCL-1 and BCL-X_L. (Fig. 4.11). Again, it is worth nothing that partial cytochrome *c* release occurs in the HCT116 WT but not in 8KO cells following inhibition of BCL-X_L with A-1331852. This suggests that these cells commit to apoptosis through cytochrome *c* release following inhibition of just one of the pro-survival proteins, perhaps suggesting distinct pools of cells with different dependencies on the BCL-2 family with a population.

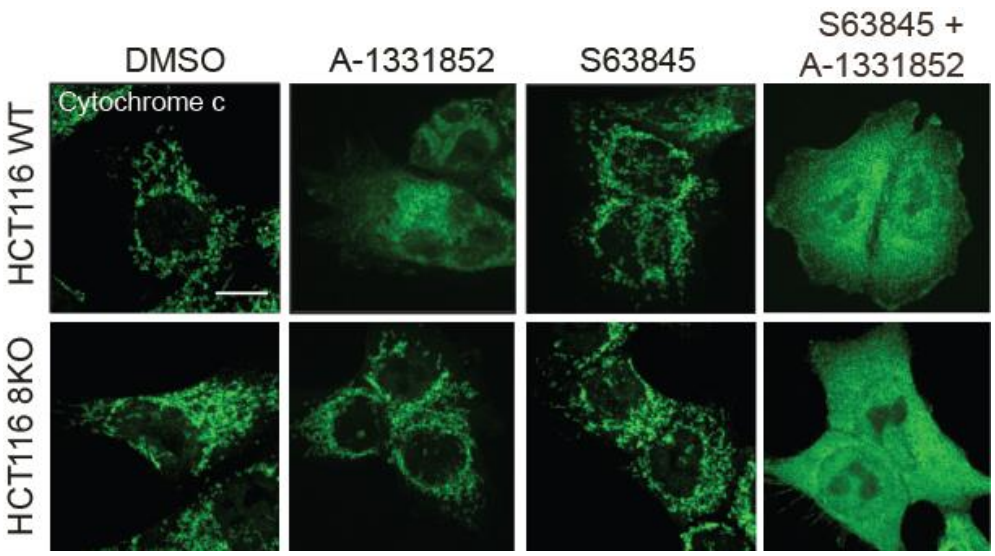


Figure 4.11. Cytochrome *c* can be released by pharmacological inhibition of MCL-1 and BCL-X_L independently of BH3-only proteins. HCT116 WT and 8KO cells were exposed to 100 nM of the indicated inhibitors for 2 h, fixed and stained with an anti-cytochrome *c* antibody to assess its localisation. Scale bar - 10 μm.

4.13 BAX is not restrained directly by MCL-1 and BCL-X_L or released following BH3 mimetics in HCT116 WT or 8KO cells.

Since BAX appears to be absolutely necessary to induce death by BH3 mimetics in HCT116 cells, it could be suggested that BAX is directly sequestered by MCL-1 and BCL-X_L and thus, when cells are exposed to BH3 mimetics, BAX could be displaced from the anti-apoptotic proteins and is somehow activated, moving to the mitochondrial membrane and initiating the apoptotic pathway, in a 'mode 1' style activation model.

While the interactions of BAK with anti-apoptotic proteins, especially MCL-1, have been described[314,315], interactions between BAX and anti-apoptotic proteins are poorly documented. Some reports have shown that BCL-2 and BAX can interact[316,317]

however there is little evidence of MCL-1 or BCL-X_L interacting with BAX directly. BAX is typically thought to be a very dynamic protein however, moving between different compartments of the cell (mostly the cytosol and mitochondria) according to cellular status. To test the hypothesis of a BAX-MCL-1 and/or BCL-X_L interaction mediating the BH3 mimetic-induced death in the HCT116 8KO cells, MCL-1 and BCL-X_L were immunoprecipitated from both HCT116 WT and 8KO cells pre-treated with Z-VAD-FMK and then combination of S63845 and A-1331852 (both at 100 nM) and tested for interactions with BAX. As a positive control for the BAX band as well as a control for BAX activation by BH3 mimetics, an anti-BAX N-terminal antibody was also used to immunoprecipitate BAX.

Despite significant levels of BCL-X_L and MCL-1 being immunoprecipitated, as shown by their respective blots, no BAX could be seen bound to either MCL-1 or BCL-X_L in either HCT116 WT or 8KO cells (Fig. 4.12A and B). The pulldown of BAX using the anti-N-terminal BAX antibody demonstrated that BAX was easily detectable in the blots however, confirming that the lack of BAX bands in the MCL-1/BCL-X_L immunoprecipitation experiments was not a result of poor immunoblotting. From this data, it is reasonable to suggest that neither HCT116 WT not 8KO cells undergo apoptosis following exposure to BH3 mimetics as a result of a displacement of BAX from pro-survival proteins. This is not a surprising result, as even if MCL-1/BCL-X_L did interact with BAX, the simple release of BAX from these proteins should not be enough to induce apoptosis, as the proposed bimodal activation of BAX requires another step of activation (typically by BH3-only proteins). This, along with the other data here, therefore suggests that other factors are in play than the currently known BCL-2 family.

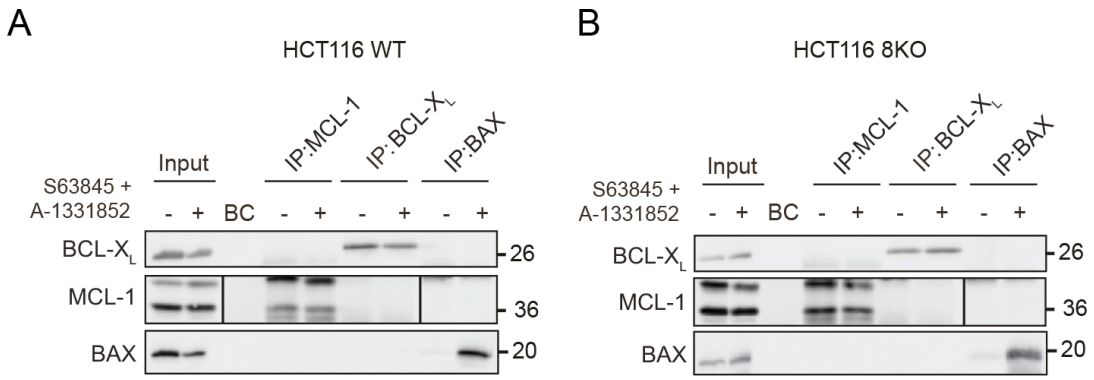


Figure 4.12. Pro-survival proteins do not interact directly with BAX in HCT116 cells to prevent its pro-apoptotic activity. (A) HCT116 WT and (B) 8KO cells were pre-treated with Z-VAD-FMK then exposed to 100 nM A-1331852 and S63845 for 4 h. Collected cells were lysed and immunoprecipitation performed against MCL-1 and BCL-X_L, then immunoblotting for the indicated proteins to detect any interacting BAX. An anti-BAX N-terminal antibody immunoprecipitation was performed as a positive control for treatment and efficient pulldown/immunoblotting of BAX. BC represents bead control and IP indicates immunoprecipitation.

4.14 Discussion

Until recently it had been thought that the major BH3-only proteins were essential to altering the fate of a cell and pushing it towards apoptosis. The study by O'Neill *et al.*[303] however disproved this by showing that HCT116 cells devoid of the eight key BH3 only proteins could still undergo apoptosis following suppression of pro-survival proteins – an observation which was confirmed by data here showing that HCT116 WT and 8KO cells undergo similar levels of apoptosis following concurrent knockdown of MCL-1 and BCL-X_L. While this work was a very important and remarkable challenge to the accepted dogma of apoptosis, it did not address whether suppression of pro-survival proteins using highly specific BH3 mimetics, which are designed to displace pro-survival proteins from their pro-apoptotic counterparts within the cell, could still induce apoptosis in the absence of the 8 key BH3-only proteins.

Remarkably, it was demonstrated here that HCT116 8KO cells can still undergo apoptosis following exposure to S63845 and A-1331852, inhibitors of MCL-1 and BCL-X_L respectively, suggesting that BH3 mimetics can function independently of BH3-only proteins to activate effector proteins and induce cell death (Fig. 4.13). Again, it is worth noting that the knockdown of MCL-1 appears to be incomplete as only the top band is knocked down, while the bottom band remains. While it is not clear why HCT116 cells display only two of the three major bands of MCL-1, it could be suggested that this lower band is in fact the mitochondrial matrix form, which would not play a role in apoptosis, which would explain why extensive apoptosis is seen with the combination of this knockdown of MCL-1 with a BCL-X_L knockdown. Future work should aim to use a pool of MCL-1 siRNAs to maximise the knockdown so that this is not an issue, while also assessing the knockdown efficiency of MCL-1 at earlier time-points, given the high turnover rate of MCL-1. Furthermore, apoptosis should be observed in the HCT116 WT and 8KO cells following S63845 and A-1331852 treatment at 24, 48 and 72 hours in order to draw better comparisons between the inhibitor and siRNA treatments (where apoptosis assays were performed at 72 hours).

The apoptosis in the HCT116 cells was also BAX- but not BAK-dependent, highlighting the importance of BAX in BH3 mimetic-induced apoptosis. While BAX underwent its typical process of translocating from the cytosol to the mitochondria, oligomerising and releasing cytochrome *c*, how exactly BAX was being activated was not evident. It was clear that BAX was not being restrained by MCL-1 and BCL-X_L directly (and therefore being released and activated following addition of BH3 mimetics), thus another mechanism must be in place in the HCT116 8KO cells. It is possible that these cells are following the indirect model of apoptosis where BH3-only

proteins are not displaced from pro-survival proteins to activate BAX/BAK but instead, directly disrupt the interactions of pro-survival proteins and BAX instead. However, simply freeing BAX/BAK from pro-survival proteins is not enough to lead to their activation. Instead, a bimodal activation is proposed to occur, where BH3-only proteins are considered essential to activation of BAX/BAK. This, taken together with the data here, suggests that the underlying mechanism of effector protein activation may be even more complex than previously known. Of course, cells with normal levels of BH3-only proteins are likely to use BH3-only proteins to activate effector proteins and subsequent apoptosis, as this is likely the most efficient route, however this data reveals that there may be more flexibility in execution of the apoptotic pathway.

While no interaction between BAX and MCL-1 or BCL-X_L could be detected using immunoprecipitation, it is known that BAX is an extremely dynamic protein and therefore, could be interacting with an array of proteins, including the pro-survival proteins, in a transient manner. BAX is typically considered to be a cytosolic protein under non-apoptotic conditions, however recent work has suggested that it actually cycles between different compartments of cells, in particular the cytosol and the mitochondria[318]. An aggregation of mitochondrial BAX is clearly not an ideal situation for a cell that is not fated for apoptosis however, therefore it has been proposed that mechanisms exist to retro-translocate BAX to the cytosol in order to prevent mitochondrial BAX accumulation, auto-activation and subsequent apoptosis[319]. Multiple reports have indeed implicated the pro-survival BCL-2 family proteins in this retrotranslocation process, with suppression of MCL-1 and BCL-X_L resulting in impaired shuttling of BAX back to the cytosol[318–322]. In this case, in the absence of the BH3-only proteins, BAX retro-translocation could be reduced following inhibition of MCL-1 and BCL-X_L activity, leading to its accumulation at the mitochondria, perhaps

initiating spontaneous activation of BAX by the outer mitochondrial membrane[303], leading to the apoptosis seen in the HCT116 8KO cells. Of note, it has been established that some detergents can induce conformational changes within BAX (and indeed other BCL-2 family proteins) that alter interactions with other BCL-2 family members and can even aberrantly activate BAX[300]. Such detergents have been used extensively to study effector protein behaviour *in vitro*. As an organelle which is densely populated by lipids which form the typical bilayer of biological membranes, the mitochondrial membrane shares a degree of properties with detergents, therefore this could support the notion of the mitochondrial membrane being able to activate BAX/BAK under certain conditions. Other studies have suggested that detergent-activated BAX does not assume the typical form of BAX capable of instigating pore-formation in the mitochondrial membrane however, and even suggest that BAX remains a monomer following activation by a detergent[323]. Thus, the capacity of the mitochondrial membrane to activate BAX in the absence of BH3-only proteins following neutralisation of pro-survival proteins therefore remains to be proven and will require extensive exploration and an improved understanding of the mitochondrial membrane during apoptosis. Furthermore, assessing the dynamic behaviour of BAX in both its translocation and potential interactions with MCL-1 and/or BCL-X_L is important, particularly since it was only assessed at single time-points here. Live cell imaging of a fluorescently tagged BAX in conjunction with a mitochondrial tracker and tagged MCL-1/BCL-X_L could provide further information regarding how BAX truly behaves in the absence of the BH3-only proteins.

While the mitochondrial membrane represents a compelling potential activator of effector proteins in the absence of BH3-only proteins, it is certainly not the only explanation for a BH3-only protein-independent death. It could also be suggested that

an unknown BH3-only-like protein is activating BAX to induce apoptosis in the HCT116 8KO cells. In the case of such a 'Protein X' scenario, exposure of the HCT116 8KO cells to BH3 mimetics might cause the dissociation of BCL-X_L and/or MCL-1 from the so-called 'Protein X' which might then activate BAX, thus initiating apoptosis. Indeed, as described in Chapter 1, the BH3 motif of BCL-2 family proteins is not a set sequence, rather a series of particular types of amino acids giving rise to a certain protein structure. Thus, identifying proteins with a BH3 motif is not straight-forward, as the BH3 motif sequence can take many forms. This could suggest that many proteins exist with potential BH3-like motifs which could mediate interactions with members of the BCL-2 family and induce apoptosis following pro-survival protein neutralisation in HCT116 8KO cells.

Further adding to the complexity of BH3-only protein-independent apoptosis and the possibility of a 'Protein X', it was remarked that induction of apoptosis following MCL-1 and BCL-X_L suppression varied dramatically in dependence on the BH3-only proteins. Inhibition of BCL-X_L but not MCL-1 by both pharmacological and genetic approaches resulted in increased apoptosis in the HCT116 WT but not 8KO cells, suggesting that apoptosis regulated by BCL-X_L depends on the availability of BH3-only proteins. However, this dependence on BH3-only proteins was overcome when both MCL-1 and BCL-X_L were inhibited together. This could further support the notion of a 'Protein X' scenario, in which such a protein perhaps interacts specifically with MCL-1. Thus, there appears to be something inherently different about the interactions between BCL-X_L and its partner proteins and MCL-1 and its partner proteins during regulation of apoptosis in the absence of the BH3-only proteins (and perhaps in normal apoptosis too). This could potentially be the existence of a 'Protein

X’ which specifically binds MCL-1 and, during apoptosis, is released to activate BAX – a concept which is explored in the next chapter.

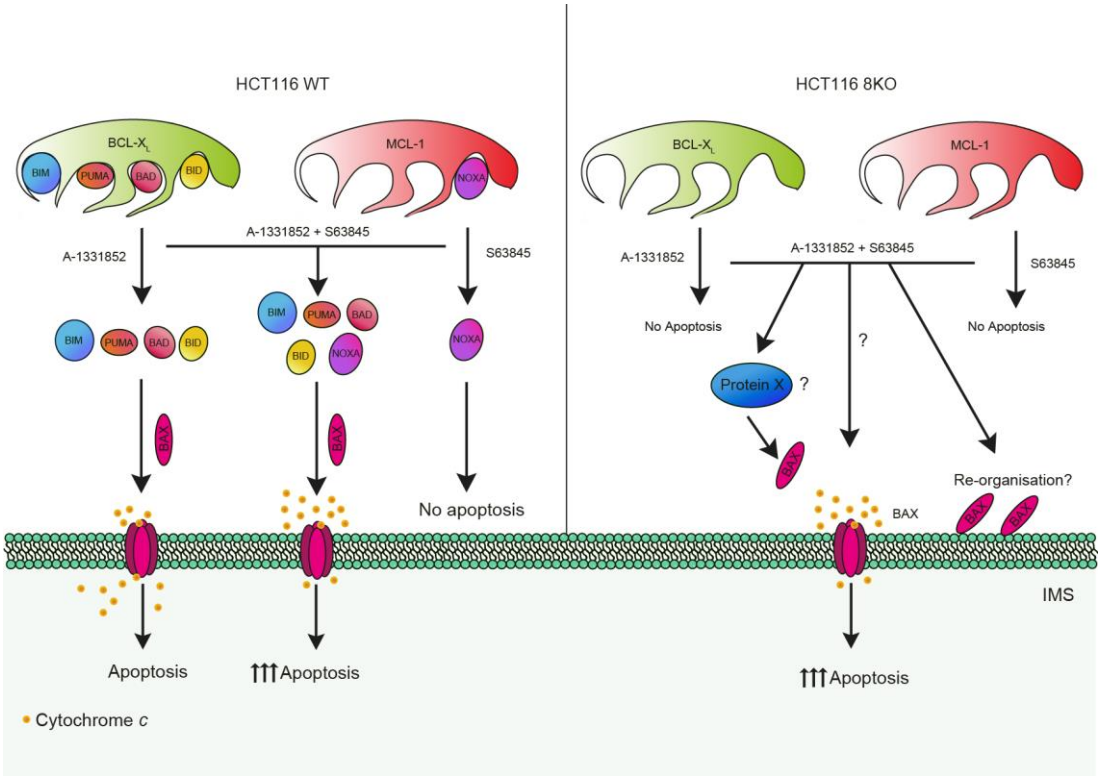


Figure 4.13 – Apoptosis in HCT116 8KO cells may occur through non-conventional routes, perhaps involving an unknown BH3-only protein that activates BAX. BCL-2 family members differ in their dependence on pro-apoptotic BH3-only members to exert their anti-apoptotic functions. In HCT116 cells, multiple BH3-only proteins, such as BIM, BID, BAD and PUMA are sequestered by BCL-X_L, whereas NOXA is the only BH3-only protein bound to MCL-1. While the displacement of BH3-only proteins from BCL-X_L following A-1331852 is sufficient to induce apoptosis in these cells, displacement of NOXA from MCL-1 following S63845 failed to induce apoptosis. However, the combination of A-1331852 and S63845 released all the BH3-only proteins from the anti-apoptotic counterparts and resulted in pronounced apoptosis. In the absence of all eight BH3-only proteins, neither A-1331852 nor S63845 alone resulted in apoptosis. However, a combination of A-1331852 and S63845 still resulted in extensive apoptosis, even in the absence of all BH3-only proteins. This could be due to the release of another pro-apoptotic BH3 motif-containing 'Protein X' from MCL-1 and/or BCL-X_L, which in turn could activate BAX in the 8KO cells. Alternatively, BH3 mimetics could indirectly result in the accumulation of BAX (either by inhibition of retrotranslocation or by passive diffusion, following the neutralization of the anti-apoptotic members) in the outer mitochondrial membrane, which in turn could result in BAX activation by the OMM.

Chapter 5

Identifying novel MCL-1 interacting proteins.

Contents

5.1 Background

In the previous chapter, it was demonstrated that cells lacking the eight key BH3-only proteins can still undergo apoptosis following suppression of MCL-1 and BCL-X_L by either RNAi or BH3 mimetics, suggesting that the known BH3-only proteins are dispensable for activation of BAX and subsequent apoptosis. Furthermore, neutralisation of MCL-1 or BCL-X_L individually induced strikingly different levels of apoptosis. Inhibition of BCL-X_L, but not MCL-1, induced ~50% apoptosis in HCT116 cells in a BH3-only protein-dependent manner. Unlike BCL-X_L, MCL-1 appeared to antagonise apoptosis in a manner independent of the known BH3-only proteins, as dual inhibition of MCL-1 and BCL-X_L together could induce apoptosis in the absence of all known BH3-only proteins. Various hypotheses were proposed to explain this BH3-only protein-independent apoptosis, including the possibility of the existence an unknown, BH3-only-like protein, or even multiple proteins, which may function as activators of BAX in the HCT116 8KO. This protein (or collection of proteins), herein referred to as 'Protein X', might also have a binding preference for MCL-1 over BCL-X_L, since it appeared that the BH3-only proteins were required for apoptosis when only BCL-X_L was inhibited.

As discussed in Chapter 1, MCL-1 is quite a promiscuous member of the anti-apoptotic BCL-2 proteins. It has several known binding partners within the BCL-2 family including BIM and NOXA (shown both in the literature and in Chapter 3), in addition to various other non-BCL-2 family member protein binding partners including SUFU[324], CDK1[177] and many more. MCL-1 also possesses a unique, elongated structure with more domains (namely PEST regions) than other BCL-2 family members[76], as described in Chapter 1. Thus, it has been proposed that MCL-1

may have a more complex biology than other anti-apoptotic proteins, regulating much more than just survival. It is not unrealistic therefore, to imagine that a number of MCL-1 interacting partners remain undiscovered and perhaps one of these could be a 'Protein X' which can promote apoptosis in the absence of the 8 key BH3-only proteins. The BH3 motif, the key and universal unit of BCL-2 family proteins, is proposed to be extremely variable in nature and therefore may exist in various distinct sequences which permit interactions between the pro- and anti-apoptotic BCL-2 family members[65], thus it is not unlikely that proteins with undiscovered potential BH3-like motifs exist which could bind to a number of the BCL-2 family proteins. Indeed, some studies have highlighted the existence of BH3-like motifs in non-BCL-2 family member proteins (for example SUFU[324]), suggesting that novel BH3-only-like proteins could potentially be displaced from MCL-1 by BH3 mimetics to induce the canonical model of apoptosis.

To explore this hypothesis, novel MCL-1 interacting proteins were explored here using subcellular fractionation, immunoprecipitation and mass spectrometry to identify novel interactions which could potentially be mediating the BH3 mimetic-induced apoptosis seen in HCT116 8KO cells.

5.2 MCL-1 is present as three major complexes of different molecular weights in multiple cell lines.

To first explore whether MCL-1 exists as distinct complexes interacting with different protein subsets within the cell, gel filtration chromatography was performed to separate protein lysates according to molecular weight. H23 and H929 cells were lysed and fractionated on Superose 6 and Superdex 200 gel filtration columns, allowing for broad separation of protein pools from 2 megadaltons (MDa) to < 30 kilodaltons (kDa)

with extremely good resolution of distinct protein pools. The eluted fractions were mixed with loading dye and subjected to immunoblotting using antibodies raised against distinct epitopes of MCL-1.

In H23 cells, MCL-1 appeared to exist in what seemed to be three major complexes of vastly different molecular weights (Figure 5.1A). The largest detected complex (termed the high molecular weight (HMW) complex) was approximately 1700-2000 kDa in size, as marked by the red arrow beginning at the 2000 kDa molecular weight marker. A second complex (termed the medium molecular weight (MMW) complex) was detected at a lower molecular weight of around 500-700 kDa, however MCL-1 was in fact detectable in all fractions between the 2000 kDa and 600 kDa fractions, albeit at varying levels. The third complex of MCL-1 was detectable at a much lower molecular weight (and thus termed the low molecular weight (LMW) complex) of around 60-150 kDa and small amounts of MCL-1 were also present, most likely as monomeric MCL-1, at around 30 kDa. The HMW MCL-1-containing fractions indicate that MCL-1 is perhaps in a large complex with many other proteins, whereas the LMW 150 kDa fraction is perhaps indicative of some of the known interactions of MCL-1, such as complexes with proteins like BIM and NOXA. The different isoforms of MCL-1, proposed to be linked to distinct functions of MCL-1 and observed typically as three bands of distinct molecular weights, varied in expression levels across the different fractions, with at least two of the isoforms being detectable in most fractions.

Similar to the complexes observed in H23 cells, MCL-1 also appeared to exist in a large 2000 kDa HMW, a MMW and a LMW complex in the H929 cells (Fig 5.1B). The relative distribution of MCL-1 in these complexes was slightly different between the H23 and H929 cells however, with more MCL-1 present in the HMW complex in

H929 cells. Additionally, little monomeric MCL-1 was detectable in H929 cells. These complexes were also detectable in a panel of other cancer cell lines from different kinds of tumours, suggesting that the existence of MCL-1 in these complexes may be universal (data not shown).

To further examine the MCL-1 complexes and determine if distinct complexes might be linked to distinct roles of MCL-1, pools of pro-apoptotic BCL-2 family members were also identified. This revealed that the two effector proteins, BAX and BAK, co-eluted in the HMW and MMW MCL-1 complexes. BAK is a well-known interacting partner of MCL-1 so it is not surprising to see co-elution of BAK with the two large MCL-1 complexes. These interactions are likely to occur on the outer mitochondrial membrane since this is where the majority of BAK localises[59]. Meanwhile, BAX could be detected in multiple fractions of different molecular weights – including at the lowest molecular weights, indicative of monomeric BAX as previously described. Surprisingly, BAX could also be seen at similar levels in the MMW fractions and at low levels in the HMW complex, co-eluting with MCL-1. This could perhaps be indicative of the retrotranslocation of BAX by the BCL-2 family members – it is proposed that BAX cycles constantly between the mitochondria and cytosol, with shuttling back to the cytosol mediated, at least in part, by multiple members of the anti-apoptotic BCL-2 family[319,321,322].

In addition to the effector proteins, several known interacting BH3-only protein partners of MCL-1 also co-eluted with multiple MCL-1 complexes. BID, known to interact with MCL-1 in its truncated form[325], was not detected co-eluting with any of the major MCL-1 complexes and was exclusively found at very low molecular weight fractions, suggesting it was predominantly monomeric. This is not surprising since little tBID should be present in basal conditions. Meanwhile, BIM, could be seen co-

eluting with MCL-1 in both the HMW and MMW complexes. PUMA was only detectable at low levels but could also be observed co-eluting with MCL-1 in all three of the major MCL-1 complexes. Furthermore, NOXA, a key MCL-1 interacting protein and regulator, could be seen co-eluting with all three of the major MCL-1 complexes, suggesting NOXA might be bound to most pools of MCL-1. This is not surprising, since NOXA is believed to play an essential role in the stability and turnover of MCL-1[123,285].

Overall, this suggested that all the major complexes of MCL-1 may in fact be linked to its anti-apoptotic activity through interactions with various BH3-only proteins. The interactions with BIM or BAK in the high molecular weight complexes do not account for the total molecular weight (for example 2 MDa) of the larger complexes however, suggesting that MCL-1 may be in complex with various other proteins alongside the BH3-only proteins, perhaps in a scaffold structure that facilitates the diverse functions of MCL-1. As previously mentioned, MCL-1 is believed to be linked to multiple functions within the cell from its obvious roles in promoting cell survival, to regulation of lipid metabolism and DNA repair[184,326]. It could therefore be envisioned that each major complex, while able to regulate apoptosis, might be linked to different subcellular localisations and therefore functions of MCL-1. Protein X, which could be mediating the BH3-only protein independent apoptosis in HCT116 cells, could be present in any of these three major complexes given their molecular weights, therefore exploring the complexes and their subcellular localisation further could reveal the pool(s) where Protein X exists.

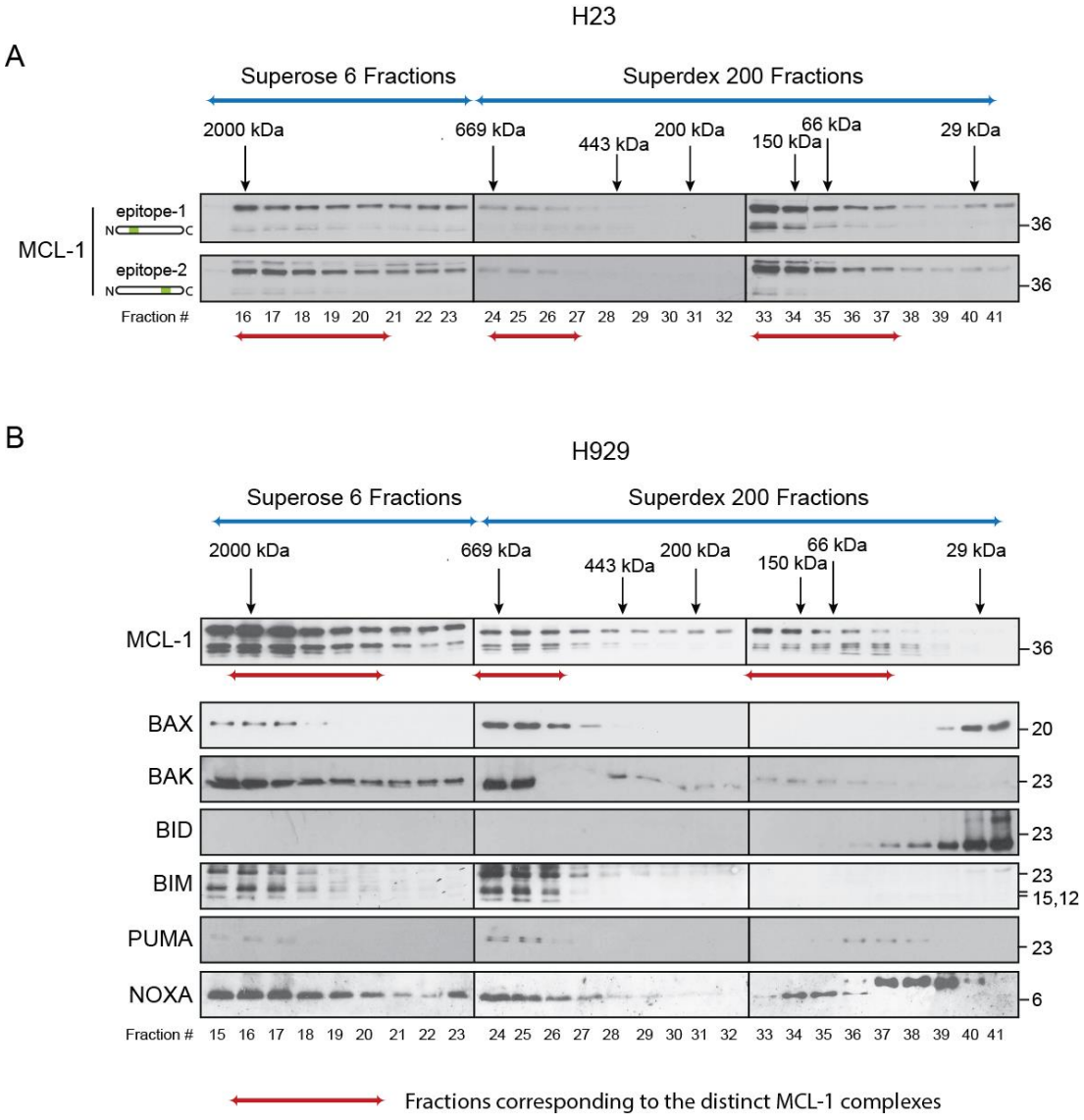


Figure 5.1. MCL-1 exists in three major complexes and a monomeric form in MCL-1-addicted cell lines. Lysates from the MCL-1-addicted cell lines H23 (A) and H929 (B) were separated using gel filtration with the indicated columns and the fractions containing protein complexes of different molecular weights were subjected to immunoblotting to detect where MCL-1 and associated BCL-2 family proteins were present. Approximate molecular weights of fractions are indicated above the blots and molecular weight markers of the immunoblots to the right. Red arrows indicate the three major MCL-1 complexes identified. Vertical lines between immunoblots indicate separate gels, with Superose 6 and Superdex 200 fractions loaded over 3 different gels.

5.3 MCL-1 is present in all the major subcellular fractions of HELA cells.

MCL-1 is an important regulator of the cell, not just in anti-apoptotic roles but also in multiple other roles as previously described. Moreover, MCL-1 has been proposed to have broad subcellular localisation supporting the multi-faceted nature of its roles within the cell. To explore the notion of different MCL-1 pools being linked to different subcellular localisations, four types of HELA cell fractions, generated by subcellular fractionation, were probed to explore the distribution of MCL-1 complexes. The fractions used were an 'extranuclear' fraction (including everything within the cell apart from the nucleus), a nucleus-enriched fraction ('nuclear'), a cytosolic ('S100') fraction (named after the 100,000 x *g* spin used to generate it[327]) and a mitochondria-enriched fraction ('mitochondria'). These fractions were purchased pre-prepared from Ipracell (Ipratech, Belgium) as generating large amounts of these different fractions, particularly the mitochondrial fraction, in the laboratory would require extremely large numbers of cells and hence, would not be cost-effective. To first ensure that the fractions were indeed enriched for the components described, each fraction was lysed in 1% CHAPS buffer and protein markers specific to each compartment detected to demonstrate purity. MCL-1 was also detected to identify where it localises. BAP31 and GM130 were used to detect the endoplasmic reticulum[328] and Golgi apparatus[329] respectively, while cytoskeletal components tubulin[330] and vimentin[331] were used as markers for the cytosol. NDUFA9 and ATPB, two components of the mitochondria-localised electron transport chain[332,333], were used to detect mitochondria and poly-ADP ribose polymerase (PARP)[334] was used to validate the nuclear fraction (Fig 5.2). Both BAP31 and GM130 were detectable in the extranuclear and mitochondrial fractions, suggesting that the mitochondrial fractions are not exclusively mitochondria and may also contain some heavy

membranes from the ER and Golgi apparatus. Meanwhile, tubulin and vimentin were enriched in the extranuclear and S100 fractions, with very small amounts detectable in the nucleus and mitochondria, indicating that the S100 fraction is indeed cytosolic and that the nuclear and mitochondrial fractions are not contaminated with cytosolic proteins. Similarly, NDUFA9 and ATPB were present in large amounts in both the extranuclear and mitochondrial fractions, suggesting that the mitochondrial fraction is indeed mitochondrially-enriched, and the nuclear and cytosolic fraction contain little contaminating mitochondria. Finally, PARP was detectable in the nuclear fraction and a small amount in the extranuclear fraction, suggesting it is a pure nuclear fraction and indicating that the other fractions do not contain nuclear proteins.

MCL-1 was detectable in all of the subcellular fractions at relatively similar levels, although with some stark differences in the presence of the three major isoforms. In the extranuclear fraction, the three typical isoforms relating to the different functions of MCL-1 (as described previously) were identified as expected and the same was seen for the mitochondrial fraction. Moreover, in the extranuclear fraction the two upper isoforms were more dominant, whereas in the mitochondrial fraction, there was a more even distribution of the three isoforms, with the middle isoform being the most dominant. In the nuclear fraction, all three isoforms were also detectable and, while the two lower bands appeared at a similar molecular weight as seen for the extranuclear fraction, the upper band seemed to run slightly higher than in any of the other fractions, possibly indicative of a post-translational modification of the upper band required for MCL-1 to be in the nucleus. While MCL-1 has previously been detected in the nucleus and is proposed to play roles in regulation of DNA repair[177,184,326], the nuclear-localised isoform(s) of MCL-1 are not well described, however this may shed some light on the characteristics of how MCL-1 behaves in the

nucleus. Further differences were detectable in the cytosolic S100 fraction, where the two larger bands of MCL-1 were detectable, but the lower band was not present at all, suggesting this isoform of MCL-1 does not function in the cytosol. Indeed, this lower isoform has been proposed to be localised to the mitochondria[175,183], thus it is not surprising that it is not identifiable in the cytosol, however its presence in the nucleus remains a mystery. With MCL-1 present in all of the major subcellular compartments of the cell and in at least 3 distinct molecular weight complexes, it is not unreasonable to imagine that it has a vast array of binding partners, with a potential Protein X candidate existing in one or multiple fractions in partnership with MCL-1.

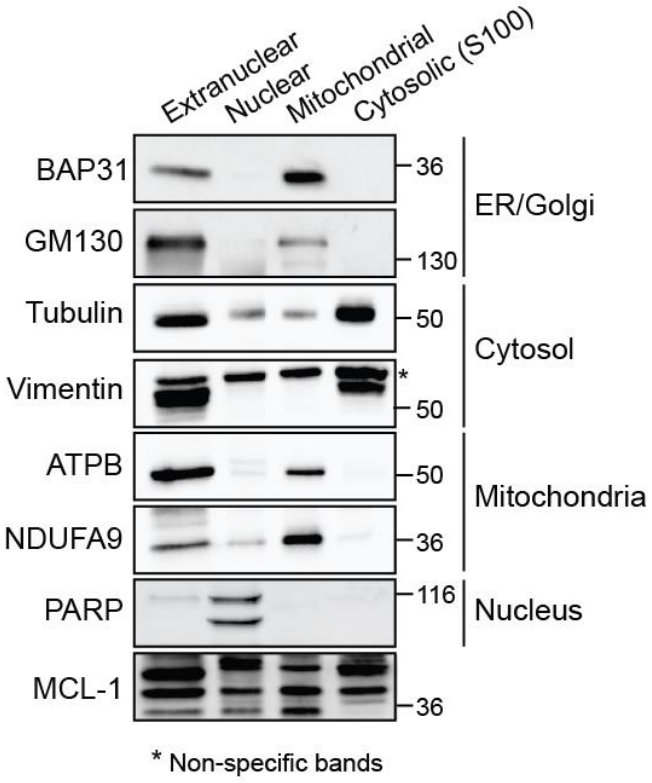


Figure 5.2. MCL-1 isoforms are detectable in all of the major subcellular compartments of HELA cells. The indicated subcellular fractions were lysed and immunoblotting performed to detect markers of the major subcellular compartments alongside MCL-1. Approximate molecular weights of bands are indicated to the right of the immunoblots. The vimentin band identified is the lower band indicated by the asterisk, while the double band of PARP is likely to represent whole and cleaved PARP.

5.4 MCL-1 complexes vary in different subcellular compartments.

Once the subcellular fractions had been validated, each one was loaded on to Superose 6 and Superdex 200 columns as performed previously to identify MCL-1 complexes. Gel filtration requires a large volume of starting material in order to detect protein in immunoblotting and, since the mitochondrial fraction was limited in quantity and large volumes also required for subsequent experiments, gel filtration was not performed on the mitochondrial fraction.

In the tested fractions, MCL-1 was detectable in various complexes and to different extents in each of the subcellular compartments (Fig 5.3). In the extranuclear lysate, the HMW and MMW complexes of MCL-1 (2000 kDa and 500-700 kDa) were readily detectable and the complex at the 150 kDa range was detectable although at lower levels. Little monomeric MCL-1 was detectable in the extranuclear fraction. This reflects the data seen previously when identifying the MCL-1 complexes and further confirms the existence of distinct pools of MCL-1 with dramatically different molecular weights. Meanwhile in the nuclear fraction, no HMW MCL-1 complex was detectable, while moderate levels of the MMW and LMW complexes were observed. No monomeric MCL-1 was detected. This suggested that the HMW complex might be present outside of the nucleus, confirmed by the fact that this complex was detectable in the extranuclear fraction. It also suggested that monomeric MCL-1 might be present at low levels in HELA cells as it was not detectable here or in the extranuclear fraction.

In the cytosolic fraction however, MCL-1 (especially the uppermost band) was detectable in almost all fractions from very high to very low molecular weight equally, but unlike the other subcellular compartments, only the largest isoform of MCL-1 was

detectable in most fractions (as suggested by the presence of the higher bands), with the lower band being detectable at very low levels in some cases. These results indicate various possibilities about the localisations of the MCL-1 complexes in each subcellular compartment. Firstly, the 'missing' HMW MCL-1 complex is likely present in bulk on the mitochondria and other membranous organelles, as it is detectable at high levels in the extranuclear fraction, moderate levels in the cytosolic fraction and not at all in the nuclear fraction. The second band is especially lacking in the nuclear and cytosolic fractions in the HMW complex, perhaps suggesting that it is mostly present on membranes, likely the mitochondria, where it can efficiently antagonise death[175]. Secondly, it would seem that nuclear MCL-1 is present in lower molecular weight complexes compared to other organelles, although it may still be associated with multiple proteins in the MMW complex which could be linked to the nuclear functions of MCL-1 and its associations with various DNA repair proteins[177,184,335]. Finally, the presence of the upper band of MCL-1 across all fractions in the cytosolic compartment could suggest that MCL-1 is a very versatile protein with many possible interacting partners in the cytosol. Monomeric MCL-1 is also readily detectable only in the cytosolic fraction, suggesting that perhaps some MCL-1 remains free in the cytosol in these cells to sequester pro-apoptotic proteins and prevent unwanted cell death.

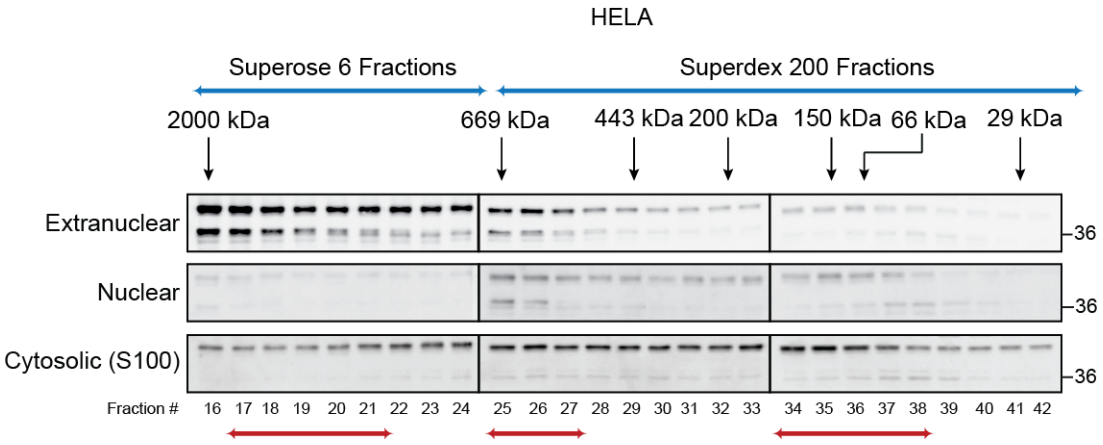


Figure 5.3. The presence of MCL-1 complexes varies in different subcellular compartments of HELA cells. Subcellular fractions of HELA cells were lysed and separated on gel filtration columns then subjected to immunoblotting to detect MCL-1 complexes, indicated by red arrows. Approximate molecular weights of fractions are indicated above the blots and molecular weight markers to the right. Red arrows indicate the major complexes of MCL-1 previously identified.

5.5 Mass spectrometry strategies for identifying novel MCL-1 interacting partners.

From the data above, it is clear that MCL-1 has a broad subcellular distribution. This, in combination with the multi-faceted roles of MCL-1, supports the idea of a potentially vast network of MCL-1-interacting proteins. Thus, it is highly likely that novel MCL-1 interacting partners exist which have not yet been detected and these interacting partners could potentially be the Protein X that was previously alluded to in Chapter 4. Given the data in Chapter 4, this Protein X could be bound to MCL-1 and, in the absence of the 8 key BH3-only proteins in the HCT116 8KO cells, be released following exposure of the cells to BH3 mimetics (or genetic suppression of the pro-survival proteins), allowing BAX activation and thus inducing apoptosis.

To explore the MCL-1 interaction network and potentially identify a Protein X candidate, mass spectrometry was utilised to analyse MCL-1-interacting proteins in the different MCL-1 complexes and subcellular compartments. Two strategies were developed to isolate and explore MCL-1-interacting proteins. The first involved immunoprecipitation of endogenous MCL-1 from each of the major subcellular compartments (extranuclear, nuclear, mitochondria, cytosolic) and analysis of the proteins which interact with MCL-1 using mass spectrometry (Figure 5.4A). The second strategy was designed to isolate the three major MCL-1 complexes (as identified in Figure 5.1) by pooling the lysates of the different MCL-1 complex fractions. For example, the first major MCL-1 complex appeared in fractions 16-20 of the Superose 6 column. These fractions would be pooled, the endogenous MCL-1 immunoprecipitated and the eluate studied by mass spectrometry, thus identifying the different interacting partners in each complex (Figure 5.4B).

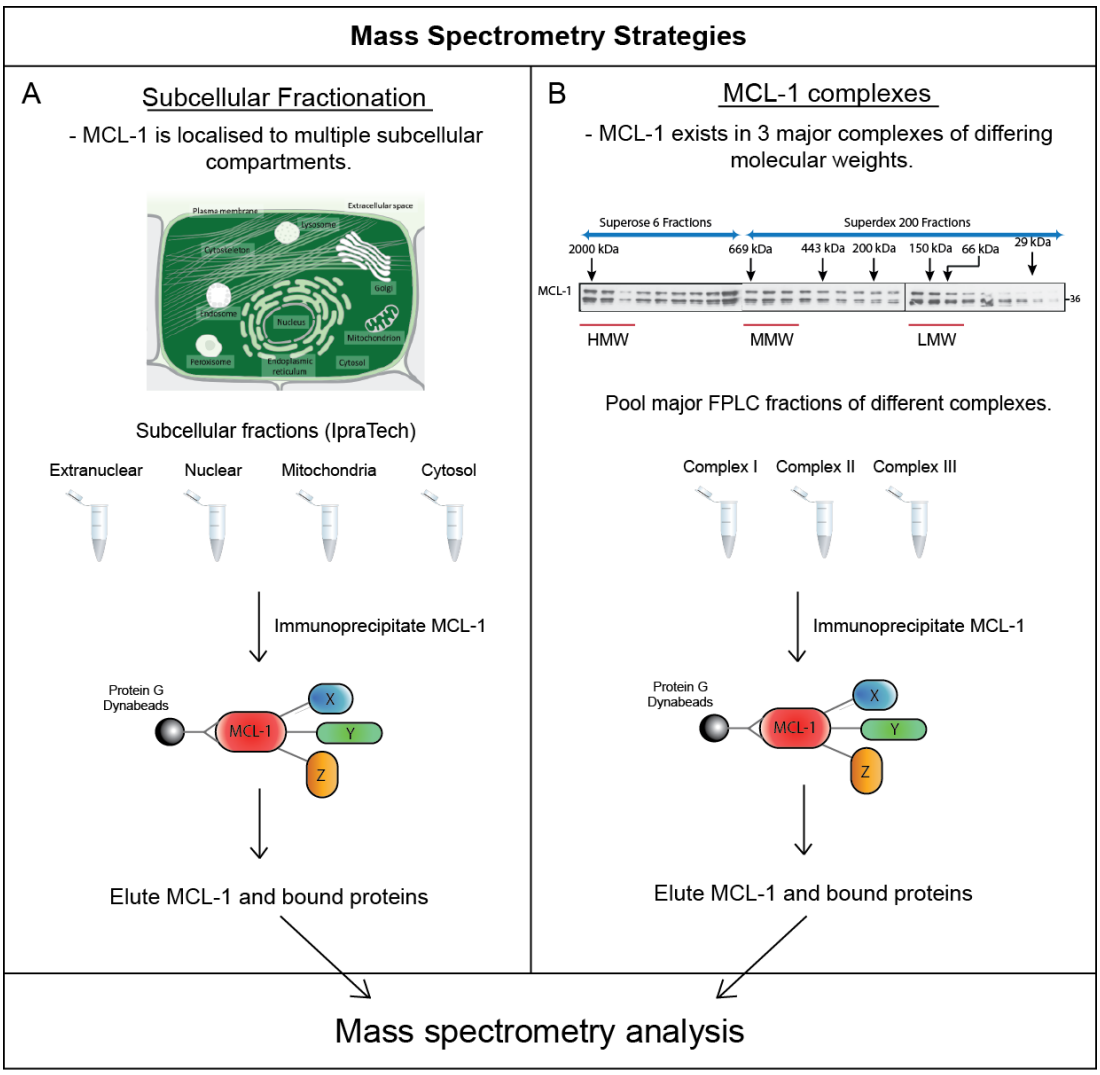


Figure 5.4. Two approaches to isolating distinct pools of MCL-1 and its interacting partners for mass spectrometry analysis. (A) Endogenous MCL-1 was isolated from four subcellular fractions by immunoprecipitation and eluted to detect interacting proteins. (B) Fractions of lysates ran on gel filtration columns were pooled according to the major MCL-1 complexes and endogenous MCL-1 immunoprecipitated to detect interacting proteins in immunoblotting and mass spectrometry. MCL-1 subcellular localisation image modified from https://www.genecards.org/cgi-bin/carddisp.pl?gene=MCL1

5.6 MCL-1 can be immunoprecipitated from different subcellular pools and its interacting proteins detected by Coomassie labelling.

Prior to mass spectrometry, optimisation experiments were carried out for both the immunoprecipitation of MCL-1 from the different subcellular pools of MCL-1. Since MCL-1 had previously successfully been immunoprecipitated in Chapters 3 and 4, the same antibody (Abcam Y-37) was used, while minor changes were made to the normal immunoprecipitation protocol for mass spectrometry, as outlined in Chapter 2. To optimise pulldown of MCL-1 in the pre-prepared subcellular fractions, the extranuclear fraction was subjected to MCL-1 immunoprecipitation and the amount of MCL-1 immunoprecipitated detected by both immunoblotting and EZBlue (Coomassie) staining (Figure 5.5A). This experiment confirmed that (1) immunoprecipitation in the Ipratech fractions could successfully pull out MCL-1 and (2) MCL-1-interacting proteins could also be pulled down and detected in Coomassie gels, suggesting that ample peptides would be present for identification of new MCL-1-interacting proteins in mass spectrometry.

For the MCL-1 complex immunoprecipitation approach, a large-scale gel filtration of HELA cells using Superose 6 and Superdex 200 columns was performed and lysates pooled into the appropriate complexes for subsequent immunoprecipitation. A small volume of lysate was also subjected to immunoblotting to ensure that the expected MCL-1 complexes were present (data not shown). While multiple steps had been undertaken to maintain protein-protein interactions in the gel filtration, it was important to identify whether MCL-1 could still be immunoprecipitated from pooled fractions of the MCL-1 complex experiments and furthermore, whether other proteins were pulled with it, as seen for the subcellular immunoprecipitation optimisation. MCL-1 could be readily immunoprecipitated from the pooled fractions of the HMW

complex of MCL-1 (~2 MDa) and furthermore, a higher level of global proteins is detectable in the immunoprecipitation compared to the bead control of the Coomassie gel (Figure 5.5B), suggesting that MCL-1 interacting proteins are indeed co-immunoprecipitating and that, therefore, this approach should yield many MCL-1 interacting proteins in mass spectrometry.

In addition to optimisation of the immunoprecipitation protocol, optimisation was carried forward to mass spectrometry. One sample from each immunoprecipitation strategy was subjected to mass spectrometry in order to confirm detection of peptides and therefore validate the approach for future studies. MCL-1 (and many other proteins) could be readily detected in mass spectrometry from the immunoprecipitated eluate of the extranuclear fraction of the subcellular approach. This was encouraging enough to pursue similar analysis for other subcellular fractions. However, preliminary optimisation studies using the HMW fractions of HELA cell lysates did not detect MCL-1 by mass spectrometry, therefore subsequent experiments focused on the subcellular compartment localisations of MCL-1 (data not shown). All mass spectrometry analysis was carried out by Deborah Simpson of the mass spectrometry facility at The Centre for Proteome Research at The University of Liverpool.

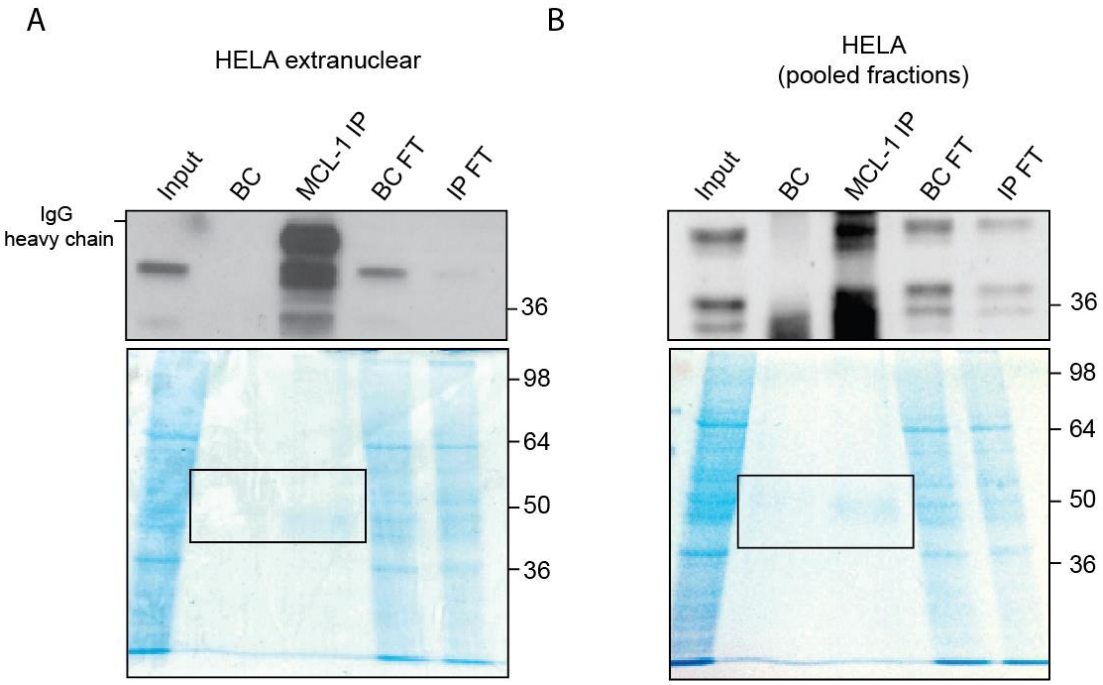


Figure 5.5. Immunoprecipitation of MCL-1 is effective at isolating MCL-1 and co-immunoprecipitated protein is detectable on Coomassie gels. MCL-1 was immunoprecipitated from either (A) the extranuclear subcellular fraction of HELA cells or (B) pooled FPLC fractions of the HMW complex of HELA cells and two gels ran – one to detect MCL-1 by immunoblotting and one to detect global protein pulldown using a Coomassie dye. Immunoprecipitation (IP) samples were compared to a bead control (BC) to ensure no non-specific pulldown was occurring and flow-through (FT) ran on gels to show immunoprecipitation efficiency. Approximate molecular weights are indicated to the right of the immunoblots. The box on the whole protein gels highlights where the most protein was detectable.

5.7 Many proteins co-immunoprecipitate with MCL-1 in the extranuclear fraction of HELA cells.

To identify the proteins which interact with MCL-1 and potentially identify candidates for Protein X, eluates from MCL-1 immunoprecipitations in the different subcellular fractions were submitted, alongside bead controls, to the mass spectrometry facility at The Centre for Proteome Research at The University of Liverpool. Three replicates were submitted for each individual sample. The resulting data was returned as a list of identified peptides, along with other information including the peptide counts (the number of times a particular peptide was detected, along with unique peptides for a particular protein), confidence scores of each individual protein, *p* values for significance over three replicates and *q* values – an adjusted *p* value to incorporate a false detection rate due to the high peptide numbers found. Maximum fold changes that highlight the difference in the levels of immunoprecipitated peptides compared to the bead control were also included.

In the case of the extranuclear lysate, a staggering number (>800) of peptides were identified (Table 5.1). Encouragingly, MCL-1 was identified in the peptide list as a reliable hit – found to be 33-fold higher in the immunoprecipitation than the bead control with a peptide count of 12 and a unique peptide count of 10. This suggested that the pulldown was effective and reliable. Moreover, to further support the efficacy of the immunoprecipitation, several known MCL-1-interacting partners were also identified with high confidence, including BIM (205-fold higher in the immunoprecipitation versus bead control) and NOXA (181-fold higher in immunoprecipitation versus bead control). A full list of proteins identified in the extranuclear fraction is available in Supplementary Table 5.1.

Due to the nature of immunoprecipitation and mass spectrometry analysis, it was proposed that many of the peptides identified may have appeared as false-positives, partly owing to the 'sticky' nature of many proteins. Thus, in order to isolate significant hits and better identify true potential MCL-1 interacting partners, multiple stringent triages were performed. The first filter applied to the peptide list excluded any peptides which appeared to be higher in the bead control samples compared to the immunoprecipitation samples which would indicate non-specific binding to the beads. Subsequently, only proteins with two or more spectral counts (meaning they were detected at least twice) were considered. Next, the peptide list was compared to the CRAPome (http://crapome.org/) - an online protein database which has data from over 400 mass spectrometry experiments[336]. Comparing the peptide lists generated from immunoprecipitation of MCL-1 to a repository of mass spectrometry peptide lists allowed identification of proteins which are repeatedly found in mass spectrometry analyses and are, therefore, likely to be contaminating proteins which are not actually proteins of interest. Using a cut-off of 10%, meaning that only peptides which occurred in less than 10% of mass spectrometry experiments in the CRAPome database were accepted, the peptide list was reduced from 598 to 203. The cut-off point of 10% CRAPome frequency was utilised following successful identification of a novel MCL-1 interacting partner in similar experiments performed previously[183] and further stringent filters applied in this article were also applied to the data here. Of the proteins remaining following CRAPome analysis, only those with a 5-fold change in the immunoprecipitation versus bead control were accepted, further reducing the list to 198. Finally, only proteins with a *q* value, which incorporates a false discovery rate in experiments with large volumes of data, were included, leaving 196 proteins identified in the extranuclear fraction in total.

Table 5.1. Many MCL-1 interacting proteins are detectable in the extranuclear fraction of HELA cells. Analysis of the eluate of an MCL-1 immunoprecipitation in the extranuclear fraction of HELA cells yielded 813 protein hits in total. The protein list was reduced to 196 by a series of triages as indicated in the table.

Fraction: Extranuclear	**Peptides**
Total proteins identified	813
Maximum fold change > 2	812
> 2 spectral counts	598
< 10% CRAPome frequency	203
> 5-fold change IP vs BC	198
< 0.02 FDR/q value	196

Since the list of candidate proteins was still extensive even after removing many non-specific hits, the peptide list was subjected to a pathway analysis using the Reactome (https://reactome.org/) - an online tool which groups proteins based on major cellular functions[337]. This analysis revealed that MCL-1 may play a role in, or at least be linked to, many different aspects of cellular activity through its interacting partners, from protein and lipid metabolism to the cell cycle and membrane trafficking (Figure 5.6). Indeed, as described earlier, MCL-1 is known to function in more than just the apoptotic pathway. For example, MCL-1 was recently linked to lipid metabolism through an interaction with VLCAD[183]. Furthermore, MCL-1 is believed to have a

regulatory role in the cell cycle through its interaction with CDK1[177]. Many of the major pathways enriched in extranuclear mass spectrometry analysis are linked to the mitochondria or are mitochondrially-localised, such as the citric acid cycle, fatty acid metabolism and mitochondrial biogenesis. Since key roles for MCL-1 at the mitochondria in apoptosis have been demonstrated, this data could suggest that MCL-1 may in fact be responsible for regulation of much more mitochondrial activity than currently known. Indeed, some reports have linked MCL-1 and mitochondrial oxidative phosphorylation and dynamics, showing that MCL-1 is critical to the dynamics of the mitochondria and ATP production, amongst other functions[175,338]. Thus, MCL-1 may have a range of mitochondrial roles, some of which it may exert via the proteins enriched in the pathways identified here.

Since the focus of this study was on the role of MCL-1 in apoptosis, the proteins identified in cell death pathways by the Reactome analysis were of particular interest. These four proteins were BIM, NOXA, STAT3 and NMT1. As BH3-only proteins, it is already well known that BIM and NOXA interact with MCL-1[194,339] (also shown in Chapter 3). These two proteins were therefore not of interest as a Protein X, since they are (1) known BH3-only proteins and (2) already knocked out in the HCT116 8KO cells therefore could not mediate the apoptosis in these cells.

Meanwhile, the transcription factor, STAT3 has also previously been linked to MCL-1. It has been suggested that STAT3 can upregulate the transcription of MCL-1[168,340], thereby promoting cell survival. STAT3 has also been shown to directly interact with MCL-1 in MCF-7 cells and this interaction is proposed to alter the epithelial-mesenchymal transition by increasing apoptosis[341]. Since it is believed that the association of MCL-1 and STAT3 increases apoptosis, it is an unlikely candidate for a Protein X, which would in theory be displaced from MCL-1 following exposure of

a cell to BH3 mimetics to then activate BAX. Finally, N-myristoyltransferase-1 (NMT1) regulates the addition of fatty acid myristate groups to a protein as a post-translational modification. While no direct links between MCL-1 and NMT-1 exist, it has been predicted that MCL-1 is an extremely likely candidate for myristoylation due to the domains it contains and that this could promote the caspase cleavage of MCL-1, leading to apoptosis[342]. Other work has shown that NMT1 can also negatively regulate apoptosis however – downregulation of NMT1 can lead to increased apoptosis[343]. While many interesting MCL-1 interacting candidates were identified in this fraction, the four proteins identified that have been linked to apoptosis did not represent hits that we wanted to pursue in this study. Of course, this is not to say that many of the interactions could be validated and later linked to apoptosis or perhaps other functions of MCL-1. However, for an initial study, it was decided that only proteins with prior links to apoptosis would be focused on.

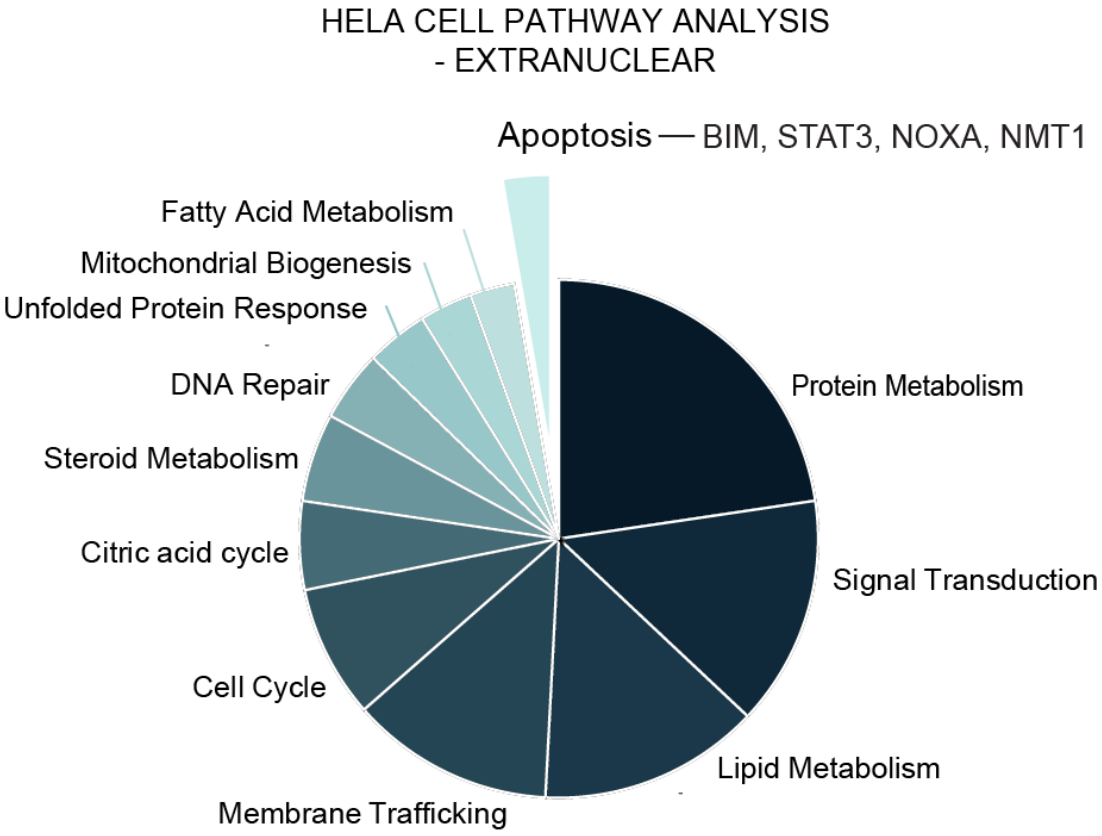


Figure 5.6. Candidates for MCL-1 interacting partners are wide-ranged in function in the extranuclear subcellular fraction of HELA cells. Filtered peptide lists from mass spectrometry analysis of an MCL-1 pulldown in the extranuclear lysate of HELA cells were grouped in a pathway analysis using the Reactome online database, revealing the major potential subcellular roles that MCL-1 may be linked to through its various interacting proteins.

5.8 Only MCL-1 can be identified as a significantly pulled-down protein in the nuclear fraction of HELA cells.

While many potential MCL-1-interacting proteins and Protein X candidates were revealed in the extranuclear lysate fraction, considerably fewer proteins were identified in the nuclear fraction. The exact same process was carried out for the immunoprecipitation and mass spectrometry experiments in the nuclear subcellular fractions as performed for the extranuclear fraction and the same parameters applied to the resulting peptides during triage and analysis (Table 5.2).

160 proteins were identified overall in the nuclear fraction as potential MCL-1-interacting partners, however this list was reduced to 31 following exclusion of proteins with a fold change of < 2 in the immunoprecipitation versus the bead control. Once processed by the CRAPome, only 3 proteins remained: MCL-1, Methyltransferase-Like 3 (METTL3) and SP100. Subsequent triage, however, meant that the only protein identified in the nuclear fraction which could be considered significant was MCL-1 itself. While this did confirm that the pulldown of MCL-1 was effective and that the approach was valid, the lack of significantly co-immunoprecipitated proteins suggested that MCL-1 does not have many, if any, nuclear-localised interaction partners in HELA cells under basal conditions.

Table 5.2. – No candidate MCL-1 interacting partners were identified in the analysis of the nuclear extract of HELA cells. Analysis of the eluate of an MCL-1 immunoprecipitation in the nuclear fraction of HELA cells yielded 160 proteins in total. The protein list was reduced to 1 by a series of triages as indicated in the table.

Fraction: Nuclear	**Peptides**
Total proteins identified	160
Maximum fold change > 2	31
> 2 spectral counts	19
< 10% CRAPome frequency	3
> 5-fold change IP vs BC	3
< 0.02 FDR/q value	1

5.9 The cytosol contains a small number of candidates for novel MCL-1-interacting proteins in HELA cells.

For the cytosolic S100 fraction, mass spectrometry and analysis of the resulting peptides were carried out as done for the previous fractions. Like the nucleus, the cytosolic fraction yielded considerably fewer potential MCL-1-interacting proteins compared to the extranuclear fraction (Table 5.3)

Initially, 156 proteins were revealed as potential interacting partners of MCL-1, which was reduced to 154 following exclusion of peptides with a fold change of < 2. Removing proteins which were only identified by a single peptide further reduced the number to 125, while analysis of the peptide list using the CRAPome database left just

23 proteins, including MCL-1. Further triage (as previously performed and indicated in Table 5.3) reduced the final list of cytosolic MCL-1-interacting candidates to just 5 proteins which are described in Supplementary Table 5.3. None of these proteins had been previously linked to MCL-1 although some may play a role in regulation of the cell cycle (AURKA[344]) and the autophagic response (TFEB[345]), both of which are closely associated with apoptosis. No known MCL-1 interacting proteins were identified in the cytosolic fraction, suggesting that, despite MCL-1 being present in the cytosol, the interactions of MCL-1 with known BCL-2 family members (such as BIM and NOXA) may occur on membranes, particularly the mitochondrial membrane.

Table 5.3 – Few proteins are detectable as candidate novel MCL-1 interacting partners in the cytosolic fraction of HELA cells. Analysis of the eluate of an MCL-1 immunoprecipitation in the cytosolic fraction of HELA cells yielded 156 proteins in total. The protein list was reduced to 5 by a series of triages as indicated in the table.

Fraction: Cytosol	**Peptides**
Total proteins identified	156
Maximum fold change > 2	154
> 2 spectral counts	125
< 10% CRAPome frequency	23
> 5-fold change IP vs BC	16
< 0.02 FDR/q value	5

5.10 Many potential MCL-1 interacting proteins can be identified from the mitochondrially-enriched fraction of HELA cells.

While few MCL-1-interacting proteins were reliably identified in the cytosolic and nuclear fraction mass spectrometry analysis, a strikingly vast number was found in the mitochondrially enriched fraction, which may come as no surprise, since large amounts of MCL-1 are found at the mitochondria, linked to both its apoptotic and non-apoptotic roles

Overall, exactly 1000 proteins were identified in the mass spectrometry, reduced to 998 following selection of peptides with at least a 2-fold change in the immunoprecipitation versus bead control and furthermore to 668 by removing proteins identified by a single peptide. Subsequent CRAPome analysis (again, with a cut-off of 10% frequency), left 315 proteins, while looking at only proteins with a minimum of a 5-fold change left 308. Finally, considering only the proteins with a *q* value of <0.02 left 296 potential MCL-1 interacting proteins, which was evidently still an enormous number of proteins to assess (Table 5.4). MCL-1 itself was readily detectable while several known MCL-1 interacting proteins, including BID[325] and DAD1[346] were also identified, giving confidence to the immunoprecipitation. A full list of the proteins identified is available in Supplementary Figure 5.4.

To functionally group proteins, pathway analysis was performed using the Reactome (Fig. 5.7). Proteins were grouped similarly to that seen previously for the extranuclear lysate analysis – protein and lipid metabolism were enriched pathways while membrane trafficking proteins and signal transduction proteins were also enriched.

Table 5.4 – Many proteins are detectable as possible novel MCL-1 interacting partners in the mitochondrial fraction of HELA cells. Analysis of the eluate of an MCL-1 immunoprecipitation in the mitochondrial fraction of HELA cells yielded an enormous 1000 proteins in total. The protein list was reduced to 296 by a series of triages as indicated in the table.

Fraction: Mitochondria	**Peptides**
Total proteins identified	1000
Maximum fold change > 2	998
> 2 spectral counts	668
< 10% CRAPome frequency	315
> 5-fold change IP vs BC	308
< 0.02 FDR/q value	296

In the pathway analysis, four proteins (plus MCL-1) which had previously been linked to apoptosis were identified: BID, STAT3, DRP-1 (also known as DNM1L) and BAP31 (also known as BCAP31). BID is a BH3-only protein, known MCL-1 interacting partner[325] and furthermore, knocked out in the HCT116 8KO cells and therefore was disregarded as a Protein X candidate. Furthermore, as previously discussed, STAT3 is a transcriptional regulator and known interacting partner of MCL-1. This left BAP31 and DRP-1 as two potential candidate MCL-1 interacting partners/Protein X with prior links to apoptosis.

DRP-1 is the major regulator of mitochondrial fission and was proposed to be a curious potential MCL-1-interacting partner and Protein X candidate for multiple reasons: (1) mitochondrial fission and DRP-1 are known to be important to apoptosis[347] (2) MCL-

1-mediated regulation of mitochondrial dynamics has been linked to apoptosis[338] and (3) DRP-1 is known to associate with BAX[348]. Thus, DRP-1 has strong prior links to apoptosis and regulation of mitochondrial integrity. Meanwhile, BAP31, an endoplasmic reticulum-localised protein, also presented a potentially interesting hit, especially as it has been shown to form a complex with BCL-2/BCL-X_L and pro-caspase-8 that, during FAS-induced apoptotic signalling, can be cleaved and then induce apoptosis, possibly by further regulation of caspase-8[349,350]. Further studies have suggested that BAP31 mediates ER-mitochondria cross-talk during apoptosis and also provides feedback during ER-stress[351]. Thus, both DRP-1 and BAP31 have strong links to apoptosis and could be envisioned as MCL-1-interacting proteins which might get displaced following BH3 mimetic exposure to mediate apoptosis in the absence of the 8 key BH3-only proteins.

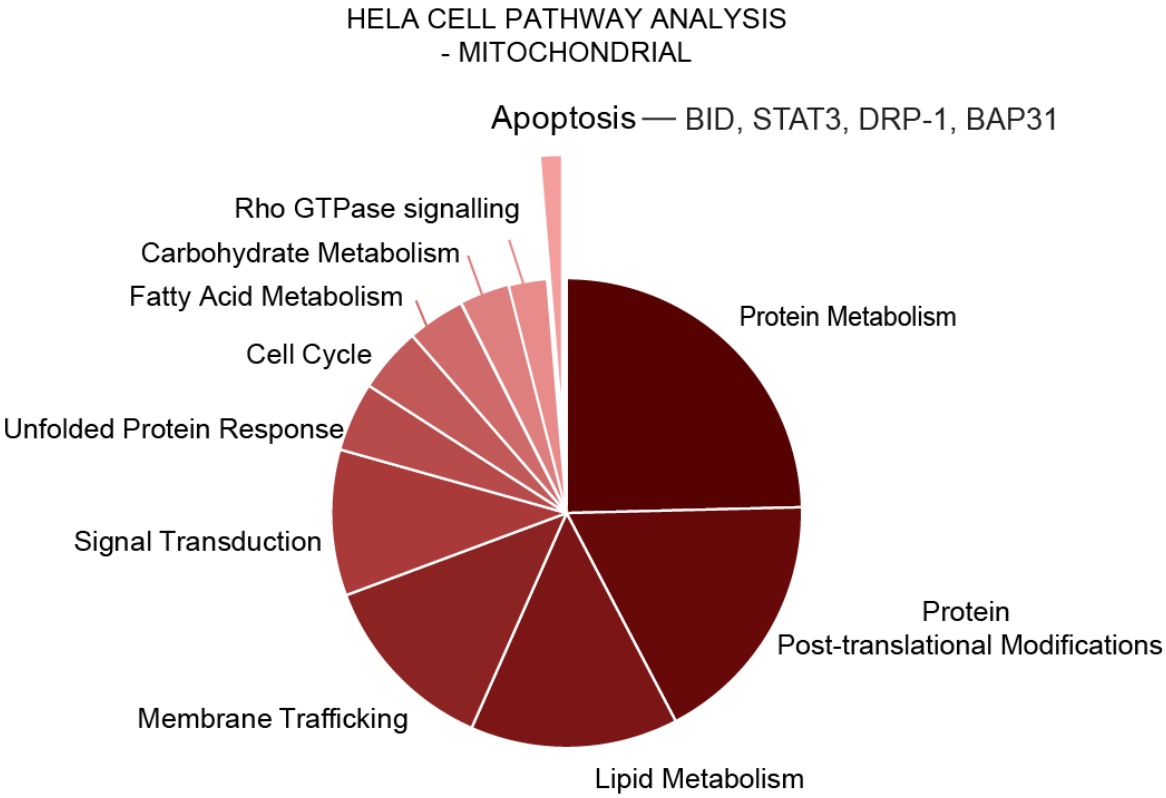


Figure 5.7. Candidates for MCL-1 interacting partners are wide-ranged in function in the mitochondrial fraction of HELA cells. Filtered peptide lists from mass spectrometry analysis of an MCL-1 pulldown in a mitochondria-enriched fraction of HELA cells were grouped following a pathway analysis using the Reactome, revealing further potential subcellular roles that MCL-1 may be linked to through its various interacting proteins.

5.11 Validation of the MCL-1-DRP-1 and MCL-1-BAP31 interactions.

As described above, the two most enticing novel MCL-1-interacting partners and Protein X candidates were BAP31 and DRP-1. In order to validate the interactions observed in mass spectrometry between MCL-1 and DRP-1/BAP31, MCL-1 was immunoprecipitated in H1299 cells and the eluate subsequently subjected to immunoblotting to detect MCL-1, BAP31 and DRP-1 (Fig. 5.8).

MCL-1 was pulled very efficiently, as demonstrated by the large amount of MCL-1 present in the MCL-1 immunoprecipitation lane compared to the bead control and furthermore, by the lack of MCL-1 in the immunoprecipitation flow-through. Both DRP-1 and BAP31 appeared to co-immunopreciptate with MCL-1 as shown in the immunoprecipitation lanes for each protein, validating the interactions identified in mass spectrometry. The amount of DRP-1 and BAP31 pulled appeared to be significant, however the amount of DRP-1 and BAP31 remaining in the flow-through is relatively high compared to the bead control flow-through, suggesting that there are other pools of these proteins which MCL-1 is not bound to. Nevertheless, both interactions with MCL-1 were validated, confirming both BAP31 and DRP-1 as novel MCL-1 interacting proteins and potential Protein X candidates.

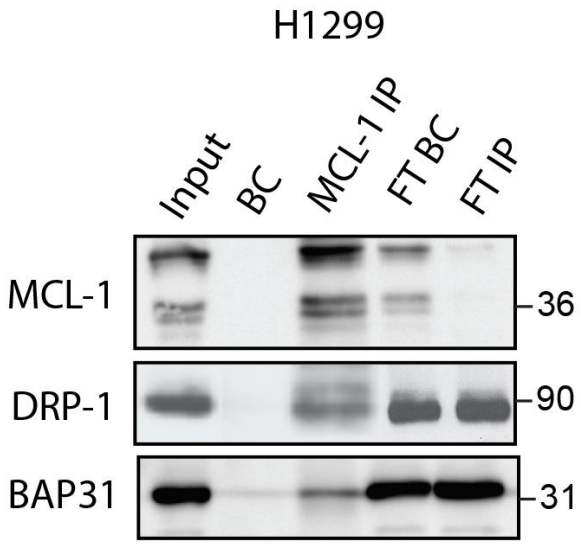


Figure 5.8. MCL-1 interacts with both DRP-1 and BAP31. MCL-1 was immunoprecipitated in H1299 cells and the eluate subjected to immunoblotting to detect interactions with DRP-1 and BAP31. Input represents whole cell lysate, BC represents bead control, IP indicates immunoprecipitation, FT BC represents the bead control flow-through, FT IP represents the IP flow-through. Approximate molecular weights of bands are indicated to the right of the immunoblots.

5.12 BAP31 is not required for BH3 mimetic-induced apoptosis in H1299 cells.

It was demonstrated that BAP31 can bind to MCL-1, however the functional consequences of this interaction and whether BAP31 might be a Protein X, along with its potential role in BH3 mimetic-induced apoptosis, remained unclear. In order to explore the function of the MCL-1-BAP31 interaction in this context, BAP31 was knocked down in H1299 cells using RNA interference and the cells exposed to a combination of S63845 and A-1331852. Assessment of the extent of apoptosis revealed that knockdown of BAP31 alone induced a very slight increase in apoptosis, increasing ~10% compared to the control siRNA (Fig. 5.9). Knockdown of BAP31

under BH3 mimetic conditions did not alter the levels of apoptosis, however, with similar levels of apoptosis observed between control and BAP31 siRNA conditions following S63845 and A-1331852 treatment. If BAP31 was to function like a Protein X, a decrease in apoptosis following BH3 mimetic exposure would be expected when BAP31 was removed – as is the case following depletion of BH3-only proteins[352–354]. Thus, this suggested that BAP31 does not play a role in BH3 mimetic-induced apoptosis in H1299 cells, further implying that it may not be the anticipated Protein X. The interaction between MCL-1 and BAP31 is of great interest, however. BAP31 is an ER resident protein[328] and has been suggested to contain a BH3-like motif and interact with other BCL-2 family members under particular conditions[349,350]. With the growing connections between the mitochondria and endoplasmic reticulum during apoptosis[355], future exploration of this interaction might reveal some very exciting results about ER-mitochondria junctions, as well as a role for MCL-1 in mediating this cellular function.

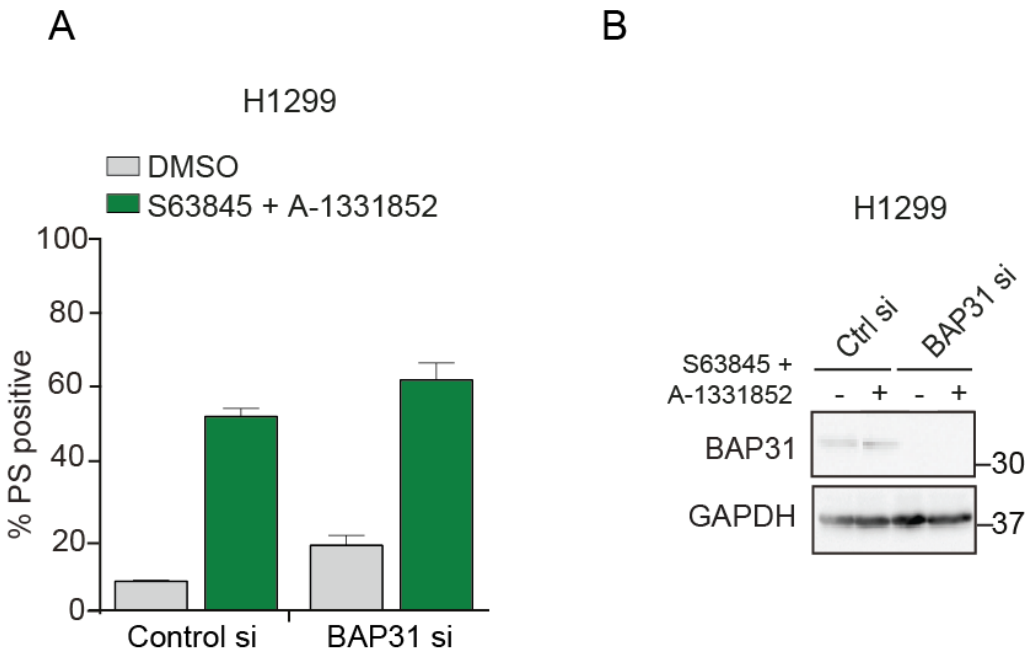


Figure 5.9. BAP31 does not appear to play a significant role in mediating BH3 mimetic induced apoptosis in H1299 cells. (A) H1299 cells were incubated with either a control siRNA or siRNA against BAP31 for 72 h then treated with 100 nM each of both S63845 and A-1331852 for 4 h before being collected for assessment of apoptosis by PS externalisation in flow cytometry. (B) Knockdown of BAP31 was validated by immunoblotting with GAPDH as a loading control. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

5.13 DRP-1 is required for BH3 mimetic-induced apoptosis in H1299 cells.

The DRP-1-MCL-1 interaction was explored further in the same way as the MCL-1-BAP31 interaction but yielded very different results. Again, in theory, removing DRP-1 from the cell should function like removal of a BH3-only protein - if DRP-1 functions as a Protein X, this would result in abrogated levels of apoptosis following BH3 mimetics. As mentioned in the mass spectrometry results, previous studies have already linked DRP-1 to apoptosis. Early work demonstrated that DRP-1 can

translocate from the cytosol to the mitochondria to induce mitochondrial fission during apoptosis and furthermore, inhibition of DRP-1 activity abrogates cytochrome *c* release induced by staurosporine[356]. This effect could not be overcome by overexpression of BAX, suggesting that DRP-1 performs a function that is essential to apoptosis[356]. The role for DRP-1 in apoptosis is extremely complex and somewhat controversial however, as it has also been shown that inhibition of DRP-1-mediated mitochondrial fragmentation does not completely block apoptosis - instead it causes a delay of cytochrome *c* release via modulation of OPA1 activity[357]. Further adding to the complexity of DRP-1 in apoptosis, other studies have shown that inhibiting DRP-1 can sometimes enhance apoptosis[358]. In support of an interaction between MCL-1 and DRP-1, during the preparation of this study, it was shown that MCL-1 can interact with DRP-1 in multiple reports in both apoptotic and non-apoptotic scenarios[182,338]. The studies suggested that MCL-1 interacts with DRP-1 in order to regulate mitochondrial dynamics, with MCL-1 potentially functioning as a scaffold on the mitochondrial membrane for DRP-1 translocation in both cancer cells and stem cells[182,338]. Thus, DRP-1 is clearly an important interacting partner of MCL-1, affirming its potential as a Protein X that may be able to induce apoptosis in the absence of the major BH3-only proteins.

To explore the MCL-1/DRP-1 functional axis in apoptosis, DRP-1 was knocked down in H1299 cells for 72 h and the cells were subsequently exposed to either DMSO or S63845 and A-1331852 (both at 100 nM) for 4 h (Figure 5.10A). The combination of S63845 and A-1331852 resulted in ~65% apoptosis in the control siRNA cells compared to the DMSO control with less than 10% apoptotic cells. Knockdown of DRP-1 alone did not induce significant apoptosis in the H1299 cells and remarkably, knockdown of DRP-1 in combination with the BH3 mimetics could partially reduce

apoptosis, with ~35% of cells undergoing apoptosis in the combination treatment. This suggested that DRP-1 is required for BH3 mimetic induced apoptosis in H1299 cells and could therefore potentially function like a Protein X. Knockdown of DRP-1 was validated in both control and treated conditions, demonstrating complete loss of DRP-1 in both cases (Figure 5.10B).

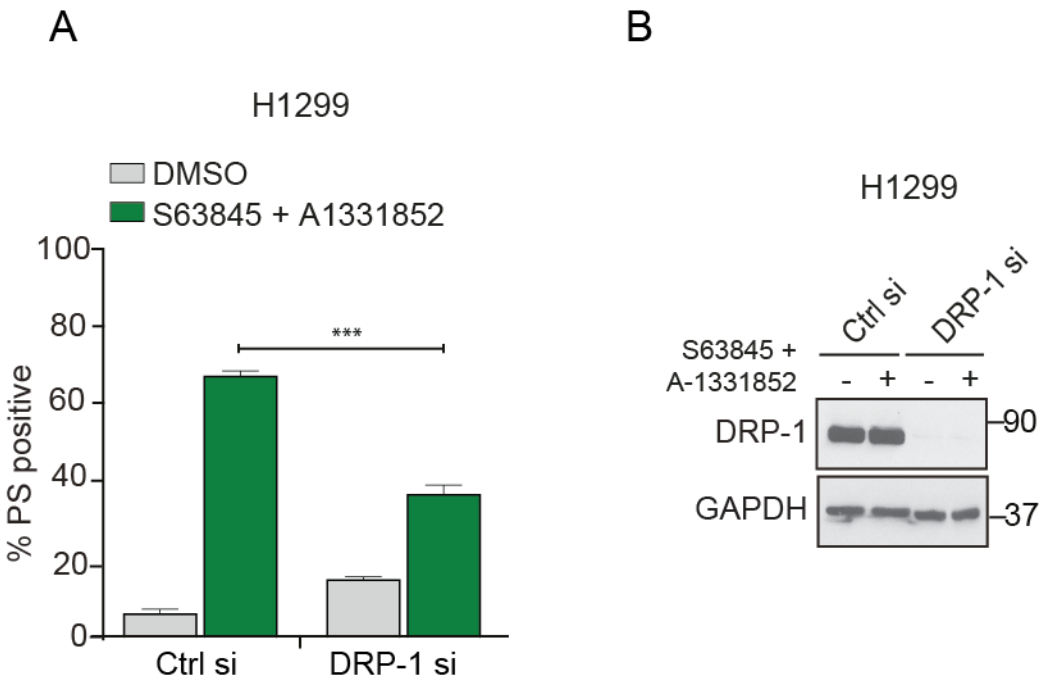


Figure 5.10 Knockdown of DRP-1 partially rescues BH3 mimetic-induced apoptosis in H1299 cells. (A) H1299 cells were incubated with either a control siRNA or siRNA against DRP-1 for 72 h then treated with 100 nM each of S63845 and A-1331852 for 4 h before being collected for assessment of apoptosis by PS externalisation in flow cytometry. (B) Knockdown of DRP-1 was validated by immunoblotting with GAPDH used as a loading control. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

5.14 DRP-1 co-elutes with MCL-1 and re-distributes to high molecular weight fractions following exposure to BH3 mimetics.

To further explore the interaction between MCL-1 and DRP-1 and assess which of the previously identified MCL-1 complexes contain DRP-1, H1299 cells were exposed to BH3 mimetics, lysed and fractionated using Superose 6 and Superdex 200 FPLC columns. MCL-1 appeared in its expected fractions (HMW, MMW, LMW), while DRP-1 seemed to elute between 150-600 kDa, suggesting the DRP-1 identified bound to MCL-1 in the mass spectrometry and immunoprecipitation may be linked to the LMW MCL-1 complex under control conditions (Fig 5.11). When treated with the MCL-1 inhibitor A-1210477 however, the distribution of both MCL-1 and DRP-1 was vastly different. Considerably less MCL-1 was detected in the HMW complex, while virtually no MCL-1 was detected in the two lower molecular weight complexes. Instead, a large amount of very low molecular weight, possibly monomeric MCL-1 was detected. While MCL-1 became less distributed following exposure to an MCL-1 inhibitor, DRP-1 became more widely distributed across the different molecular weight fractions, with a large portion of DRP-1 apparently shifting to where the HMW 2 MDa complex of MCL-1 can typically be found. This could suggest that following BH3 mimetic treatment, MCL-1 and DRP-1 dissociate and DRP-1 oligomerises in a HMW complex to mediate death and perhaps, activate BAX. Indeed, DRP-1 has previously been shown to facilitate BAX translocation to/oligomerisation at the mitochondria during apoptosis[348,359,360], supporting a role for DRP-1 in mediating aspects of BAX forming pores in the mitochondrial membrane during apoptosis. It is also known that DRP-1 forms high molecular weight oligomers during mitochondrial fission as it forms rings to constrict the mitochondria[361], therefore, it could be

envisaged that the HMW fraction of DRP-1 detected following exposure to A-1210477 is indicative of these oligomers and thus, mitochondrial fission.

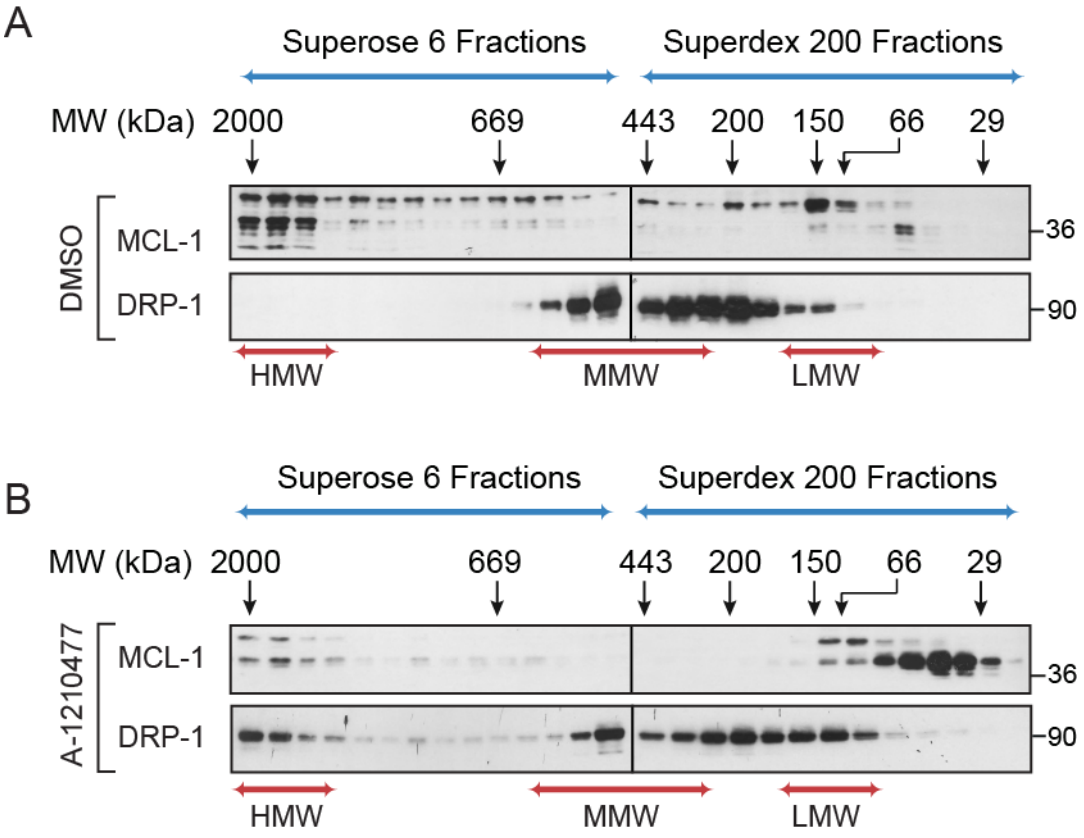


Figure 5.11. MCL-1 shifts to a low molecular weight and DRP-1 to a high molecular weight following treatment with an MCL-1 inhibitor. H1299 cells were exposed to DMSO or 10 µM A-1210477 for 4 h and cells lysed and ran on Superose 6 and Superdex 200 FPLC columns. Collected fractions were subjected to immunoblotting to detect MCL-1 and DRP-1. Approximate molecular weights of fractions are indicated above the blots and molecular weight markers of bands are indicated to the right. Red arrows indicate the 3 major MCL-1 complexes identified.

5.15 Inhibition of MCL-1 enhances DRP-1-mediated mitochondrial fission.

The previous data suggested that following inhibition of MCL-1 by BH3 mimetics, DRP-1 moves to a HMW complex, perhaps indicating that DRP-1 is forming an oligomer. It is well known that oligomers of DRP-1 form a constrictive ring around the mitochondria during mitochondrial fission[361]. Interestingly, previous studies have alluded to MCL-1 having a role in regulating mitochondrial dynamics and bioenergetics[309,338]. Furthermore, putative inhibitors of MCL-1 have been shown to induce extensive mitochondrial fission[294,362,363]. Therefore, it could be suggested that the interaction between MCL-1 and DRP-1 is one way by which MCL-1 could be involved in mitochondrial dynamics. In order to test this hypothesis, H1299 cells were plated on coverslips and DRP-1 knocked down using RNAi. The cells were then exposed to A-1210477, fixed and labelled with a HSP60 antibody (a mitochondrial marker[294]) to assess the state of the mitochondrial network. As expected, under control conditions, the mitochondrial network was highly filamentous, while A-1210477 induced extensive mitochondrial fission, as shown by the punctate appearance of the mitochondrial network in the treated cells (Fig. 5.12A). Knockdown of DRP-1 alone resulted in a hyperfilamentous mitochondrial network and, remarkably, the mitochondrial fission seen following MCL-1 inhibition was significantly prevented with a DRP-1 knockdown, suggesting that the BH3 mimetic-induced mitochondrial fission is DRP-1-dependent. Quantification of the extent of fission revealed that the knockdown of DRP-1 almost completely diminished fission induced by the inhibition of MCL-1, with the extent of fission dropping from ~95% to less than 20% following DRP-1 knockdown (Figure 5.12B).

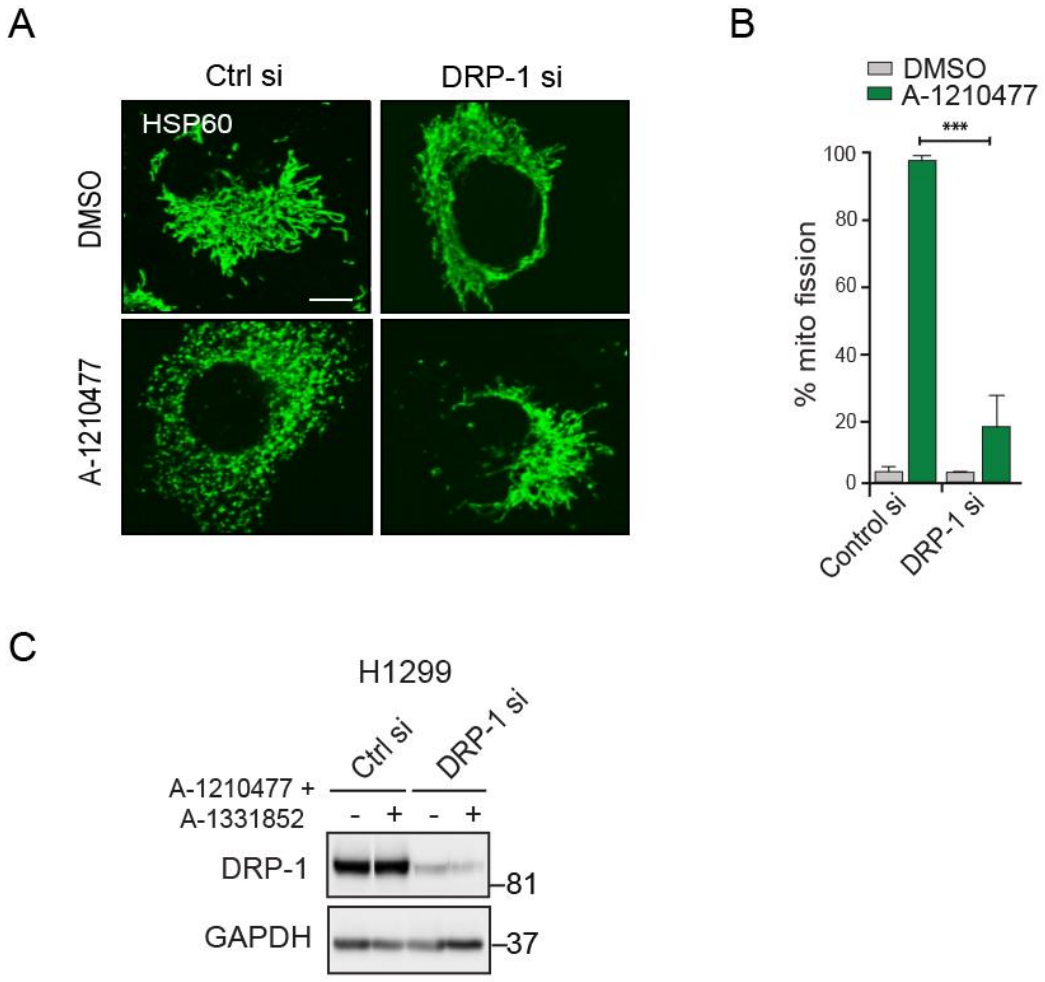


Figure 5.12. Inhibition of MCL-1 induces mitochondrial fission in a DRP-1 dependent manner. (A) H1299 cells were grown on coverslips in the presence of either a control siRNA or DRP-1 siRNA for 72 h and subsequently treated with 10 μM A-1210477 for 2 h and mitochondrial structure assessed by labelling of cells with HSP60. (B) The extent of fission or rescue in cells was assessed by counting the number of cells with mitochondrial fission over 3 independent replicates. (C) Knockdown of DRP-1 was assessed by immunoblotting. Approximate molecular weight markers of bands are indicated to the right of the blots.

5.16 DRP-1 contains a BH3-like motif but the MCL-1-DRP-1 interaction does not appear to occur *via* the BH3-motif.

Previous data has demonstrated that (1) MCL-1 interacts with DRP-1 and (2) DRP-1 moves to a HMW complex, away from its co-elution with MCL-1, following exposure to the MCL-1 inhibitor A-1210477. This suggested that A-1210477 facilitated the disruption of the MCL-1/DRP-1 interaction, and the subsequent release and oligomerisation of DRP-1, thus placing DRP-1 as a potential candidate for Protein X. For DRP-1 to function like a BH3-only protein, it would be assumed that it may contain a domain or motif which is structurally similar to the BH3 motif, which would allow it to heterodimerise with MCL-1 and perhaps other BCL-2 family proteins. Thus, the DRP-1 protein sequence was analysed to identify any regions which might indicate a potential BH3 motif and the sequence compared to that of the known BH3-only proteins, PUMA and NOXA. Indeed, sequence alignment revealed that DRP-1 appears to contain region resembling a BH3 motif (Figure 5.13A), with a conserved glutamine residue, a conserved hydrophobic residue (isoleucine) and conserved basic residues (arginine and lysine). As previously described, the BH3 motif is highly flexible in which amino acids it may contain and there is even sequence variation between isoforms of the same protein (for example PUMA, as shown in Figure 5.13A) therefore the variability in the sequence of DRP-1 compared to the other BH3-only proteins is not surprising. Thus, the presence of these distinct amino acids with hydrophobic and basic properties suggests this domain may indeed be a BH3-like motif and therefore, MCL-1 and DRP-1 could interact via this motif.

To further explore whether DRP-1 and MCL-1 interact in a BH3-dependent manner, immunoprecipitation of MCL-1 was performed, as previously described (Fig. 5.8), using both control and A-1210477-treated H1299 cells. DRP-1 could clearly be seen

co-immunoprecipitating with MCL-1 in the control fraction, while the activity of A-1210477 was confirmed by stabilisation of MCL-1 in the treated samples (Fig. 5.13B). Surprisingly however, exposure of cells to A-1210477 did not result in decreased MCL-1/DRP-1 interaction as expected. Instead, it appeared that more DRP-1 was detectable bound to MCL-1 following A-1210477 treatment, though this could be a result of more MCL-1 being pulled due to its stabilisation. Thus, DRP-1 and MCL-1 do not dissociate following inhibition of MCL-1 with a BH3 mimetic, at least not in the context of an immunoprecipitation. While this may argue against DRP-1 being the Protein X candidate, it is evident from the previous data that DRP-1 and MCL-1 interact and that this interaction might change under conditions of BH3 mimetics, which may potentially affect mitochondrial fission and/or apoptosis. Therefore, it is quite possible that DRP-1 and MCL-1 interact with each other in a highly dynamic manner, which could make it difficult to detect their dependence on BH3 motifs for such an interaction, at least using current techniques. Alternatively, it could also be proposed that MCL-1 and DRP-1 interact via a motif distinct of this proposed BH3 motif.

A

PUMA	E	Q	W	A	R	E	I	G	A	Q	L	R	R	M	A	D	D	L	N	A
PUMA 2A	E	Q	W	A	R	E	I	G	A	Q	A	R	R	M	A	D	D	L	N	A
NOXA A	A	E	L	P	P	E	F	A	A	Q	L	R	K	I	G	D	K	V	Y	C
DRP-1	V	S	F	E	L	L	V	K	R	Q	I	K	R	L	E	E	P	S	L	R

B

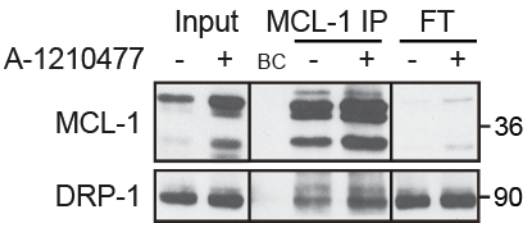


Figure 5.13. DRP-1 contains a BH3-like motif but the MCL-1-DRP-1 interaction occurs in a BH3-independent manner. (A) Sequence alignment of a BH3-like motif in DRP-1 compared to the BH3 motifs of known BH3-only proteins. Orange indicates conserved glutamine residues, yellow indicates a conserved hydrophobic residue and green, conserved basic residues. (B) MCL-1 was immunoprecipitated in H1299 exposed to 10 µM A-1210477 for 4 h and the eluate subjected to immunoblotting to detect DRP-1. Input represents whole cell lysate, BC represents bead control, IP indicates immunoprecipitation, FT represents flow-through. Approximate molecular weights are indicated to the right of the immunoblots.

5.17 DRP-1 plays a very different role in BH3 mimetic-induced apoptosis in HCT116 cells.

DRP-1 is required in H1299 cells for apoptosis, suggesting it could potentially behave, in part, like a BH3-only protein, though its potential as a Protein X as alluded to previously remained unclear. While DRP-1 appears to contain a BH3-like domain, it seemed that the interaction between MCL-1 and DRP-1, surprisingly, does not occur via the BH3 regions, as the BH3 mimetic A-1210477 could not displace DRP-1 from MCL-1. Regardless of this, to assess if DRP-1 could still be a potential candidate for Protein X, HCT116 WT and 8KO cells were incubated with a control or DRP-1 siRNA then exposed to a combination of S63845 and A-1331852 for 4 h and apoptosis assessed by PS externalisation in flow cytometry. The rationale behind this study was that DRP-1 would still interact with MCL-1 in the 8KO cells and could therefore drive apoptosis following BH3 mimetics in these cells, even in the absence of all 8 BH3-only proteins. In other words, silencing of DRP-1 should technically abolish BH3 mimetic-induced apoptosis in the 8KO cells, similar to the results previously observed in H1299 cells.

In HCT116 WT cells, S63845 and A-1331852 induced ~75% apoptosis as expected, with basal death ~10% (Figure 5.14A). Remarkably, knockdown of DRP-1 exacerbated cell death and could even induce apoptosis alone (~60% apoptosis), which was significantly enhanced to ~90% death when these cells were further exposed to a combination of S63845 and A-1331852. This is in direct contrast with what was seen for H1299 cells and other studies, where knockdown of DRP-1 protected cells from BH3 mimetic-induced death. Similar results were observed in the HCT116 8KO cells (Figure 5.14B). Again, as expected, a combination of S63845 and A-1331852 induced significant death of ~65% under control siRNA conditions, compared to less than 10%

in the DMSO treated cells. Knockdown of DRP-1 alone in the HCT116 8KO also induced significant apoptosis of almost 40%, although remarkably this was much lower than that seen in the HCT116 WT cells, suggesting that the siDRP-1-induced death may be somewhat dependent on the BH3-only proteins in the HCT116 cells. When knockdown of DRP-1 was combined with S63845 and A-1331852 however, apoptosis exceeded 80% in the 8KO cells, reflecting the results seen in the HCT116 WT cells, suggesting that the requirement of the BH3-only proteins for DRP-1-induced death is overcome when MCL-1 and BCL-X_L are inhibited. Knockdown efficiency of DRP-1 was confirmed by immunoblotting and showed that almost all DRP-1 was knocked down in both cell lines (Figure 5.14C and D). This data, taken together with previous data, suggested that DRP-1 is not a likely Protein X candidate and is, instead, critical for survival in the HCT116 cells.

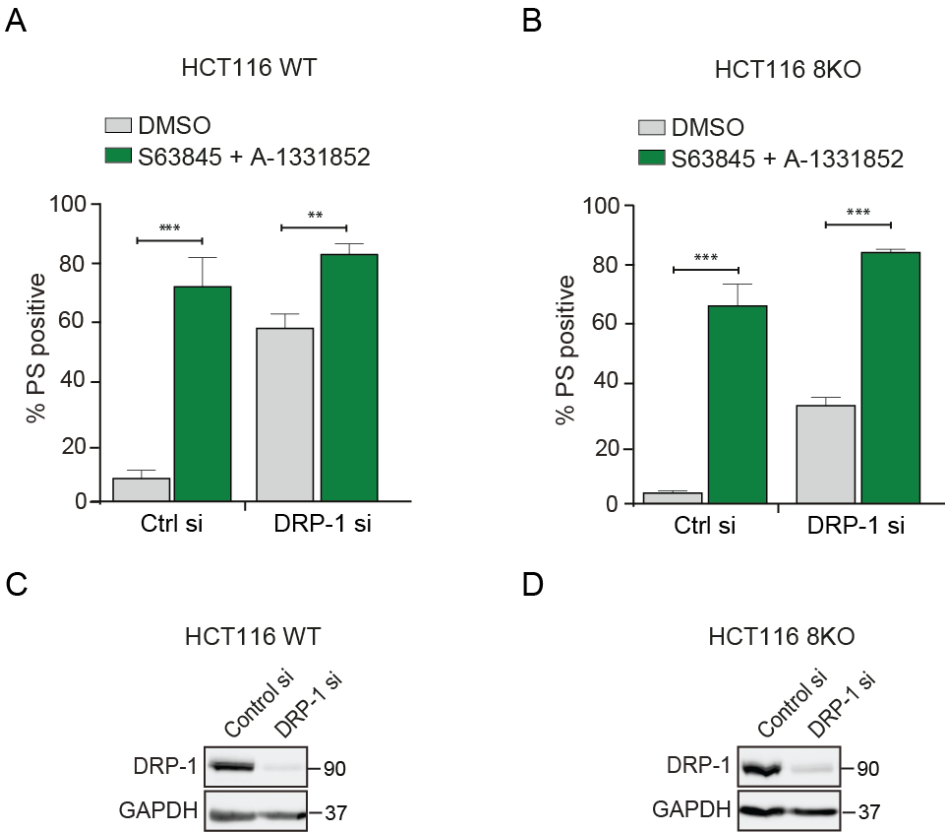


Figure 5.14 Loss of DRP-1 induces apoptosis in HCT116 cells. (A) HCT116 WT and (B) 8KO cells were incubated with either control or DRP-1 siRNA for 72 h then exposed to DMSO or a combination of 100 nM S63845 and A-1331852 for 4 h before being collected for assessment of apoptosis by PS externalisation in flow cytometry. (C, D) Knockdown of DRP-1 was validated by immunoblotting with GAPDH as a loading control. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

5.18 Apoptosis induced by DRP-1 downregulation is BAX-dependent.

It was previously established in Chapter 4 and in other studies that HCT116 cells rely on BAX and not BAK for apoptosis[310]. Since depletion of DRP-1 could induce apoptosis in both HCT116 WT and 8KO cells (Fig 5.14) and since BAX and DRP-1 have previously been linked[348,359,360], it was asked whether apoptosis induced by DRP-1 downregulation requires BAX. For this, HCT116 BAX-/- and HCT116 DKO (lacking BAX and BAK) were incubated with a control or DRP-1 siRNA for 72 h then exposed to S63845 and A-1331852 (both at 100 nM) and analysed for apoptosis (Figure 5.15A). In the absence of BAX, it was clear that neither BH3 mimetics nor the DRP-1 siRNA could induce significant apoptosis in these cells (Figures 5.15A and C), though a slight increase in apoptosis was seen in the BAX-/- cells following knockdown of DRP-1. This may suggest that knockdown of DRP-1 was inducing low levels of BAX-independent, non-specific death. Knockdown efficiency of DRP-1 was confirmed by immunoblotting and showed that almost all DRP-1 was knocked down in both cell lines (Figure 5.15B and D). This suggested that the apoptosis seen in the HCT116 cells following DRP-1 downregulation is therefore largely BAX-dependent and that perhaps DRP-1-BAX can indeed regulate each other but not in the anticipated manner.

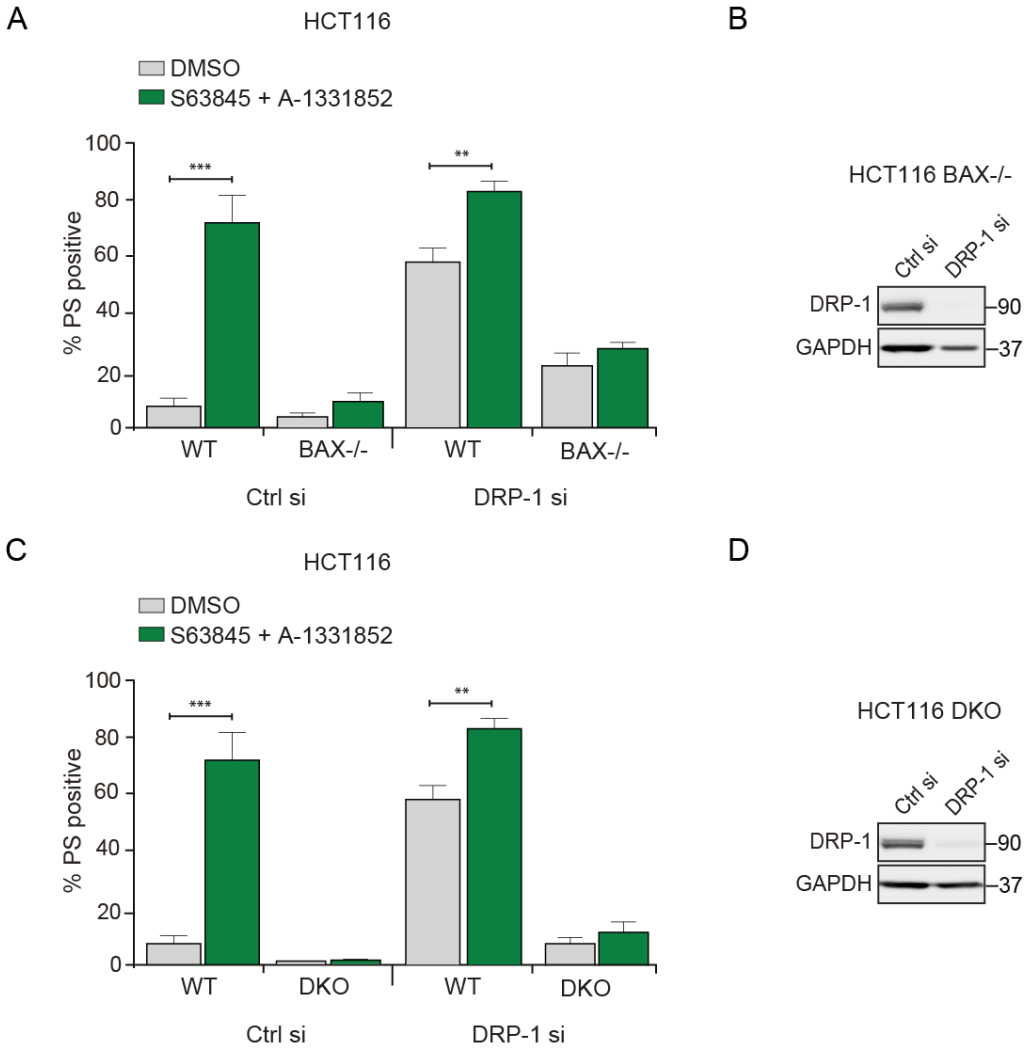


Figure 5.15. Knockdown of DRP-1 induces death in a BAX-dependent manner in HCT116 cells. HCT116 BAX-/- (A) and DKO cells (C) were incubated with either control or DRP-1 siRNA for 72 h then exposed to DMSO or a combination of 100 nM S63845 and A-1331852 for 4 h before being collected for assessment of apoptosis by PS externalisation in flow cytometry. (B, D) Knockdown of DRP-1 was validated by immunoblotting with GAPDH as a loading control. Error bars = Mean ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

5.19 Discussion

MCL-1 is a unique and complex member of the BCL-2 protein family which likely has a biology that is even more intricate than currently known. Here it was shown that not only is MCL-1 identifiable in all of the major subcellular compartments of the cell but it also exists in novel, distinct complexes of varying molecular weight which may be indicative of its multi-faceted functions. Indeed, MCL-1 has been previously shown to have diverse functions which would require distinct cellular localisations, including regulation of DNA repair in the nucleus[177,179,184,326,335] and regulation of respiration and lipid metabolism on the inner mitochondrial membrane[175,183]. Studying the different pools of MCL-1 also revealed differences in the dominant localisations of the controversial MCL-1 isoforms. MCL-1 is detected in immunoblotting, under reducing conditions, as at least three bands corresponding to its different isoforms, which have all been attributed to its varying molecular functions. Although no article exists which clearly attributes each individual isoform to a particular function, it has been proposed that the longest isoform is the major apoptotic regulator of the three bands. In contrast, the shortest isoform is suggested to be localised to the IMM where it mediates non-apoptotic mitochondrial activity[172,175,181]. Indeed, here, the shortest isoform was expressed most highly in the mitochondrial fraction and was not present at all in the cytosolic fraction. Interestingly, a seemingly modified version of the longest MCL-1 isoform was detectable in the nuclear fraction, running slightly above the weights of the other three bands in the other isoforms. Previous reports have linked the short forms of MCL-1 to its nuclear activities[177] while a long, modified form has not been previously described in the nucleus, however this could suggest that some isoforms of MCL-1 must be modified to enter the nucleus, perhaps undergoing a phosphorylation event which alters its molecular weight. While the full extent of the

role of MCL-1 in the nucleus remains obscure, it is clear from this data that all three bands (perhaps in modified forms) are present and thus may be performing some of the described nuclear roles of MCL-1. With further layers of complexity being added to the existence of MCL-1 isoforms, it is imperative that future studies focus on defining these isoforms and their roles within the cell.

In order to further explore the complexity of MCL-1 pools with the goal of identifying potential candidates for a Protein X in the HCT116 8KO cells, mass spectrometry was utilised to assess the protein members of different pools of MCL-1. This study attempted to use two approaches – the first being immunoprecipitation of MCL-1 from the major subcellular compartments and the second being immunoprecipitation of MCL-1 from the novel MCL-1 complexes identified through FPLC. Despite these two strategies using the same approach and same antibodies, the outcome for each was quite different, namely because in the resulting peptide list of the subcellular fraction, MCL-1 was readily identifiable in each subcellular compartment along with multiple known MCL-1 interacting proteins. However, in the mass spectrometry involving fractions corresponding to distinct MCL-1 complexes (HMW, MMW, LMW), MCL-1 was not detectable in the peptide lists during optimisation, despite many proteins being apparently pulled down with it. This result was disappointing as identifying the differences between the major MCL-1 complexes may have revealed a great deal about the biology of MCL-1, including its various pools, novel interacting partners and potentially some candidates for Protein X. It will be important to establish whether the MCL-1 complexes can be isolated in another approach which allows for maximal maintenance of protein-protein interactions *in situ*, perhaps using a recombinant, tagged MCL-1 system.

Despite the lack of success in the mass spectrometry involving fractions corresponding to distinct MCL-1 complexes, MCL-1 was readily identifiable along with many candidate interacting proteins in the subcellular fractionation approach. In both the extranuclear and mitochondrial fractions, the initial number of peptides identified by mass spectrometry was very high, meaning that stringent sorting of the peptide lists was required. As described, a recent article identified a novel MCL-1 interacting protein (VLCAD) using a similar approach (albeit a pull-down of tagged MCL-1 rather than immunoprecipitation)[183]. Therefore, the same stringencies applied to their list of peptides was applied here. This greatly reduced the numbers of peptides in the two fractions and allowed efficient grouping of identified peptides into pathways using the Reactome pathway analysis tool. In order to simplify the approach of identifying significant Protein X candidates, the resulting enriched pathways were analysed for proteins linked to cell death – this is how DRP-1 and BAP31 were identified. It is almost certain, however, that some, if not many, of the other identified proteins might be novel MCL-1 interacting partners, which could be linked to either the anti-apoptotic roles of MCL-1 or perhaps its more diverse roles such as regulation of metabolism. Notably, VLCAD was identified as an MCL-1 interacting partner in the mass spectrometry performed here, supporting the principle of the technique and subsequent analysis. Many of the other proteins previously identified could be associated with the metabolic regulatory roles of MCL-1, such as ATP5F1, part of the ATP synthase structure. MCL-1 has previously shown to promote the assembly of the ATP synthase complex[175] therefore a possible interaction may shed new light on this mechanism. Proteins associated with post-translational modifications were also enriched in both the extranuclear and mitochondrial extracts, such as YES, a tyrosine kinase[364]. Exploration of such proteins may reveal previously unreported post-translational

modifications of MCL-1 and perhaps, some more unexpected links – MCL-1 has been linked to regulation of tyrosine kinase signalling during metastasis, further widening its potential diverse roles within the cell[365]. Future work should aim to identify the significant proteins from these enriched pathways, validate the interactions and add to the understanding of the complex biology of MCL-1. It is worth nothing that significant numbers of proteins were not co-immunoprecipitated with MCL-1 in the nuclear or cytosolic fraction, particularly in the case of the nucleus where MCL-1 has been proposed to interact with highly abundant proteins, such as γ-H2AX[184]. The lack of co-immunoprecipitated proteins in these cases may be a result of the state of the cells – these interactions are linked to various types of DNA repair and if low levels of DNA repair were being carried out at the time of cell collection, the interactions may not be identifiable by mass spectrometry. Future experiments investigating MCL-1 interactions should aim to validate many of the potential candidate interacting partners identified here, perhaps using improve control conditions and a different approach to isolating MCL-1. An isotype control and an immunoprecipitation in MCL-1-null cells would help identify non-specific candidate interacting proteins. Moreover, generating individual subcellular fractions in the lab would allow for precise control of conditions of the experiment from start to finish, including lysis of cells and storage conditions. This may allow for improved maintenance of endogenous interactions and minimal presence of interactions induced by processing of samples, which may have potentially occurred during processing of the purchased subcellular fractions. A further improvement would be to use a tagged, immobilised MCL-1 target, rather than MCL-1 isolated by IP.

The two validated, novel MCL-1 interacting proteins identified here are DRP-1 and BAP31, with the functional consequences of the MCL-1-DRP-1 interaction explored

further. Following validation of the interaction between MCL-1 and DRP-1, it was shown that DRP-1 co-elutes with the MMW complex of MCL-1. Furthermore, inhibition of MCL-1 seemed to cause DRP-1 to shift to a high molecular weight, suggesting that BH3 mimetics can cause the dissociation of MCL-1 and DRP-1 and that this, in turn, might be what is mediating the excessive mitochondrial fission observed during apoptosis following MCL-1 inhibition. The proposed mechanism of action of DRP-1 involves formation of oligomers at the mitochondrial membrane which form rings to physically constrict and pinch off mitochondrial membranes from one other, leading to fissed mitochondria[361]. This is an important event not just in the context of mitochondrial fission and maintenance of mitochondrial membrane dynamics, but also during apoptosis as mitochondrial fission has been implicated as a possible prerequisite for the efficient execution of the intrinsic pathway of apoptosis[356]. In agreement, DRP-1 oligomerisation resulted in not just mitochondrial fission but also apoptosis in H1299 cells, as cells devoid of DRP-1 underwent significantly less apoptosis following BH3 mimetics. However, it must be noted that BH3 mimetic-mediated mitochondrial fission and apoptosis can also be uncoupled as the fissed mitochondrial phenotype observed following A-1210477 in H1299 cells does not necessarily result in death. These cells depend not just on MCL-1 but also on BCL-X_L for survival, and as a result, inhibition of MCL-1 and the ensuing mitochondrial fission is not sufficient to induce apoptosis, unless BCL-X_L is also neutralised. Thus, it seems that DRP-1-mediated mitochondrial fission plays a central role in apoptosis but is not a direct inducer of apoptosis, at least in these cells. This goes against DRP-1 being a candidate Protein X, despite it seemingly having a BH3-like domain, interacting with MCL-1 and leading to decreased apoptosis when downregulated.

One surprising result observed was that despite the apparent dissociation of MCL-1 and DRP-1 in gel filtration analysis, A-1210477 could not disrupt the interaction between MCL-1 and DRP-1 in immunoprecipitation. One possible explanation for this could be the fact that DRP-1 is an extremely dynamic protein which moves continuously between the mitochondria and cytosol. It could therefore be proposed that in this immunoprecipitation experiment, DRP-1 had already carried out its fission activity at the mitochondrial membrane and rapidly translocated back to its previous interacting partners – meaning that the fission process was perhaps not 'caught' at the right time. Thus, it is not impossible that MCL-1 and DRP-1 dissociate in immunoprecipitation approaches but to detect this, alternative strategies such as time-lapse high-resolution confocal microscopy, might be needed to capture the MCL-1-DRP-1 interactions at the right moment.

In addition to validating the physical interaction between MCL-1 and DRP-1 and observing its dissociation in gel filtration following inhibition of MCL-1, the interaction was also functionally validated. DRP-1 is required for BH3 mimetic induced apoptosis in H1299 cells but how it precisely functions in the apoptotic signalling pathway is unclear and whether this is linked to a proposed 'Protein X' function is unclear. What is clear is the fact that in both the HCT116 WT and 8KO cells, loss of DRP-1 itself induces extensive apoptosis. In this case, it almost appears that DRP-1 functions like a pro-survival protein rather than a pro-apoptotic BH3-only like protein, though there are many potential ways that loss of DRP-1 could induce death. Perhaps HCT116 cells are very sensitive to perturbations of their mitochondrial network and therefore a loss of mitochondrial fission results in altered mitochondrial bioenergetics which in turn induces high levels of stress and, therefore apoptosis. The DRP-linked death appears to be BAX dependent however, demonstrating that the

death seen is organised and not a type of necrosis or other form of cell death. Some reports have indeed suggested that, in opposition to the majority of the literature, inhibiting DRP-1 by pharmacological or genetic approaches can enhance apoptosis induced by BH3 mimetics in specific cell lines (melanoma) while inhibiting apoptosis in other types of cancer cell lines (breast cancer)[358]. Thus, the role of DRP-1 in apoptosis may be dependent upon the type and status of a cell and thus, colon carcinoma cells like HCT116 require DRP-1 for survival.

This study reveals that the complexities of the BCL-2 protein family and their interacting proteins in apoptosis extend way beyond what is currently known. Future therapeutic targeting of the BCL-2 family must be approached with care until the nexus of interactions and functional consequences is fully understood, particularly when it comes to MCL-1 now that there is a strong possibility that it could be targeted in the clinic in the imminent future using the novel MCL-1 inhibitors.

Chapter 6

General Discussion

Contents

6.1 The changing shape of the MCL-1 inhibitor field

The pro- and anti-apoptotic proteins of the BCL-2 family are the major regulators of cell survival, whose expression levels play an extremely important role in the development of various diseases, including cancer. The interactions between the proteins in this family are believed to be what dictates the survival or death of a cell. As such, an emphasis has been placed on developing compounds which target these interactions so that inhibitors can be used in therapeutic strategies to induce apoptosis in cancer cells and therefore aid in the clearance of tumours. Years of development have yielded several highly specific and potent inhibitors of the BCL-2 family (termed BH3 mimetics), including ABT-263[246] and the now FDA-approved ABT-199[256] as well as various MCL-1 inhibitors, such as the novel S63845[269] which was assessed in this study. S63845, has proven itself as a very promising MCL-1 inhibitor with great therapeutic potential, in addition to use as a tool to study MCL-1, both here and in other studies[202,269,271,366]. The promise of potent and highly specific MCL-1 inhibitors is incredibly exciting to the cancer therapy field, as MCL-1 is among the top ten of the most amplified genes across many cancer types[195]. In addition to S63845, during the preparation of this manuscript, another novel, highly potent and specific MCL-1 inhibitor, AZD5991[367] was published, which gave extremely promising results and further adds another agent to the BH3 mimetic armamentarium. MCL-1 has never before been in such a strong position as a druggable target in cancer, thus further development of these inhibitors which have the potential to enter the clinic are likely to greatly improve chemotherapeutic regimens as both single agents and in combination with other therapies.

6.2 The dispensability of BH3-only proteins in apoptosis

BH3 mimetics, including S63845, are proposed to induce apoptosis by disrupting the interactions between the BCL-2 family members, including the BH3-only proteins and their pro-survival counterparts. Thus, the role of the BH3-only proteins in BH3 mimetic-induced apoptosis has been firmly cemented in the field by a plethora of studies (reviewed in [61]). Recent work, however, has suggested that the BH3-only proteins may be dispensable for apoptosis following suppression of the pro-survival proteins[220,303,304], with the so called 'octa-knockout' cells (lacking the 8 major BH3-only proteins) delivering a massive blow to the accepted models of activation of BAX and BAK[303].

Here, using highly specific BH3 mimetics, it was first established that the mere interaction between a pro-survival protein and a pro-apoptotic protein may not necessarily dictate the sensitivity of a cell to specific BH3 mimetics. For example, in a cell line that depends on either BCL-2 or MCL-1 for survival, like the MV-4-11 cell line (which undergoes extensive apoptosis with either ABT-199 or S63845), one would expect similar binding patterns of the BH3-only members to both BCL-2 and MCL-1. In this cell line, BIM was largely bound to BCL-2 with relatively little bound to MCL-1. Furthermore, ABT-199 failed to release BIM from BCL-2 whereas S63845 appeared to release a small amount of BIM from MCL-1. Despite this discrepancy, both of these inhibitors, individually, induced extensive apoptosis in these cells. This pattern was observed in several suspension and adherent cell lines from haematological and solid cancers. This suggested that BIM may not be the critical deciding factor in dictating whether the cells should undergo apoptosis, and in fact multiple BH3-only members may have to act together in making that critical decision.

Having raised questions about the importance of the BH3-only proteins in determining sensitivity of a cell to undergo apoptosis, the HCT116 8KO cells were utilised to further explore how BH3 mimetics induce apoptosis. The validity of the HCT116 8KO cells was confirmed in this study and it was demonstrated that concurrent knockdown of both MCL-1 and BCL-X_L in these cells could induce the same extent of apoptosis in the 8KO cells as in the wild-type cells, suggesting a dispensability of the BH3-only proteins in apoptosis, as originally reported[303].

It was next of interest to assess the apoptosis of the HCT116 8KO cells with respect to BH3 mimetics that induce rapid apoptosis (< 4 h compared to the ~72 h that is normally required to silence expression of proteins) and are proposed to displace pro-apoptotic proteins (which are missing in the 8KO cells) from their pro-survival counterparts to induce apoptosis. This was made possible by the recent introduction of S63845 as a *bona fide* MCL-1 inhibitor. Remarkably, despite the proposed mechanism of BH3 mimetic action, the cells that lack all the 8 major BH3-only proteins could still undergo BH3 mimetic-induced apoptosis, confirming previous studies which have implied that some, if not all, of the major BH3-only proteins, are dispensable for apoptosis induction induced by various stimuli[220,301,304]. Despite the on-going controversies of effector protein activation[219,220,301,304], this concept goes against the central dogma of apoptosis and the mechanism of BH3 mimetic action, where BH3-only proteins, in some way or another, promote MOMP by activating BAX/BAK.

One theory put forward to explain the dispensability of the BH3-only proteins in apoptosis was the possibility of a passive interaction of BAX with the mitochondrial membrane, which undergoes activation due to yet-to-be-characterised mechanism(s). As previously discussed, the interaction of BAX with the OMM is believed to be

prevented by the pro-survival proteins[217] and it is believed that BAX is being constantly cycled between the cytosol and OMM (translocation and retrotranslocation) by pro-survival proteins[319–322]. Thus, when the pro-survival proteins are suppressed, BAX may accumulate at the OMM and could somehow be activated by the OMM. Indeed, as previously alluded to, the lipid-rich mitochondrial membrane may have the capacity to activate BAX under certain conditions, as lipid detergents have previously been shown to be capable of activating BAX[300,323]. Therefore, perhaps as a result of re-organisation of BAX by the OMM and the fact that certain activation domains of BAX are no longer sequestered by anti-apoptotic proteins[86], BAX can indeed become activated and form pores in the absence of BH3-only proteins. Indeed, experiments in a further modified cell line of the HCT116 8KO cells supported this. Forced expression of BAX under control of a tetracycline-inducible system in the presence of Z-VAD-FMK in the 'allKO' cells (where all major members of the BCL-2 family including BAX and BAK were knocked out) resulted in BAX being largely mitochondrially-localised, suggesting spontaneous interaction of BAX with the OMM when neither pro- nor anti-apoptotic proteins are present[301].,

If BAX accumulation at the mitochondria following suppression of BCL-X_L and MCL-1 is the rate-limiting step in the induction of apoptosis, then it is reasonable to speculate that BAX would interact with BCL-X_L, and/or MCL-1 under control conditions and BH3 mimetics would disrupt this interaction to allow BAX to accumulate at the mitochondria. In support of this hypothesis, cytosolic and/or extra-mitochondrial pools of BCL-X_L and MCL-1 have been characterised both in this study and other reports[170,368–370]. However, in this study, BAX did not bind MCL-1 or BCL-X_L either under control conditions or following BH3 mimetics, thus casting doubts on potential interactions of BAX with the anti-apoptotic BCL-2 family members, at least

in this context. Having said that, it must be noted that these conclusions were drawn from immunoprecipitation studies, and the nature of BAX's interaction with any protein other than itself appears to be extremely dynamic. Therefore, it is possible that other strategies such as *in vitro* assessment of binding or, perhaps proximity labelling assays must be used to study these dynamic interactions in the future.

6.3 Novel BH3-only-like proteins could regulate BH3 mimetic-induced apoptosis in the absence of the major BH3-only proteins

An alternative conclusion to these results would be that perhaps another mechanism is in place which mediates the BAX-dependent but BH3-only protein independent BH3 mimetic-induced apoptosis in the HCT116 8KO cells. One such mechanism was proposed to be the existence of proteins with BH3-like, pro-apoptotic qualities which might activate BAX to facilitate apoptosis in the absence of the 8 major BH3-only proteins. Indeed, since the characterisation of the major BH3-only proteins (like BIM, PUMA, NOXA), more proteins have been identified with BH3-like motifs which can bind to different pro-survival proteins. For instance, SUFU can bind to MCL-1, BCL-2 and BCL-X_L via a BH3 motif and be displaced from the pro-survival proteins following BH3 mimetic exposure. However, SUFU has been shown to have no roles to play in BH3 mimetic-mediated apoptosis[324]. Thus, a scenario where novel BH3 motif-containing proteins, other than the 8 characterised BH3-only members, can bind to anti-apoptotic proteins and regulate BH3 mimetic-induced death is not unlikely.

Identification of a novel BH3-only-like protein is not straightforward however – partly owing to the fact that the BH3 motif is not a well-defined sequence[65] and partly due to the promiscuous binding patterns of the BCL-2 family. While the individual pro-survival proteins all perform the same function by promoting cell survival, they also

share binding partners. For instance, BIM can bind multiple pro-survival BCL-2 family members while NOXA specifically binds MCL-1 and BCL-2A1, as described in Chapter 1. Therefore, if a novel BH3-only-like protein that drives BH3 mimetic-mediated apoptosis exists, then it may bind more than one of the pro-survival BCL-2 family members. The data here, however, supported the notion of a novel BH3-only-like protein interacting specifically with MCL-1 in the HCT116 8KO cells, since apoptosis observed following the inhibition of BCL-X_L alone appeared to depend on the presence of one or more of the 8 BH3-only proteins. Strikingly, this requirement of the BH3-only proteins was mitigated when MCL-1 was simultaneously inhibited in these cells, suggesting that the two anti-apoptotic proteins may regulate death by different mechanisms, at least in HCT116 cells. It was therefore hypothesised that, while BCL-X_L mediates apoptosis through its typical BH3-only protein binding partners (BAD, BIM etc), MCL-1 might have novel interacting proteins ('Protein X') which, when displaced following BH3 mimetic exposure, might mediate the apoptosis seen in HCT116 8KO cells. The study therefore focused on novel MCL-1-interacting proteins and their function in BH3 mimetic-induced apoptosis.

6.4 MCL-1 – a key pro-survival protein with multi-faceted functions

Here, MCL-1 was found to exist in all the major cellular compartments with distinct localisations of its three major isoforms. For example, the IMM-specific 36 kDa isoform[175,183] of MCL-1 is not present in the cytosol but could clearly be detected in other subcellular compartments. This suggested that the three major isoforms of MCL-1 may each have their own unique expression patterns within the cell, further underscoring the many layers of the complicated biology of MCL-1. This study also demonstrated, for the first time, the existence of distinct molecular weight pools of MCL-1, termed MCL-1 complexes. This observation suggested that MCL-1 exists in

large pools with other proteins, which are perhaps linked to its diverse functions. Indeed, the existence of the complexes seemed to vary between major subcellular fractions, suggesting each MCL-1 complex might have distinct subcellular localisations. This supported the notion of novel MCL-1-interacting proteins and implied that MCL-1 might exist in multiple high molecular pools with a large number of other proteins. Analysis of MCL-1 interacting partners by mass spectrometry revealed that MCL-1 has a vast potential interaction network, especially in the mitochondria, where hundreds of purported interacting proteins were identified, even after stringent filtering. Pathway analysis of the peptides identified in the mitochondrial and whole cell lysate subgroups supported the idea of MCL-1 being an extremely multi-faceted protein. Not only were known MCL-1-interacting partners linked to both its pro-survival functions as well as its more diverse functions identified (VLCAD[183]), but many other proteins linked to cellular roles where MCL-1 has been implicated were identified, such as proteins linked to the ATP synthase subunits[175].

The striking numbers of proteins linked to key mitochondrial functions identified as potential interacting partners further supports the need for intensive exploration of MCL-1 outside of its pro-survival roles. It is very possible that MCL-1 has essential, non-pro-survival related functions in certain cell types which could be altered by inhibition of MCL-1 with potent inhibitors such as S63845. It has been proposed that non-apoptotic, mitochondrial functions of MCL-1 are important to maintenance of cardiac tissue in mice[276], as suppression of MCL-1 leads to severe mitochondrial abnormalities. Inhibition of MCL-1 could therefore, like BCL-X_L in platelets, have adverse effects on the healthy cells. While the S63845 pilot study found no toxic side effects of pharmacological inhibition of MCL-1 in mice[202], whether this translates to

humans over an extended period of treatment (potentially in combination with other therapies) remains unclear.

It could be suggested that, since several of the non-apoptotic mitochondria-associated roles of MCL-1 are attributed to the shortest MCL-1 isoform (also termed 'matrix MCL-1'), pharmacological inhibition of MCL-1 might not affect these functions, since BH3 mimetics target the protein-protein interactions of proteins bound by the BH3 motif. However, it has been shown that at least some of the novel interaction partners MCL-1 bind to MCL-1 *via* its BH3 domain, including VLCAD[175,183]. Thus, MCL-1 inhibitors could disrupt this interaction and negatively influence the mitochondrial matrix functions of MCL-1. Moreover, the fact that MCL-1 inhibitors could clearly bind to and stabilise all the major MCL-1 isoforms and also induce mitochondrial fragmentation clearly demonstrates the potential for activity of MCL-1 inhibitors that may lie outside the apoptotic spectrum. Thus, it is important to understand the entire complex biology of MCL-1, especially the non-apoptotic mitochondrial roles, before compounds like S63845 are used extensively in cancer therapy.

6.5 The DRP-1 and MCL-1 interaction in regulation of apoptosis

Mitochondrial dynamics, including fission and fusion, are closely related to apoptosis. DRP-1, the major mitochondrial fission regulator, has been controversially linked to apoptosis for many years. Early studies suggested that loss of DRP-1-mediated mitochondrial fission can protect against apoptosis[356]. Multiple studies supported this and suggested it occurred as a result of the loss of mitochondrial fission, leading to decreased OPA1 release and subsequently meaning cytochrome *c* is not efficiently released from the mitochondria into the cytosol as a result of altered cristae remodelling[357,371–373]. It was also highlighted that loss of DRP-1 simply delays but

does not block the onset of apoptosis. This suggested that mitochondrial fission and apoptosis were distinct and that the former was not a pre-requisite for the latter.

DRP-1 has also been associated with apoptosis at the level of BAX oligomerisation on mitochondrial membranes[348,359,360.] Mass spectrometry analysis of the mitochondrial pool of MCL-1 identified DRP-1 as a potential interacting partner, thus warranting further studies into this interaction and its physiological functions. [348,359,360] Initial exploration of DRP-1 as an MCL-1 interacting partner and Protein X candidate was promising. The MCL-1-DRP-1 interaction was validated (and was further validated by reports published during the production of this study[182,338]), while knockdown of DRP-1 in H1299 cells presented significant protection against BH3 mimetic-induced apoptosis. This suggested that DRP-1 could possibly be a factor required for BAX/BAK activation and, as such, a Protein X. This is in line with previous studies demonstrating the cytoprotective nature of DRP-1 deficiency[356,357,371,372]. However, whether this was a result of DRP-1 functioning like Protein X, with BH3-only protein-like qualities, remained unclear. Subsequent data showed that a BH3 mimetic-induced dissociation of the DRP-1/MCL-1 complex, can lead to the translocation of DRP-1 to very HMW fractions in H1299 cells. This supported the idea of BH3 mimetics being able to displace DRP-1 from MCL-1, potentially thereby activating BAX to result in apoptosis. This shift in molecular weight to an oligomeric complex could also be associated with the role of oligomeric DRP-1 in facilitating BAX translocation/oligomerisation at the mitochondria, however.

Surprisingly, in immunoprecipitation experiments where the MCL-1-DRP-1 interaction was interrogated directly, although a convincing interaction between MCL-1 and DRP-1 was observed, this was not disrupted by BH3 mimetics, suggesting that

the interaction was not dependent on the BH3 motif of MCL-1 or proposed BH3-like motif of DRP-1. This observation further suggested that DRP-1 could not be a candidate for Protein X as for DRP-1 to be Protein X it had to meet three major requirements: (1) It should interact with MCL-1, (2) it should be displaced from MCL-1 following exposure to S63845 indicating a BH3-motif dependency and (3) upon release, it should drive apoptosis in cells. While DRP-1 appeared to fit the first and third requirement, its inability to displace from MCL-1 following S63845 diminished its possibility of being a suitable candidate for Protein X.

However, if a Protein X scenario is to be considered, it could be suggested that DRP-1, MCL-1 and BAK/BAX are in a transient complex, which can be disrupted by addition of BH3 mimetics with two possible outcomes. When only MCL-1 is inhibited, the MCL-1-DRP-1-BAK/BAX complex is disrupted, thereby somehow allowing DRP-1 to form oligomers on the mitochondrial membrane, resulting in fission. Apoptosis is not seen following this fission however, as BAX/BAK can be attenuated by counterpart anti-apoptotic proteins, in this case, BCL-X_L. Thus, when both MCL-1 and BCL-X_L are inhibited, the effector proteins are no longer sequestered by anti-apoptotic proteins and DRP-1 is no longer restrained by MCL-1. This could mean DRP-1 facilitates oligomerisation of BAX/BAK which are then be free to form pores in the mitochondria, with downstream events being further facilitated by DRP-1. This hypothesis would require DRP-1 to directly activate BAX/BAK however, or, perhaps demarcate sites on the mitochondria where BAX/BAK accumulate, leading to autoactivation and oligomerisation. Extensive studies would have to be carried out to support this hypothesis, first starting by demonstrating an interaction between DRP-1 and the effector proteins and observing whether DRP-1 can indeed activate BAX rather than just simply provide sites for its translocation[348,360]

While the above scenario is not impossible, it is also reasonable to argue that DRP-1 simply modulates BH3-mimetic-induced apoptosis through its previously proposed mechanisms. In such a case, DRP-1 may be aiding the re-modelling of the cristae to tease out cytochrome *c*[357,371], thus when knocked down, apoptosis is attenuated. This may seem like a more enticing mechanism since studies have already demonstrated this, however it remains a controversial topic and does not account for the link between MCL-1 and DRP-1. Perhaps MCL-1 and DRP-1 associate and mediate cristae remodelling together, as previously implied[338].

Overall, DRP-1 is clearly important to BH3 mimetic-induced apoptosis in solid cancer cell lines but is unlikely to serve as our proposed Protein X. In further support of this, studies in the HCT116 WT and 8KO cells revealed even more complex behaviour of DRP-1 in apoptosis. In WT and 8KO cells, knockdown of DRP-1 induced significant cell death on its own, while further enhancing BH3 mimetic-induced apoptosis. Moreover, this death appeared to be at least partially BH3-only protein- and BAX-dependent, as BAX KO cells did not undergo as significant apoptosis following DRP-1 siRNA or BH3 mimetic exposure. It therefore appears that DRP-1 plays a critical role in maintaining viability of HCT116 cells. It is not clear why HCT116 cells undergo such extensive apoptosis following knockdown of DRP-1, however the induction of apoptosis as a result of loss of DRP-1 has been observed in multiple cell types in previous studies, suggesting the protective role of DRP-1 in apoptosis may be cell type-dependent[374,375]. It could be suggested that knockdown of DRP-1 leads to a hyperfilamentous mitochondria in the HCT116 cells which might alter their metabolism and lead to stress and in turn, BAX-dependent apoptosis. Indeed, previous studies have shown that knockdown of DRP-1 does indeed negatively alter oxidative phosphorylation, resulting in decreased oxygen consumption in complex II of the

electron transport chain[357]. This could be linked to the death seen in the HCT-116 cells. Remarkably, the apoptosis seen following knockdown of DRP-1 could also be associated with replication stress and genome instability. Lack of mitochondrial fission caused by knockdown of DRP-1 has been shown to influence replication stress and DNA damage during the cell cycle, as normal mitochondrial fission/fusion cycles are essential to genome stability in some cell lines[374].

Thus, it appears that the roles of DRP-1 in apoptosis cannot be defined by looking at specific cell types as it has distinctly opposite roles in two cell types studied here, further supported by previous literature. Future assessment of DRP-1 in cell death pathways should consider multiple types of cells and try to establish what causes the difference in response following loss of DRP-1.

6.6 Implications and future studies

Despite the complexities surrounding the BCL-2 family and BH3 mimetics, the entry of BH3 mimetics into the clinic for cancer treatment represents a huge milestone in the field. In particular, the development of S63845 and other MCL-1 inhibitors should greatly impact not only the BH3 mimetic field but also other chemotherapeutic approaches, since upregulation of MCL-1 is a key factor in drug resistance mechanisms in various therapeutic approaches in multiple cancers[172,203,204,209]. By exploring and understanding the BCL-2 family to the greatest extent, these therapeutic agents can be refined to a very high calibre and will almost certainly help improve the lives of cancer patients in the future.

While these compounds are likely to be excellent additions to the chemotherapeutic armamentarium, their mechanism of action has been challenged by this study, alongside various recently published articles, highlighting that the apoptotic signalling

pathway, particularly at the level of BCL-2 protein family interactions, is still not completely understood. Determining the full scale and dispensability of BCL-2 family interactions in apoptosis is of great importance. In particular, further developing models of 8KO cell lines to other cell types and to have additional knockouts of proteins of interest, is essential. Exploring the recently proposed mechanism of retro-translocation of BAX/BAK by the pro-survival proteins in the 8KO cells is also of great interest and could support the idea of suppression of MCL-1/BCL-X_L leading to diminished cycling of BAX/BAK, therefore allowing it to be activated by the OMM. Moreover, how the OMM might lead to activation of BAX/BAK without the known BH3-only proteins is an absolutely essential question to answer and will require some complex membrane studies. Establishing more intricate models of BAX/BAK activation will vastly improve our understanding of apoptosis and the dispensability of BH3-only proteins.

Finally, identifying other novel interacting partners of the BCL-2 protein family, should reveal more about the complex nature of the master regulators of apoptosis, particularly the increasingly complex biology of MCL-1 which has proven itself as not just a regulator of apoptosis but also many other cellular functions. This study has focused on just a few targets based on identified proteins having previous links to apoptosis, however with the multi-faceted nature of MCL-1, it is likely that there is a lot more to uncover. Understanding these functions is key to its targeting and may help establish MCL-1 as a prominent therapeutic target in the clinic.

6.7 Concluding remarks

The major findings of this study contribute multiple novel points to the field of apoptosis:

- S63845 is a highly potent and specific MCL-1 inhibitor which can be combined with other BH3 mimetics to induce rapid apoptosis in many types of cancer cell lines.
- The interactions of pro-survival proteins with distinct pro-apoptotic BH3-only proteins might not always dictate sensitivity of a cell line to specific BH3 mimetics.
- The eight key BH3-only proteins are dispensable for apoptosis following suppression of MCL-1 and BCL-X_L however BAX remains critical.
- BH3-only proteins are indispensable for induction of apoptosis induced by inhibition of BCL-X_L, however this can be overcome by further inhibition of MCL-1.
- MCL-1 exists in complexes of distinct molecular weights which may be linked to its various subcellular roles.
- The subcellular roles of MCL-1 may be even more extensive than what is currently known as it has a vast number of potential unidentified interacting partners.
- DRP-1 is one such novel interacting partner with critical roles to play in BH3 mimetic-induced apoptosis.

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Appendix

Supplementary Table 5.1 – The full list of candidate interacting proteins identified in the extranuclear lysate detected in mass spectrometry. Fold change, *p* values and *q* values are indicated for each protein, while known interacting partners of MCL-1 are shown in bold font.

Protein Identifier	Protein name	Fold change	p value	q value
CEP85	Centrosomal protein of 85 kDa	55.9	0.0002	0.0024
CARS	Cysteine--tRNA ligase, cytoplasmic	42.5	0.0002	0.0024
FBXO2	F-box only protein 2	32.2	0.0001	0.0024
KTN1	Kinectin	49.3	0.0005	0.0035
CEBPZ	CCAAT/enhancer-binding protein zeta	36.5	0.0006	0.0041
CPT1A	Carnitine O-palmitoyltransferase 1, liver isoform	24.7	0.0006	0.0041
SP100	Nuclear autoantigen Sp-100	253.6	0.0009	0.0042
BCL2L11	***Bcl-2-like protein 11***	***205.8***	***0.0011***	***0.0042***
NOMO2	Nodal modulator 2	174.9	0.0010	0.0042
ESF1	ESF1 homolog	60.2	0.0008	0.0042
SEC61B	Protein transport protein Sec61 subunit beta	13.5	0.0010	0.0042
RBM4B	RNA-binding protein 4B	98.5	0.0015	0.0045
AURKA	Aurora kinase A	70.5	0.0016	0.0045
FARSB	Phenylalanine--tRNA ligase beta subunit	26.9	0.0021	0.0045
DHCR7	7-dehydrocholesterol reductase	20.9	0.0017	0.0045
STT3B	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B	19.1	0.0021	0.0045
IDH2	Isocitrate dehydrogenase [NADP], mitochondrial	13.2	0.0014	0.0045
GALK1	Galactokinase	9.8	0.0013	0.0045
IPO4	Importin-4	49.9	0.0022	0.0046
SRPRB	Signal recognition particle receptor subunit beta	20.0	0.0023	0.0046
CPS1	Carbamoyl-phosphate synthase [ammonia], mitochondrial	14.4	0.0027	0.0046
TMED9	Transmembrane emp24 domain-containing protein 9	14.3	0.0024	0.0046
STT3A	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	12.6	0.0028	0.0046
UGDH	UDP-glucose 6-dehydrogenase	9.6	0.0028	0.0046
APOBEC3C	DNA dC->dU-editing enzyme APOBEC-3C	103.8	0.0044	0.0047
FAM171A2	Protein FAM171A2	48.3	0.0037	0.0047
BZW2	Basic leucine zipper and W2 domain-containing protein 2	30.9	0.0036	0.0047
ZMPSTE24	CAAX prenyl protease 1 homolog	25.7	0.0037	0.0047
FANCI	Fanconi anemia group I protein	24.1	0.0030	0.0047
NOC2L	Nucleolar complex protein 2 homolog	23.8	0.0041	0.0047
DHCR24	Delta(24)-sterol reductase	12.1	0.0038	0.0047
DNAJA3	DnaJ homolog subfamily A member 3, mitochondrial	11.2	0.0041	0.0047
ATP5H	ATP synthase subunit d, mitochondrial	10.8	0.0043	0.0047
FDFT1	Squalene synthase	78.9	0.0053	0.0049
RRP8	Ribosomal RNA-processing protein 8	1666.7	0.0064	0.0049
DDX24	ATP-dependent RNA helicase DDX24	113.2	0.0068	0.0049
CUL1	Cullin-1	67.4	0.0070	0.0049

HEXIM1	Protein HEXIM1	51.1	0.0065	0.0049
TRMT112	Multifunctional methyltransferase subunit TRM112-like protein	42.5	0.0067	0.0049
MCL1	***Induced myeloid leukaemia cell differentiation protein Mcl-1***	***33.9***	***0.0062***	***0.0049***
CSNK2A2	Casein kinase II subunit alpha'	28.4	0.0057	0.0049
TMEM33	Transmembrane protein 33	23.3	0.0060	0.0049
A2M	Alpha-2-macroglobulin	11.9	0.0058	0.0049
HLA-A	HLA class I histocompatibility antigen, A-68 alpha chain	10.7	0.0070	0.0049
BSG	Basigin	5.8	0.0061	0.0049
NCLN	Nicalin	22.6	0.0071	0.0050
EDC3	Enhancer of mRNA-decapping protein 3	13.0	0.0077	0.0050
EIF2B2	Translation initiation factor eIF-2B subunit beta	122.6	0.0089	0.0051
C7orf50	Uncharacterized protein C7orf50	117.7	0.0091	0.0051
SQLE	Squalene monooxygenase	99.2	0.0087	0.0051
AP2M1	AP-2 complex subunit mu	84.6	0.0092	0.0051
HLTF	Helicase-like transcription factor	69.9	0.0085	0.0051
UPF1	Regulator of nonsense transcripts 1	43.2	0.0091	0.0051
TJAP1	Tight junction-associated protein 1	39.2	0.0081	0.0051
SRP9	Signal recognition particle 9 kDa protein	32.5	0.0091	0.0051
PTRH2	Peptidyl-tRNA hydrolase 2, mitochondrial	27.4	0.0095	0.0051
CPNE3	Copine-3	26.1	0.0087	0.0051
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	24.9	0.0093	0.0051
VAPB	Vesicle-associated membrane protein-associated protein B/C	13.6	0.0085	0.0051
ATP5D	ATP synthase subunit delta, mitochondrial	11.0	0.0096	0.0051
RBM42	RNA-binding protein 42	7.9	0.0093	0.0051
VAPA	Vesicle-associated membrane protein-associated protein A	6.2	0.0093	0.0051
ATP5F1	ATP synthase F(0) complex subunit B1, mitochondrial	10.4	0.0099	0.0052
EIF2AK2	Interferon-induced, double-stranded RNA-activated protein kinase	373.1	0.0108	0.0053
DDX52	Probable ATP-dependent RNA helicase DDX52	248.8	0.0104	0.0053
CIAO1	Probable cytosolic iron-sulfur protein assembly protein CIAO1	30.9	0.0107	0.0053
LRRC47	Leucine-rich repeat-containing protein 47	15.2	0.0108	0.0053
PMAIP1	***Phorbol-12-myristate-13-acetate-induced protein 1***	***181.0***	***0.0114***	***0.0054***
SLC7A5	Large neutral amino acids transporter small subunit 1	5.1	0.0115	0.0054
LMAN1	Protein ERGIC-53	15.0	0.0118	0.0055
NDUFA4	Cytochrome c oxidase subunit NDUFA4	14.3	0.0119	0.0055
SEC11A	Signal peptidase complex catalytic subunit SEC11A	10.2	0.0117	0.0055
TMEM97	Transmembrane protein 97	7.8	0.0122	0.0056
NDUFS3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	15.0	0.0126	0.0056
TRIM25	E3 ubiquitin/ISG15 ligase TRIM25	183.6	0.0129	0.0056
NMT1	***Glycylpeptide N-tetradecanoyltransferase 1***	***170.1***	***0.0135***	***0.0056***
STAT3	***Signal transducer and activator of transcription 3***	***119.3***	***0.0136***	***0.0056***

RNH1	Ribonuclease inhibitor	15.1	0.0139	0.0057
PFKM	ATP-dependent 6-phosphofructokinase, muscle type	29.2	0.0145	0.0057
MYO1E	Unconventional myosin-Ie	97.5	0.0152	0.0058
ABCF2	ATP-binding cassette sub-family F member 2	83.1	0.0159	0.0058
SLC16A3	Monocarboxylate transporter 4	7.4	0.0155	0.0058
RBM33	RNA-binding protein 33	99.9	0.0163	0.0059
ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	5.3	0.0178	0.0061
TATDN3	Putative deoxyribonuclease TATDN3	332.4	0.0181	0.0061
RBM4	RNA-binding protein 4	139.5	0.0182	0.0061
POLR1C	DNA-directed RNA polymerases I and III subunit RPAC1	238.8	0.0184	0.0061
NOB1	RNA-binding protein NOB1	326.3	0.0192	0.0061
LARP4B	La-related protein 4B	52.2	0.0192	0.0061
TMED10	Transmembrane emp24 domain-containing protein 10	22.4	0.0189	0.0061
GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2	13.3	0.0190	0.0061
POLR2L	DNA-directed RNA polymerases I, II, and III subunit RPABC5	111.1	0.0195	0.0062
DNAJC9	DnaJ homolog subfamily C member 9	54.0	0.0195	0.0062
TRIP13	Pachytene checkpoint protein 2 homolog	43.1	0.0196	0.0062
MARK2	Serine/threonine-protein kinase MARK2	139.5	0.0199	0.0062
ZNF598	Zinc finger protein 598	2029.7	0.0202	0.0062
SPATS2L	SPATS2-like protein	114.7	0.0204	0.0062
LSS	Lanosterol synthase	16.3	0.0203	0.0062
NIP7	60S ribosome subunit biogenesis protein NIP7 homolog	518.3	0.0213	0.0063
DHX29	ATP-dependent RNA helicase DHX29	120.9	0.0217	0.0064
ANAPC5	Anaphase-promoting complex subunit 5	299.1	0.0219	0.0064
PRKCI	Protein kinase C iota type	20.2	0.0223	0.0064
DHX16	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16	204.2	0.0229	0.0065
DIS3	Exosome complex exonuclease RRP44	85.0	0.0227	0.0065
RRM1	Ribonucleoside-diphosphate reductase large subunit	9.7	0.0234	0.0065
ORC5	Origin recognition complex subunit 5	118.7	0.0241	0.0066
GPATCH4	G patch domain-containing protein 4	34.1	0.0242	0.0066
XRN1	5'-3' exoribonuclease 1	197.6	0.0248	0.0066
PCF11	Pre-mRNA cleavage complex 2 protein Pcf11	168.6	0.0245	0.0066
LARP4	La-related protein 4	135.8	0.0247	0.0066
PPFIA1	Liprin-alpha-1	124.8	0.0251	0.0066
S100A16	Protein S100-A16	116.7	0.0250	0.0066
PCYT1B	Choline-phosphate cytidylyltransferase B	33.8	0.0248	0.0066
IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	5.6	0.0250	0.0066
CIT	Citron Rho-interacting kinase	127.4	0.0257	0.0067
DCAF7	DDB1- and CUL4-associated factor 7	484.7	0.0262	0.0068
TM9SF2	Transmembrane 9 superfamily member 2	10.1	0.0268	0.0068
AGFG1	Arf-GAP domain and FG repeat-containing protein 1	274.7	0.0270	0.0068

MUC13	Mucin-13	20.7	0.0276	0.0068
YTHDF3	YTH domain-containing family protein 3	427.9	0.0279	0.0069
WDR43	WD repeat-containing protein 43	286.9	0.0283	0.0069
TRIM31	E3 ubiquitin-protein ligase TRIM31	221.0	0.0292	0.0070
ACSL1	Long-chain-fatty-acid--CoA ligase 1	15.2	0.0293	0.0070
TOPBP1	DNA topoisomerase 2-binding protein 1	80.3	0.0299	0.0070
DPF2	Zinc finger protein ubi-d4	185.2	0.0300	0.0070
TMA16	Translation machinery-associated protein 16	154.5	0.0302	0.0070
LSG1	Large subunit GTPase 1 homolog	181.7	0.0303	0.0070
PACSIN3	Protein kinase C and casein kinase substrate in neurons protein 3	47.3	0.0305	0.0071
TYMS	Thymidylate synthase	12.6	0.0306	0.0071
SEPT10	Septin-10	391.4	0.0314	0.0071
NARS	Asparagine--tRNA ligase, cytoplasmic	13.1	0.0313	0.0071
CTNND1	Catenin delta-1	114.3	0.0317	0.0071
PAK1IP1	p21-activated protein kinase-interacting protein 1	111.4	0.0317	0.0071
NOL10	Nucleolar protein 10	60.6	0.0316	0.0071
POLR2C	DNA-directed RNA polymerase II subunit RPB3	531.3	0.0319	0.0071
CMAS	N-acylneuraminate cytidylyltransferase	63.8	0.0325	0.0071
DTX3L	E3 ubiquitin-protein ligase DTX3L	166.1	0.0333	0.0072
KRI1	Protein KRI1 homolog	106.6	0.0333	0.0072
USP10	Ubiquitin carboxyl-terminal hydrolase 10	90.1	0.0338	0.0072
NTPCR	Cancer-related nucleoside-triphosphatase	74.1	0.0340	0.0072
B2M	Beta-2-microglobulin	13.0	0.0336	0.0072
TUBGCP3	Gamma-tubulin complex component 3	41.5	0.0349	0.0073
LTA4H	Leukotriene A-4 hydrolase	7.1	0.0353	0.0073
PDCD11	Protein RRP5 homolog	142.5	0.0357	0.0073
YES1	Tyrosine-protein kinase Yes	558.2	0.0366	0.0074
TCEB2	Transcription elongation factor B polypeptide 2	47.0	0.0366	0.0074
VPS16	Vacuolar protein sorting-associated protein 16 homolog	291.5	0.0369	0.0074
NBN	Nibrin	85.7	0.0371	0.0074
ZC3H15	Zinc finger CCCH domain-containing protein 15	27.4	0.0372	0.0074
CLINT1	Clathrin interactor 1	176.6	0.0376	0.0074
TBL1XR1	F-box-like/WD repeat-containing protein TBL1XR1	105.8	0.0378	0.0075
CD3EAP	DNA-directed RNA polymerase I subunit RPA34	606.0	0.0381	0.0075
ACADVL	***Very long-chain specific acyl-CoA dehydrogenase, mitochondrial***	***9.1***	***0.0383***	***0.0075***
ZNF143	Zinc finger protein 143	123.2	0.0387	0.0075
CUX1	Protein CASP	1007.5	0.0391	0.0076
PKP3	Plakophilin-3	47.9	0.0391	0.0076
ACOT9	Acyl-coenzyme A thioesterase 9, mitochondrial	8.8	0.0390	0.0076
LARP7	La-related protein 7	8.1	0.0391	0.0076
FLII	Protein flightless-1 homolog	60.8	0.0397	0.0076
ILK	Integrin-linked protein kinase	52.3	0.0397	0.0076
SHANK2	SH3 and multiple ankyrin repeat domains protein 2	219.1	0.0403	0.0076

PPIG	Peptidyl-prolyl cis-trans isomerase G	130.6	0.0401	0.0076
TRRAP	Transformation/transcription domain-associated protein	36.3	0.0400	0.0076
MCM8	DNA helicase MCM8	20.0	0.0403	0.0076
LIMCH1	LIM and calponin homology domains-containing protein 1	316.3	0.0412	0.0076
RBM22	Pre-mRNA-splicing factor RBM22	127.3	0.0413	0.0076
PYGB	Glycogen phosphorylase, brain form	28.1	0.0415	0.0076
PWP2	Periodic tryptophan protein 2 homolog	100.8	0.0419	0.0077
EIF1	Eukaryotic translation initiation factor 1	13.4	0.0424	0.0077
CDC42BPB	Serine/threonine-protein kinase MRCK beta	19.2	0.0427	0.0078
VIL1	Villin-1	142.9	0.0433	0.0078
EIF4G2	Eukaryotic translation initiation factor 4 gamma 2	20.8	0.0447	0.0079
MED4	Mediator of RNA polymerase II transcription subunit 4	4444.3	0.0448	0.0079
POLR1E	DNA-directed RNA polymerase I subunit RPA49	841.1	0.0449	0.0079
EIF2A	Eukaryotic translation initiation factor 2A	44.6	0.0455	0.0079
CTNNA1	Catenin alpha-1	9.4	0.0454	0.0079
CDK11A	Cyclin-dependent kinase 11A	34.2	0.0456	0.0079
PPP6R3	Serine/threonine-protein phosphatase 6 regulatory subunit 3	3052.0	0.0460	0.0079
VPS18	Vacuolar protein sorting-associated protein 18 homolog	324.9	0.0459	0.0079
CWF19L2	CWF19-like protein 2	66.3	0.0461	0.0079
COPE	Coatomer subunit epsilon	23.1	0.0460	0.0079
ASS1	Argininosuccinate synthase	7.6	0.0461	0.0079
TRIP6	Thyroid receptor-interacting protein 6	59.2	0.0463	0.0080
RRS1	Ribosome biogenesis regulatory protein homolog	103.3	0.0466	0.0080
POLR1B	DNA-directed RNA polymerase I subunit RPA2	164.0	0.0467	0.0080
GTF3C1	General transcription factor 3C polypeptide 1	48.6	0.0472	0.0080
RACGAP1	Rac GTPase-activating protein 1	265.2	0.0477	0.0081
KDM1A	Lysine-specific histone demethylase 1A	73.7	0.0476	0.0081
ALKBH5	RNA demethylase ALKBH5	165.5	0.0484	0.0081
KIAA1033	WASH complex subunit 7	341.1	0.0486	0.0081
AP2A1	AP-2 complex subunit alpha-1	122.9	0.0489	0.0081
ZFC3H1	Zinc finger C3H1 domain-containing protein	96.5	0.0487	0.0081
KIF2A	Kinesin-like protein KIF2A	20.8	0.0487	0.0081
LMAN2	Vesicular integral-membrane protein VIP36	18.7	0.0491	0.0082
DHX8	ATP-dependent RNA helicase DHX8	152.2	0.0496	0.0082
CIRH1A	Cirhin	65.6	0.049648	0.0082

Supplementary Table S5.2 – The full list of candidate MCL-1 interacting proteins identified in the nuclear fraction detected by mass spectrometry. Fold change, *p* values and *q* values are indicated for each protein.

Protein Identifier	Protein name	Fold change	p value	q value

MCL-1	***Induced myeloid leukemia cell differentiation protein Mcl-1***	***10.8***	***1.96E-05***	***0.0118***

Supplementary Table S5.3 – The full list of candidate MCL-1 interacting proteins identified in the cytosolic fraction detected by mass spectrometry. Fold change, *p* values and *q* values are indicated for each protein.

Protein Identifier	Protein name	Fold change	p value	q value
CARS	Cysteine--tRNA ligase, cytoplasmic	45.2	0.000115	0.0033
TFEB	Transcription factor EB	108	0.000172	0.00353
FBXO2	F-box only protein 2	57.8	0.0005	0.008
AURKA	Aurora kinase A	81	0.002	0.019
MCL-1	***Induced myeloid leukemia cell differentiation protein Mcl-1***	***34***	***0.0037***	***0.021***

Supplementary Table S5.4 – The full list of candidate MCL-1 interacting proteins identified in the mitochondrial fraction detected by mass spectrometry. Fold change, *p* values and *q* values are indicated for each protein.

Protein Identifier	Protein name	Fold change	p value	q value
ILVBL	Acetolactate synthase-like protein	264.9	2.32E-07	4.23E-05
FADS1	Fatty acid desaturase 1	48.7	4.28E-07	5.81E-05
RETSAT	All-trans-retinol 13,14-reductase	125.6	9.71E-07	8.14E-05
RDH11	Retinol dehydrogenase 11	98.9	1.17E-06	8.14E-05
PGRMC2	Membrane-associated progesterone receptor component 2	61.5	1.79E-06	8.14E-05
STT3B	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B	55.2	1.12E-06	8.14E-05
TMEM33	Transmembrane protein 33	51.4	1.42E-06	8.14E-05
ERGIC2	Endoplasmic reticulum-Golgi intermediate compartment protein 2	34.9	1.51E-06	8.14E-05
TRIM25	E3 ubiquitin/ISG15 ligase TRIM25	23.8	9.91E-07	8.14E-05
CAPG	Macrophage-capping protein	15.3	1.86E-06	8.14E-05
SNX3	Sorting nexin-3	14.4	1.47E-06	8.14E-05
STX7	Syntaxin-7	11.2	1.85E-06	8.14E-05
DNAJB12	DnaJ homolog subfamily B member 12	97.8	2.32E-06	8.89E-05
DAD1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	45.0	3.67E-06	9.97E-05
COPZ1	Coatomer subunit zeta-1	28.5	3.66E-06	9.97E-05
PAFAH1B1	Platelet-activating factor acetylhydrolase IB subunit alpha	12.4	4.54E-06	9.97E-05
ERGIC1	Endoplasmic reticulum-Golgi intermediate compartment protein 1	64.1	5.22E-06	0.0001021

SEC61A1	Protein transport protein Sec61 subunit alpha isoform 1	43.5	5.08E-06	0.0001021
FAM171A2	Protein FAM171A2	42.1	5.09E-06	0.0001021
CISD2	CDGSH iron-sulfur domain-containing protein 2	112.8	5.53E-06	0.0001062
SIGMAR1	Sigma non-opioid intracellular receptor 1	51.8	5.76E-06	0.0001067
HEXIM1	Protein HEXIM1	49.0	5.84E-06	0.0001067
ARL1	ADP-ribosylation factor-like protein 1	45.0	6.07E-06	0.0001067
NSF	Vesicle-fusing ATPase	52.2	6.83E-06	0.0001106
TRIP13	Pachytene checkpoint protein 2 homolog	15.8	6.66E-06	0.0001106
TMED5	Transmembrane emp24 domain-containing protein 5	72.9	8.85E-06	0.000121
ARF6	ADP-ribosylation factor 6	37.2	9.07E-06	0.000121
PYGL	Glycogen phosphorylase, liver form	34.6	8.16E-06	0.000121
CPS1	Carbamoyl-phosphate synthase [ammonia], mitochondrial	29.4	9.07E-06	0.000121
TMEM97	Transmembrane protein 97	22.0	8.72E-06	0.000121
VAPB	Vesicle-associated membrane protein-associated protein B/C	62.4	9.28E-06	0.0001212
ERLIN1	Erlin-1	68.5	9.48E-06	0.000122
TMEM109	Transmembrane protein 109	77.8	1.05E-05	0.0001226
TMED9	Transmembrane emp24 domain-containing protein 9	26.1	1.01E-05	0.0001226
VIMP	Selenoprotein S	85.8	1.11E-05	0.0001262
BRI3BP	BRI3-binding protein	92.2	1.21E-05	0.0001321
HMOX2	Heme oxygenase 2	87.1	1.22E-05	0.0001321
TFEB	Transcription factor EB	187.6	1.32E-05	0.000138
UBE2K	Ubiquitin-conjugating enzyme E2 K	11.3	1.31E-05	0.000138
HLA-A	HLA class I histocompatibility antigen, A-68 alpha chain	31.6	1.35E-05	0.0001389
TBL2	Transducin beta-like protein 2	112.0	1.36E-05	0.0001392
DHCR7	7-dehydrocholesterol reductase	115.0	1.42E-05	0.0001408
TMX1	Thioredoxin-related transmembrane protein 1	54.6	1.39E-05	0.0001408
EMC1	ER membrane protein complex subunit 1	53.9	1.47E-05	0.0001408
PPME1	Protein phosphatase methylesterase 1	36.5	1.44E-05	0.0001408
TMUB1	Transmembrane and ubiquitin-like domain-containing protein 1	114.1	1.58E-05	0.0001438
CHORDC1	Cysteine and histidine-rich domain-containing protein 1	48.1	1.69E-05	0.0001447
LGALS3BP	Galectin-3-binding protein	28.6	1.66E-05	0.0001447
UGT1A6	UDP-glucuronosyltransferase 1-6	24.1	1.79E-05	0.0001474
DNM1L	***Dynamin-1-like protein***	***55.6***	***1.82E-05***	***0.0001483***
ERMP1	Endoplasmic reticulum metallopeptidase 1	41.4	1.83E-05	0.0001485
RAB14	Ras-related protein Rab-14	85.6	1.92E-05	0.0001528
STT3A	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	72.9	1.96E-05	0.0001528
CTAGE5	cTAGE family member 5	56.1	1.92E-05	0.0001528
EI24	Etoposide-induced protein 2.4 homolog	216.9	2.47E-05	0.0001725
PTRH2	Peptidyl-tRNA hydrolase 2, mitochondrial	120.1	2.39E-05	0.0001725
ECEL1	Endothelin-converting enzyme-like 1	46.7	2.34E-05	0.0001725
RAC1	Ras-related C3 botulinum toxin substrate 1	16.5	2.51E-05	0.0001725

DDRGK1	DDRGK domain-containing protein 1	138.0	2.59E-05	0.0001759
RAB34	Ras-related protein Rab-34	23.0	2.59E-05	0.0001759
TMCO1	Transmembrane and coiled-coil domain-containing protein 1	136.2	2.63E-05	0.0001773
EMC7	ER membrane protein complex subunit 7	84.5	2.88E-05	0.0001912
CD55	Complement decay-accelerating factor	12.6	3.11E-05	0.0002012
ACBD5	Acyl-CoA-binding domain-containing protein 5	118.5	3.19E-05	0.0002016
CEP85	Centrosomal protein of 85 kDa	55.5	3.21E-05	0.0002016
HSD17B12	Very-long-chain 3-oxoacyl-CoA reductase	48.0	3.19E-05	0.0002016
DHCR24	Delta(24)-sterol reductase	71.7	3.35E-05	0.0002095
TMED4	Transmembrane emp24 domain-containing protein 4	45.3	3.69E-05	0.0002219
COPE	Coatomer subunit epsilon	56.4	3.77E-05	0.0002242
LMAN1	Protein ERGIC-53	34.3	3.77E-05	0.0002242
THEM6	Protein THEM6	22.6	3.81E-05	0.0002251
RAB3GAP1	Rab3 GTPase-activating protein catalytic subunit	8.3	4.04E-05	0.0002311
MGST1	Microsomal glutathione S-transferase 1	19.9	4.15E-05	0.0002357
TXNDC5	Thioredoxin domain-containing protein 5	59.6	4.37E-05	0.0002413
ITPK1	Inositol-tetrakisphosphate 1-kinase	35.7	5.01E-05	0.000263
VAPA	Vesicle-associated membrane protein-associated protein A	72.9	5.09E-05	0.0002649
SEC31A	Protein transport protein Sec31A	43.7	5.28E-05	0.0002698
TRMT112	Multifunctional methyltransferase subunit TRM112-like protein	30.4	5.30E-05	0.0002698
MIA3	Melanoma inhibitory activity protein 3	47.6	5.40E-05	0.0002707
ATP13A1	Manganese-transporting ATPase 13A1	128.5	6.16E-05	0.000297
DNM2	Dynamin-2	35.0	6.31E-05	0.0003012
FLVCR1	Feline leukemia virus subgroup C receptor-related protein 1	9.7	6.41E-05	0.0003038
PTPN1	Tyrosine-protein phosphatase non-receptor type 1	84.6	6.46E-05	0.0003045
MOSPD2	Motile sperm domain-containing protein 2	85.8	6.51E-05	0.0003053
CSRP1	Cysteine and glycine-rich protein 1	26.5	6.53E-05	0.0003053
PFKM	ATP-dependent 6-phosphofructokinase, muscle type	28.5	6.61E-05	0.0003077
COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	29.1	6.79E-05	0.0003083
LPP	Lipoma-preferred partner	18.5	6.73E-05	0.0003083
YKT6	Synaptobrevin homolog YKT6	69.8	6.87E-05	0.0003105
SGPL1	Sphingosine-1-phosphate lyase 1	207.4	7.05E-05	0.000316
VRK1	Serine/threonine-protein kinase VRK1	111.3	7.20E-05	0.0003178
RAB18	Ras-related protein Rab-18	41.8	7.19E-05	0.0003178
NUP62	Nuclear pore glycoprotein p62	122.0	7.45E-05	0.0003235
NUP98	Nuclear pore complex protein Nup98-Nup96	45.9	7.94E-05	0.0003355
ITPR1	Inositol 1,4,5-trisphosphate receptor type 1	42.5	7.79E-05	0.0003355
GFPT1	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	29.2	8.01E-05	0.0003371
ESYT2	Extended synaptotagmin-2	42.2	8.14E-05	0.00034
EHD1	EH domain-containing protein 1	37.4	8.31E-05	0.0003401
LPCAT3	Lysophospholipid acyltransferase 5	33.0	8.37E-05	0.0003401
RAB20	Ras-related protein Rab-20	29.3	8.35E-05	0.0003401

ALDH3A2	Fatty aldehyde dehydrogenase	31.5	8.81E-05	0.0003527
GLRX3	Glutaredoxin-3	24.4	9.14E-05	0.0003569
ABCD3	ATP-binding cassette sub-family D member 3	45.5	0.0001032	0.0003906
TES	Testin	26.1	0.0001071	0.0003943
UBE2L3	Ubiquitin-conjugating enzyme E2 L3	12.9	0.0001074	0.0003943
ASS1	Argininosuccinate synthase	67.3	0.0001124	0.0004093
ARL6IP5	PRA1 family protein 3	48.7	0.0001134	0.0004093
ATP1B3	Sodium/potassium-transporting ATPase subunit beta-3	20.7	0.0001141	0.0004093
TAP1	Antigen peptide transporter 1	134.9	0.0001198	0.0004231
TMED1	Transmembrane emp24 domain-containing protein 1	34.6	0.0001198	0.0004231
SRPR	Signal recognition particle receptor subunit alpha	24.9	0.0001199	0.0004231
LPCAT1	Lysophosphatidylcholine acyltransferase 1	15.4	0.0001225	0.0004254
ABCD1	ATP-binding cassette sub-family D member 1	41.2	0.0001266	0.000437
CLPTM1L	Cleft lip and palate transmembrane protein 1-like protein	5.7	0.0001276	0.0004378
ECI2	Enoyl-CoA delta isomerase 2, mitochondrial	56.9	0.0001351	0.000451
CDK5RAP3	CDK5 regulatory subunit-associated protein 3	52.4	0.0001372	0.0004512
SEC61B	Protein transport protein Sec61 subunit beta	37.0	0.0001367	0.0004512
FAF2	FAS-associated factor 2	208.1	0.0001394	0.0004546
MOGS	Mannosyl-oligosaccharide glucosidase	29.4	0.000141	0.0004553
CHD1L	Chromodomain-helicase-DNA-binding protein 1-like	40.2	0.0001463	0.0004673
TMEM161A	Transmembrane protein 161A	20.6	0.0001481	0.0004684
SPCS2	Signal peptidase complex subunit 2	76.8	0.0001501	0.0004704
VAMP8	Vesicle-associated membrane protein 8	7.0	0.0001511	0.0004722
PGRMC1	Membrane-associated progesterone receptor component 1	107.4	0.0001576	0.0004845
SEC24C	Protein transport protein Sec24C	5.1	0.0001635	0.0004974
RRM1	Ribonucleoside-diphosphate reductase large subunit	14.9	0.0001647	0.0004992
SACM1L	Phosphatidylinositide phosphatase SAC1	71.0	0.0001682	0.0005055
ZMPSTE24	CAAX prenyl protease 1 homolog	151.1	0.0001714	0.0005078
CYP51A1	Lanosterol 14-alpha demethylase	42.3	0.0001699	0.0005078
ESF1	ESF1 homolog	144.7	0.0001727	0.0005092
ALG3	Dol-P-Man:Man(5)GlcNAc(2)-PP-Dol alpha-1,3-mannosyltransferase	36.2	0.0001767	0.0005185
NUP210	Nuclear pore membrane glycoprotein 210	38.7	0.0001847	0.0005346
ASPH	Aspartyl/asparaginyl beta-hydroxylase	103.8	0.0001917	0.000549
KTN1	Kinectin	21.6	0.0001988	0.0005621
NCLN	Nicalin	26.5	0.0002002	0.0005637
RAB9A	Ras-related protein Rab-9A	7.1	0.0002031	0.0005698
SLC33A1	Acetyl-coenzyme A transporter 1	7.6	0.0002039	0.0005704
DTX3L	E3 ubiquitin-protein ligase DTX3L	38.9	0.0002114	0.0005841
NUP88	Nuclear pore complex protein Nup88	8.5	0.0002182	0.0005954
CERS2	Ceramide synthase 2	85.3	0.0002214	0.0006024
CLINT1	Clathrin interactor 1	14.1	0.0002227	0.0006032
LONP2	Lon protease homolog 2, peroxisomal	373.6	0.0002254	0.0006044

POR	NADPH--cytochrome P450 reductase	48.4	0.0002417	0.0006373
NCEH1	Neutral cholesterol ester hydrolase 1	30.2	0.0002439	0.0006413
GYS1	Glycogen [starch] synthase, muscle	45.0	0.0002503	0.0006552
RAB13	Ras-related protein Rab-13	22.1	0.0002634	0.0006786
SPTLC2	Serine palmitoyltransferase 2	36.5	0.0002705	0.000693
PIGS	GPI transamidase component PIG-S	34.9	0.0002768	0.0007052
HTATIP2	Oxidoreductase HTATIP2	20.1	0.0002899	0.0007249
LSG1	Large subunit GTPase 1 homolog	47.5	0.0003034	0.000751
SAR1B	GTP-binding protein SAR1b	20.6	0.0003047	0.0007525
MISP	Mitotic interactor and substrate of PLK1	17.8	0.0003136	0.0007692
TMX2	Thioredoxin-related transmembrane protein 2	88.9	0.0003161	0.0007736
ANXA7	Annexin A7	27.7	0.0003179	0.0007762
JAGN1	Protein jagunal homolog 1	22.6	0.0003225	0.0007841
TRIM26	Tripartite motif-containing protein 26	28.7	0.0003338	0.0008043
SURF4	Surfeit locus protein 4	89.5	0.0003509	0.000842
SCD	Acyl-CoA desaturase	14.0	0.0003518	0.0008421
AIFM2	Apoptosis-inducing factor 2	107.8	0.0003705	0.0008754
SEC63	Translocation protein SEC63 homolog	86.1	0.0003698	0.0008754
UMPS	Uridine 5'-monophosphate synthase	37.2	0.000369	0.0008754
NPC1	Niemann-Pick C1 protein	19.4	0.0003771	0.0008873
HMOX1	Heme oxygenase 1	107.2	0.0003899	0.0009134
MCL1	Induced myeloid leukemia cell differentiation protein Mcl-1	120.2	0.0003946	0.0009186
PREB	Prolactin regulatory element-binding protein	63.8	0.0003942	0.0009186
IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	68.5	0.0004247	0.0009762
PIGU	Phosphatidylinositol glycan anchor biosynthesis class U protein	52.1	0.0004243	0.0009762
TAP2	Antigen peptide transporter 2	18.6	0.000436	0.0009938
EFHD2	EF-hand domain-containing protein D2	15.7	0.0004448	0.0010088
MYOF	Myoferlin	15.1	0.000451	0.0010191
TMBIM6	Bax inhibitor 1	185.6	0.0004574	0.0010297
SAR1A	GTP-binding protein SAR1a	11.9	0.0004739	0.0010583
NFXL1	NF-X1-type zinc finger protein NFXL1	20.4	0.0004911	0.0010854
CHP1	Calcineurin B homologous protein 1	121.8	0.0005019	0.0011007
GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2	15.1	0.0005233	0.0011405
IDI1	Isopentenyl-diphosphate Delta-isomerase 1	45.3	0.000529	0.0011505
PHLDA2	Pleckstrin homology-like domain family A member 2	62.7	0.000567	0.0012139
PEX13	Peroxisomal membrane protein PEX13	46.0	0.0005706	0.0012169
PYGB	Glycogen phosphorylase, brain form	21.6	0.0005921	0.0012504
SORD	Sorbitol dehydrogenase	40.0	0.0005964	0.0012571
MBOAT7	Lysophospholipid acyltransferase 7	56.8	0.0006047	0.0012674
SRP54	Signal recognition particle 54 kDa protein	399.1	0.000616	0.0012813
TMED2	Transmembrane emp24 domain-containing protein 2	79.2	0.0006154	0.0012813
SLC27A2	Very long-chain acyl-CoA synthetase	27.4	0.0006141	0.0012813
GPAA1	Glycosylphosphatidylinositol anchor attachment 1 protein	29.0	0.0006257	0.0012989

SOAT1	Sterol O-acyltransferase 1	86.5	0.0006331	0.0013092
RHOG	Rho-related GTP-binding protein RhoG	26.1	0.0006327	0.0013092
MAGOHB	Protein mago nashi homolog 2	188.2	0.0006454	0.0013249
TTLL12	Tubulin--tyrosine ligase-like protein 12	56.2	0.0006467	0.0013249
A2M	Alpha-2-macroglobulin	18.8	0.0006458	0.0013249
SEC23A	Protein transport protein Sec23A	97.7	0.0006545	0.0013359
SPCS3	Signal peptidase complex subunit 3	188.1	0.0006634	0.0013441
SCFD1	Sec1 family domain-containing protein 1	48.5	0.0006711	0.0013546
FLOT2	Flotillin-2	60.9	0.0006748	0.001356
BZW2	Basic leucine zipper and W2 domain-containing protein 2	51.1	0.0006817	0.001356
EPHX1	Epoxide hydrolase 1	46.1	0.0006766	0.001356
FLOT1	Flotillin-1	29.1	0.0006807	0.001356
TMED10	Transmembrane emp24 domain-containing protein 10	45.0	0.0007109	0.0013987
FA2H	Fatty acid 2-hydroxylase	64.0	0.0007264	0.001423
TYMS	Thymidylate synthase	19.5	0.000732	0.0014274
MUC13	Mucin-13	12.7	0.0007354	0.0014302
UBTF	Nucleolar transcription factor 1	94.3	0.000771	0.0014825
ITGB5	Integrin beta-5	7.1	0.0007709	0.0014825
FYTTD1	UAP56-interacting factor	90.0	0.0007903	0.0015169
CDKAL1	Threonylcarbamoyladenosine tRNA methylthiotransferase	76.6	0.0008204	0.0015637
ANP32E	Acidic leucine-rich nuclear phosphoprotein 32 family member E	14.6	0.0008597	0.0016271
STX8	Syntaxin-8	157.3	0.0008711	0.0016362
IKBIP	Inhibitor of nuclear factor kappa-B kinase-interacting protein	16.3	0.0008719	0.0016362
TMEM259	Membralin	17.2	0.0009028	0.0016826
LYN	Tyrosine-protein kinase Lyn	41.5	0.0009181	0.0017023
AGPAT6	Glycerol-3-phosphate acyltransferase 4	48.8	0.0009291	0.0017169
RHEB	GTP-binding protein Rheb	26.4	0.0009277	0.0017169
SRPRB	Signal recognition particle receptor subunit beta	15.1	0.0009457	0.0017446
MLEC	Malectin	17.1	0.0009664	0.0017799
BZW1	Basic leucine zipper and W2 domain-containing protein 1	16.4	0.00098	0.0017958
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	7.4	0.0010452	0.0019026
EMC3	ER membrane protein complex subunit 3	34.8	0.0010502	0.0019085
CCDC47	Coiled-coil domain-containing protein 47	134.2	0.0010594	0.001922
STAT3	***Signal transducer and activator of transcription 3***	***29.3***	***0.0010921***	***0.0019587***
PEX14	Peroxisomal membrane protein PEX14	5.4	0.0011405	0.0020288
SEL1L	Protein sel-1 homolog 1	28.3	0.0011817	0.0020886
NDUFA4	Cytochrome c oxidase subunit NDUFA4	134.4	0.0012173	0.0021479
B2M	Beta-2-microglobulin	9.2	0.0012355	0.002166
UFL1	E3 UFM1-protein ligase 1	18.6	0.0012444	0.0021781
SEC11A	Signal peptidase complex catalytic subunit SEC11A	39.3	0.0012515	0.0021871
TMEM214	Transmembrane protein 214	49.9	0.001265	0.0022073
SPTLC1	Serine palmitoyltransferase 1	259.0	0.0012932	0.0022459

OS9	Protein OS-9	18.3	0.0014296	0.0024475
PON2	Serum paraoxonase/arylesterase 2	64.5	0.0014544	0.0024862
DPM1	Dolichol-phosphate mannosyltransferase subunit 1	31.4	0.0014854	0.0025312
SEC62	Translocation protein SEC62	11.3	0.0015641	0.0026446
SQLE	Squalene monooxygenase	70.5	0.0015788	0.0026614
BSG	Basigin	6.9	0.0015975	0.0026846
ATL3	Atlastin-3	14.6	0.0016061	0.0026908
PTGES	Prostaglandin E synthase	50.2	0.0016433	0.0027404
NOMO3	Nodal modulator 3	67.4	0.0017506	0.0028886
VMP1	Vacuole membrane protein 1	101.2	0.0018017	0.0029638
FDFT1	Squalene synthase	135.0	0.001854	0.0030347
ABCE1	ATP-binding cassette sub-family E member 1	6.3	0.0019505	0.0031564
MYO18A	Unconventional myosin-XVIIIa	133.3	0.0019966	0.0032176
TFE3	Transcription factor E3	15.6	0.0020141	0.0032403
IGF2R	Cation-independent mannose-6-phosphate receptor	7.3	0.0020648	0.0033073
RAB3GAP2	Rab3 GTPase-activating protein non-catalytic subunit	11.0	0.002149	0.003427
MAGT1	Magnesium transporter protein 1	13.5	0.0025592	0.0039713
OXSR1	Serine/threonine-protein kinase OSR1	91.8	0.0026101	0.0040388
PARP14	Poly [ADP-ribose] polymerase 14	7.8	0.0027466	0.004238
TAPBP	Tapasin	63.4	0.002787	0.0042883
LMF2	Lipase maturation factor 2	84.2	0.0028134	0.0043168
AKR1C3	Aldo-keto reductase family 1 member C3	23.1	0.0028829	0.0044111
CPT1A	Carnitine O-palmitoyltransferase 1, liver isoform	22.4	0.002969	0.0045112
EMC2	ER membrane protein complex subunit 2	14.9	0.0029744	0.0045131
NFKB2	Nuclear factor NF-kappa-B p100 subunit	10.4	0.0029925	0.0045343
GOLPH3	Golgi phosphoprotein 3	58.2	0.0030331	0.0045896
MYO1E	Unconventional myosin-Ie	6.2	0.0030482	0.004606
SSR1	Translocon-associated protein subunit alpha	24.7	0.0031407	0.0047327
PRDX5	Peroxiredoxin-5, mitochondrial	22.9	0.0034586	0.0051269
ACSL4	Long-chain-fatty-acid--CoA ligase 4	10.1	0.0035111	0.0051837
DYNC1LI1	Cytoplasmic dynein 1 light intermediate chain 1	56.6	0.003676	0.0053907
MAN2A1	Alpha-mannosidase 2	23.9	0.0036824	0.0053907
VPS35	Vacuolar protein sorting-associated protein 35	92.0	0.0036916	0.005392
CARS	Cysteine--tRNA ligase, cytoplasmic	37.9	0.0037891	0.0055196
ANXA11	Annexin A11	29.6	0.0042039	0.0060754
YES1	Tyrosine-protein kinase Yes	15.3	0.0043549	0.0062689
BID	***BH3-interacting domain death agonist***	***50.4***	***0.0045425***	***0.0064876***
ALPP	Alkaline phosphatase, placental type	19.8	0.0045737	0.0065236
IDH2	Isocitrate dehydrogenase [NADP], mitochondrial	54.3	0.0045974	0.0065489
METTL7A	Methyltransferase-like protein 7A	10.5	0.0047632	0.0067259
ZW10	Centromere/kinetochore protein zw10 homolog	18.7	0.0049653	0.0069553
HAT1	Histone acetyltransferase type B catalytic subunit	53.4	0.0050693	0.0070681
BCAP31	***B-cell receptor-associated protein 31***	***50.5***	***0.0051163***	***0.0071212***

PIGT	GPI transamidase component PIG-T	7.8	0.0051463	0.0071538
CLPTM1	Cleft lip and palate transmembrane protein 1	140.7	0.0052642	0.0072808
ATM	Serine-protein kinase ATM	14.3	0.0052966	0.0073163
ROCK2	Rho-associated protein kinase 2	39.2	0.0054535	0.0074858
NUP35	Nucleoporin NUP53	11.8	0.0058779	0.0079684
PEX16	Peroxisomal membrane protein PEX16	12.8	0.0064354	0.0086172
ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	10.4	0.0065049	0.0086891
LDLR	Low-density lipoprotein receptor	5.7	0.0069318	0.009192
MAP2K2	Dual specificity mitogen-activated protein kinase 2	9.2	0.0071417	0.0094361
TM9SF4	Transmembrane 9 superfamily member 4	1627.4	0.0074196	0.0097673
RNF114	E3 ubiquitin-protein ligase RNF114	19.4	0.0076873	0.0100718
LRRC47	Leucine-rich repeat-containing protein 47	5.4	0.0077208	0.0101036
CYB5R3	NADH-cytochrome b5 reductase 3	39.6	0.0077599	0.0101304
TM9SF2	Transmembrane 9 superfamily member 2	10.1	0.0084791	0.0109131
SLC38A2	Sodium-coupled neutral amino acid transporter 2	12.7	0.0089263	0.0114348
NBAS	Neuroblastoma-amplified sequence	7.9	0.0092029	0.0117479
AGPS	Alkyldihydroxyacetonephosphate synthase, peroxisomal	12.2	0.0096248	0.0122153
GCLM	Glutamate--cysteine ligase regulatory subunit	52.8	0.0100256	0.0126651
STX5	Syntaxin-5	10.0	0.0107782	0.0134912
PCYT1A	Choline-phosphate cytidylyltransferase A	127.1	0.0114963	0.014292
STIM1	Stromal interaction molecule 1	550.6	0.0120895	0.0149277
ARFGAP1	ADP-ribosylation factor GTPase-activating protein 1	66.9	0.0132804	0.0162697
FNDC3A	Fibronectin type-III domain-containing protein 3A	13.8	0.0137959	0.0168446
RAB5B	Ras-related protein Rab-5B	20.5	0.0140001	0.0170369
RINT1	RAD50-interacting protein 1	47.4	0.0140272	0.0170508
ABCB6	ATP-binding cassette sub-family B member 6, mitochondrial	18.8	0.0155045	0.0187012

Cell Death & Differentiation
https://doi.org/10.1038/s41418-018-0183-7

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ARTICLE

BH3-only proteins are dispensable for apoptosis induced by pharmacological inhibition of both MCL-1 and BCL-X_L

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Received: 14 May 2018 / Revised: 23 July 2018 / Accepted: 24 July 2018

Abstract

The impressive selectivity and efficacy of BH3 mimetics for treating cancer has largely been limited to BCL-2 dependent hematological malignancies. Most solid tumors depend on other anti-apoptotic proteins, including MCL-1, for survival. The recent description of S63845 as the first specific and potent MCL-1 inhibitor represents an important therapeutic advance, since MCL-1 is not targeted by the currently available BH3 mimetics, Navitoclax or Venetoclax, and is commonly associated with chemoresistance. In this study, we confirm a high binding affinity and selectivity of S63845 to induce apoptosis in MCL-1-dependent cancer cell lines. Furthermore, S63845 synergizes with other BH3 mimetics to induce apoptosis in cell lines derived from both hematological and solid tumors. Although the anti-apoptotic BCL-2 family members in these cell lines interact with a spectrum of pro-apoptotic BH3-only proteins to regulate apoptosis, these interactions alone do not explain the relative sensitivities of these cell lines to BH3 mimetic-induced apoptosis. These findings necessitated further investigation into the requirement of BH3-only proteins in BH3 mimetic-mediated apoptosis. Concurrent inhibition of BCL-X_L and MCL-1 by BH3 mimetics in colorectal HCT116 cells induced apoptosis in a BAX- but not BAK-dependent manner. Remarkably this apoptosis was independent of all known BH3-only proteins. Although BH3-only proteins were required for apoptosis induced as a result of BCL-X_L inhibition, this requirement was overcome when both BCL-X_L and MCL-1 were inhibited, implicating distinct mechanisms by which different anti-apoptotic BCL-2 family members may regulate apoptosis in cancer.

Introduction

Abnormal cell survival through resistance to apoptosis is a cardinal feature of most malignancies and plays a key role in chemoresistance. [1, 2] As the major regulators of the intrinsic mitochondrial pathway of apoptosis, the pro-survival BCL-2 family proteins (BCL-2, BCL-X_L, MCL-1, and BCL2A1) are attractive targets for novel cancer therapeutics. [3, 4] These proteins are proposed to function by binding and sequestering the pro-apoptotic BH3-only proteins, which in turn prevents the effector proteins, BAX and BAK, from forming pores in the mitochondrial membrane. The development of inhibitors of the pro-survival BCL-2 family proteins has proven particularly difficult because their inhibition requires disruption of these protein-protein interactions. However, after many years of research, small molecule inhibitors known as BH3 mimetics, which target the anti-apoptotic BCL-2 family have now been developed. The first clinically approved BH3 mimetic was ABT-263 (Navitoclax), an orally available form of

Edited by D. Vaux

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41418-018-0183-7) contains supplementary material, which is available to authorized users.

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ABT-737, which inhibits BCL-2, BCL-X_L, and BCL-w [3, 5–7] and potently induces apoptosis in several solid and hematological cancers. This was followed by the introduction of a BCL-2-specific inhibitor, ABT-199 (Venetoclax), which has received approval for treating refractory chronic lymphocytic leukemia (CLL), where BCL-2 addiction is a key feature in the pathogenesis of the disease. [8–10] Recently, BH3 mimetics specifically targeting BCL-X_L (A-1331852) [11–15] and MCL-1 (A-1210477 and S63845) [1, 12, 16] have also been developed.

In this study, we demonstrate that S63845 is a highly potent and selective MCL-1 inhibitor, which can synergize with other BH3 mimetics, including ABT-199 and A-1331852, to induce apoptosis in a wide range of cell lines derived from different hematological malignancies and solid tumors. Furthermore, we demonstrate that BH3-only proteins are required for apoptosis induced following BCL-X_L inhibition, whereas dual inhibition of BCL-X_L and MCL-1 resulted in a BH3-only protein-independent cell death.

Materials and methods

Cell culture

Cell lines derived from mantle cell lymphoma (MAVER-1), diffuse large B cell lymphoma (U-2946), chronic myeloid leukemia (K562), acute myeloid leukemia (MOLM-13, OCI-AML-3, HL-60, MV-4-11, THP-1 and U-937), Jurkat-T-lymphocyte cell lines, wild-type and deficient in caspase-9 (from Prof. J. Borst, The Netherlands Cancer Institute-Antoni van Leeuwenhoek, Amsterdam), caspase-8 and FADD (from Prof. J. Blenis, Weill Cornell Medicine, USA), non-small cell lung carcinoma (H1299), prostate cancer (PC-3, from Prof. Y. Ke, University of Liverpool, UK) and triple negative breast cancer (MDA-MB-231 from P. Meier, Institute of Cancer Research, London, UK) were cultured in RPMI 1640 medium. H929, a multiple myeloma cell line, was cultured in the same medium supplemented with 0.02% 2-mercaptoethanol. The non-small cell lung carcinoma cell line A549 was cultured in DMEM/F12 supplemented with 1% non-essential amino acids. The pancreatic cancer cell line, SUIT-2 (from A. Mielgo, University of Liverpool, UK) was cultured in DMEM. Colon cancer cell lines HT-29 (from J. Parsons, University of Liverpool, UK) and HCT116 (wild-type, DKO and 8KO) [3, 17–22] as well as HCT116 cells deficient in BAX or BAK (from R. J. Youle, National Institute of Health, USA), were cultured in McCoys 5 A Modified media. All cell lines, unless otherwise specified, were either from DMSZ (Braunshweig, Germany) or ATCC (Middlesex, UK) and were validated using short tandem repeat profiling. Peripheral blood samples from CLL patients were obtained with patient consent and local ethics committee approval and cultured as described. [23] All media were supplemented with 10% fetal calf serum (Life Technologies Inc., Paisley, UK).

Reagents

A-1331852 and A-1210477 from AbbVie Inc. (North Chicago, IL, USA), S63845 from ActiveBiochem (New Jersey, USA), and ABT-199 and Z-VAD.FMK from Selleck Chemicals Co. (Houston, TX, USA) were used. Antibodies against BCL-X_L, BIM and BAD from Cell Signalling Technology (MA, USA), NOXA from Millipore (Watford, UK), BID and tubulin from Abcam (Cambridge, UK), MCL-1, BCL-2 and PUMA from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and BAX and cytochrome *c* (BD BioSciences, California) were used for immunoblotting. Antibodies used for immunoprecipitation were MCL-1 (Y-37) and BCL-X_L from Abcam, BCL-2 from BD Biosciences and BAX (NT) from Merck-Millipore (Burlington, MA, USA). siRNAs specific to BCL-X_L (SI03025141), MCL-1 (SI02781205) and a non-targeting control (1027310) from Qiagen (Cambridge, UK) were incubated with Interferin siRNA transfection reagent (Polyplus transfection Inc., NY, USA) and added to cells at a concentration of 10 nM for 72 h.

Differential scanning fluorimetry assay

Thermal shift assays were performed using a StepOnePlus Real-Time PCR machine (Life Technologies, Paisley, UK) with Spyro-Orange dye (Invitrogen, Paisley, UK) and purified recombinant BCL-2 (Abcam) and MCL-1 protein, as previously described. [3]

Cytochrome *c* release, BAX translocation and apoptosis measurements

To quantitate cytochrome *c* release and BAX translocation, cells were grown on coverslips and treated with the specified inhibitors following a 0.5 h pre-treatment with the pan-caspase inhibitor Z-VAD.FMK (30 μM), and then fixed with 4% (v/v) paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100 in PBS, followed by incubation with primary and fluorophore-conjugated secondary antibodies prior to visualization under a fluorescent microscope. The extent of cytochrome *c* released from the mitochondria or BAX translocation to the mitochondria was quantified by counting at least 300 cells from three independent experiments. The extent of apoptosis in cells following different

treatments was quantified using an Attune NxT flow cytometer (Thermo Fisher Scientific, Paisley, UK), as previously described. [24]

Gel filtration, immunoprecipitation and Western blotting

Recombinant MCL-1 purification, size exclusion chromatography and immunoprecipitation experiments were carried out as previously described. [3, 25] Western blotting was carried out according to standard protocols. Briefly, 50 µg of total protein lysate was subjected to SDS-PAGE electrophoresis. Subsequently proteins were transferred to nitrocellulose membranes and protein bands visualized with ECL reagents (GE Healthcare).

Clonogenic studies

Cells were seeded in 6 well plates at a density of 2000 cells/well and exposed to the specified inhibitors (100 nM) 24 h post-seeding. Cells were fixed on day 7 using methanol and acetic acid and then stained with crystal violet and colonies counted using the GelCount tumor colony counter (Oxford Optronix, Abbingdon, UK).

Statistics

For time-course studies, a two-way ANOVA was performed using Dunnet's multiple comparisons analysis and other studies were analyzed for statistical significance with one-way ANOVA using Sidak's multiple comparisons analysis. The asterisks depicted correspond to the following p values: *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$.

Results

S63845 is a potent MCL-1 inhibitor and weak inducer of apoptosis in BCL-2- but not BCL-X_L-dependent cells

Consistent with previous findings, [1] we demonstrated that S63845 is a potent MCL-1 inhibitor when compared with A-1210477, as assessed by a concentration-dependent thermal stabilization of MCL-1 in our in vitro thermal shift assay [3] (Fig. 1a). S63845 also induced rapid apoptosis in two MCL-1-dependent cell lines (H929 and U-2946), [3, 6] with an IC_{50}~100 nM, demonstrating ~100-fold higher potency than A-1210477 (Fig. 1b). To assess the specificity of S63845 to

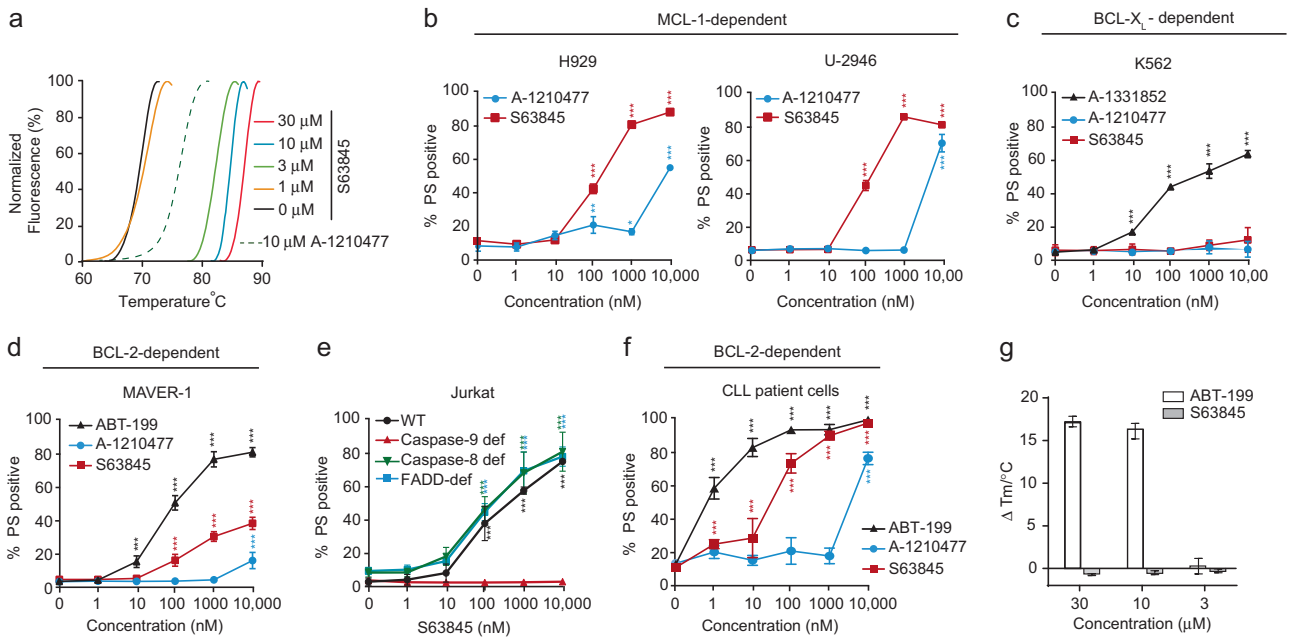

Fig. 1 S63845 binds MCL-1 and induces apoptosis in several cancer cell lines. **a** S63845 exhibits a concentration-dependent shift in MCL-1 stabilization in a thermal stability assay. **b** S63845 and A-1210477 exhibit concentration-dependent apoptosis, as assessed by PS externalization using flow cytometry, in MCL-1-dependent cell lines, H929 and U-2946 after 4 h of exposure. **c** S63845 and A-1210477 fail to induce apoptosis in a BCL-X_L-dependent cell line, K562, at 4 h, unlike A-1331852 (positive control). **d** MAVER-1, a BCL-2 dependent cell line, when exposed to the indicated BH3 mimetics for 4 h resulted in enhanced apoptosis. **e** Jurkat cells (wild type and deficient in the specified proteins), exposed to increasing concentrations of S63845 for 24 h exhibited caspase-9-dependent apoptosis. **f** Primary cells isolated from CLL patients, exposed to the indicated BH3 mimetics for 8 h, exhibited varying extents of apoptosis. **g** ABT-199 but not S63845 exhibits a concentration-dependent shift in BCL-2 stabilization in a thermal stability assay. Error bars = Mean ± SEM (standard error of the mean); *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$

induce apoptosis in a MCL-1 dependent manner, we exposed K562 cells (a BCL-X_L-dependent cell line) and MAVER-1 cells (a BCL-2-dependent cell line) to increasing concentrations of S63845 (Fig. 1c, d). As expected, S63845 (as well as A-1210477) failed to induce apoptosis in K562 cells, whereas extensive apoptosis was observed following exposure to A-1331852 (Fig. 1c). However, S63845 (as well as A-1210477) induced a concentration dependent increase in apoptosis in MAVER-1 cells, albeit at higher concentrations than ABT-199 (Fig. 1d), suggesting that S63845 could either induce death in a non-specific manner or that it could also be a weak inhibitor of BCL-2. To test this, we exposed Jurkat cells (wild type as well as deficient in FADD, caspase-8 and caspase-9) to increasing concentrations of S63845. While S63845 induced similar levels of apoptosis in wild type as well as FADD/ caspase-8 deficient Jurkat cells, cells deficient in caspase-9 were completely resistant to S63845-mediated apoptosis (Fig. 1e), thus demonstrating that S63845 both specifically induced the intrinsic pathway of apoptosis and also did not induce non-specific cell death. To identify whether S63845, in addition to binding and inhibiting MCL-1, can also potentially bind and inhibit BCL-2, we exposed primary CLL cells that exclusively depend on BCL-2 for survival, [8] to increasing concentrations of S63845 and A-1210477. While ABT-199 (positive control) induced apoptosis in these cells at low nanomolar concentrations, both S63845 and A-1210477 also induced significant apoptosis at concentrations similar to their IC_{50} values in MCL-1 dependent cells (Fig. 1f). However, we did not detect any concentration-dependent thermal stabilization of BCL-2 following S63845 in an in vitro thermal shift assay, which was in marked contrast to the thermal stabilization of BCL-2 following ABT-199 in this assay (Fig. 1g). Taken together, our data demonstrates that under these experimental conditions, S63845 does not bind BCL-2 and induces apoptosis in a MCL-1-specific manner.

S63845 is more potent than ABT-199 in inducing apoptosis in AML cell lines

Recent reports indicate that cells derived from acute myeloid leukemia (AML) patients can be effectively targeted with a BCL-2 and/or MCL-1 inhibitor, [11, 13–15] although other studies suggest that these cells depend primarily on BCL-2 for survival. [16] Indeed, MCL-1 inhibitors are currently entering clinical trials to treat AML and multiple myeloma patients (Clinical trials—NCT02979366; NCT02675452; NCT02992483). Therefore, we investigated whether S63845 alone or in combination with ABT-199 could induce apoptosis in a panel of AML cell lines. Of the six cell lines tested, MV-4-11 cells exhibited high sensitivity to both S63845 (IC_{50}~20 nM) and ABT-199 (IC_{50} ~ 50-100 nM) individually. THP-1 was much more resistant to ABT-199 but sensitive to S63845 (IC_{50}~50 nM). OCI-AML-3, U937 and MOLM-13 were completely resistant to ABT-199 but somewhat sensitive to S63845 at higher concentrations. In contrast, HL-60 cells were largely resistant to both BH3 mimetics individually (Fig. 2a). In the resistant cell lines, particularly U-937 and HL-60, BCL-2 and BCL-X_L appeared to perform redundant functions in cell survival, whereas MOLM-13 cells appeared to depend on BCL-X_L for survival, as exposure to A-1331852 alone resulted in significant apoptosis (Supplemental Fig. S1). Nevertheless, all AML cells examined were more sensitive to the combination of S63845 and ABT-199 (Fig. 2a).

Interactions between the pro and anti-apoptotic BCL-2 family members do not reflect the relative sensitivities of AML cell lines to undergo BH3 mimetic-mediated apoptosis

BH3 mimetic-induced apoptosis is usually attributed to displacement of BH3-only proteins from their anti-apoptotic partners, leading to apoptosis. Since the different AML cell lines exhibited varied responses to BH3 mimetic-mediated apoptosis, we wished to assess if this could be attributed to altered binding and/or displacement of the different BH3-only proteins to their known survival counterparts. Therefore, we immunoprecipitated the anti-apoptotic proteins required for survival and probed for each of the BH3-only proteins (Fig. 2b–d). In MV-4-11 cells, which are sensitive to both ABT-199 and S63845, BIM and PUMA bound to BCL-2 and were slightly displaced following exposure to ABT-199 (Fig. 2b). In contrast, the traces of BIM and NOXA bound to MCL-1 in these cells were readily displaced after S63845 exposure, in-line with the extent of apoptosis observed with S63845 (IC_{50}~20 nM). Neither BID nor BAD interacted with MCL-1 or BCL-2 in these cells (Fig. 2b). In OCI-AML-3, exposure to ABT-199 also resulted in a partial displacement of BIM and PUMA from BCL-2, despite the cells being largely resistant to ABT-199 (Fig. 2c). Similar to MV-4-11, BIM and NOXA bound to MCL-1 were readily displaced with S63845, and BID and BAD failed to interact with either MCL-1 or BCL-2 (Fig. 2c). In the resistant HL-60 cell line, BIM and PUMA bound to BCL-2 was not displaced following ABT-199, compatible with the cells being largely resistant to ABT-199 (Fig. 2d). BID and BAD were not bound to either MCL-1 or BCL-2. Moreover, of all the BH3-only members, only traces of NOXA appeared to interact with MCL-1 and was displaced following S63845 (Fig. 2d). Taken together, these results suggest that interactions between the pro- and anti-apoptotic BCL-2 family members may not necessarily reflect the relative sensitivities of different AML cell lines to undergo BH3 mimetic-mediated apoptosis.

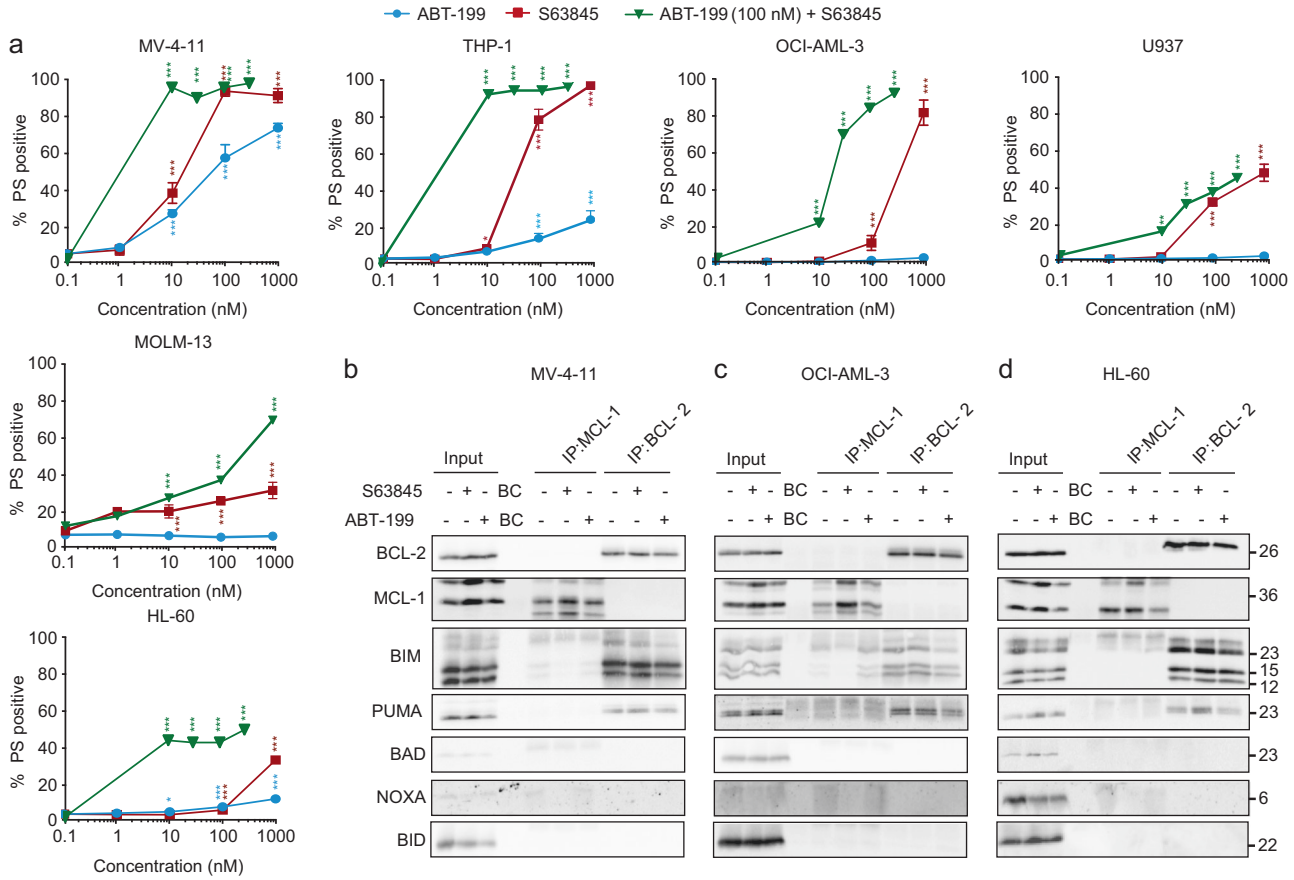

Fig. 2 S63845 can synergize with ABT-199 to induce apoptosis in AML cell lines. **a** AML cell lines were exposed to a concentration range of S63845 with ABT-199 at a constant concentration of 100 nM for 24 h and assessed for PS externalization. Immunoprecipitation of MCL-1 and BCL-2 was carried out in **b** MV-4-11, **c** OCI-AML-3, and **d** HL-60 cells, following exposure to S63845 (30 nM for MV-4-11 and 100 nM for the other 2 cell lines) or ABT-199 (50 nM for MV-4-11 and 100 nM for the other 2 cell lines) for 6 h (MV-4-11) or 8 h (OCI-AML-3 and HL60). The eluted complexes were immunoblotted for the indicated proteins. The input represents the cell lysates and BC, beads control. Error bars = Mean ± SEM; $*p \le 0.05$; $**p \le 0.01$; $***p \le 0.001$

Interactions of BCL-X_L and MCL-1 with different BH3-only members differ in solid tumor cell lines

Unlike several hematological malignancies, cancer cells derived from most solid tumors depend on both BCL-X_L and MCL-1 for survival. [3, 17, 19–22] Exposure of cells derived from a panel of solid tumors, including non-small cell lung carcinoma (H1299 and A549), pancreas (SUIT-2), colon (HCT116 and HT-29) and prostate cancer (PC-3) to a combination of S63845 or A-1331852 resulted in a marked induction of apoptosis, confirming that these cell lines depend on both BCL-X_L and MCL-1 for survival (Fig. 3a). To corroborate earlier findings in AML cell lines (Fig. 2) in non-hematological cells, we repeated immunoprecipitation studies and assessed interactions between the different BH3-only proteins with their key pro-survival counterparts, namely BCL-X_L and MCL-1. In H1299 cells, BIM and PUMA were preferentially bound to MCL-1 and BCL-X_L, respectively, and were readily displaced following exposure to the relevant BH3 mimetics (Fig. 3b). This was particularly evident following exposure to S63845, in which the newly released BIM from the MCL-1 complex exhibited enhanced binding to BCL-X_L. Similarly, exposure to A-1331852 displaced BIM from BCL-X_L, which in turn facilitated its binding to MCL-1 (Fig. 3b). Other BH3-only proteins like NOXA and BAD exhibited selectivity in binding to MCL-1 and BCL-X_L, respectively and were also displaced after exposure to the relevant BH3 mimetics. No detectable binding of BID to either BCL-X_L or MCL-1 was observed (Fig. 3b). In SUIT-2 cells, BIM was bound to both MCL-1 and BCL-X_L and was displaced to differing extents with the specific BH3 mimetics (Fig. 3c). In contrast to H1299, PUMA was not bound to either MCL-1 or BCL-X_L (Fig. 3c). However, there were some similarities, especially with NOXA being bound to MCL-1 and displaced with S63845, and BAD bound to BCL-X_L, which could be disrupted following A-1331852 (Fig. 3c). Therefore, H1299 and SUIT-2 cells exhibit significant differences in their BCL-2 family interaction

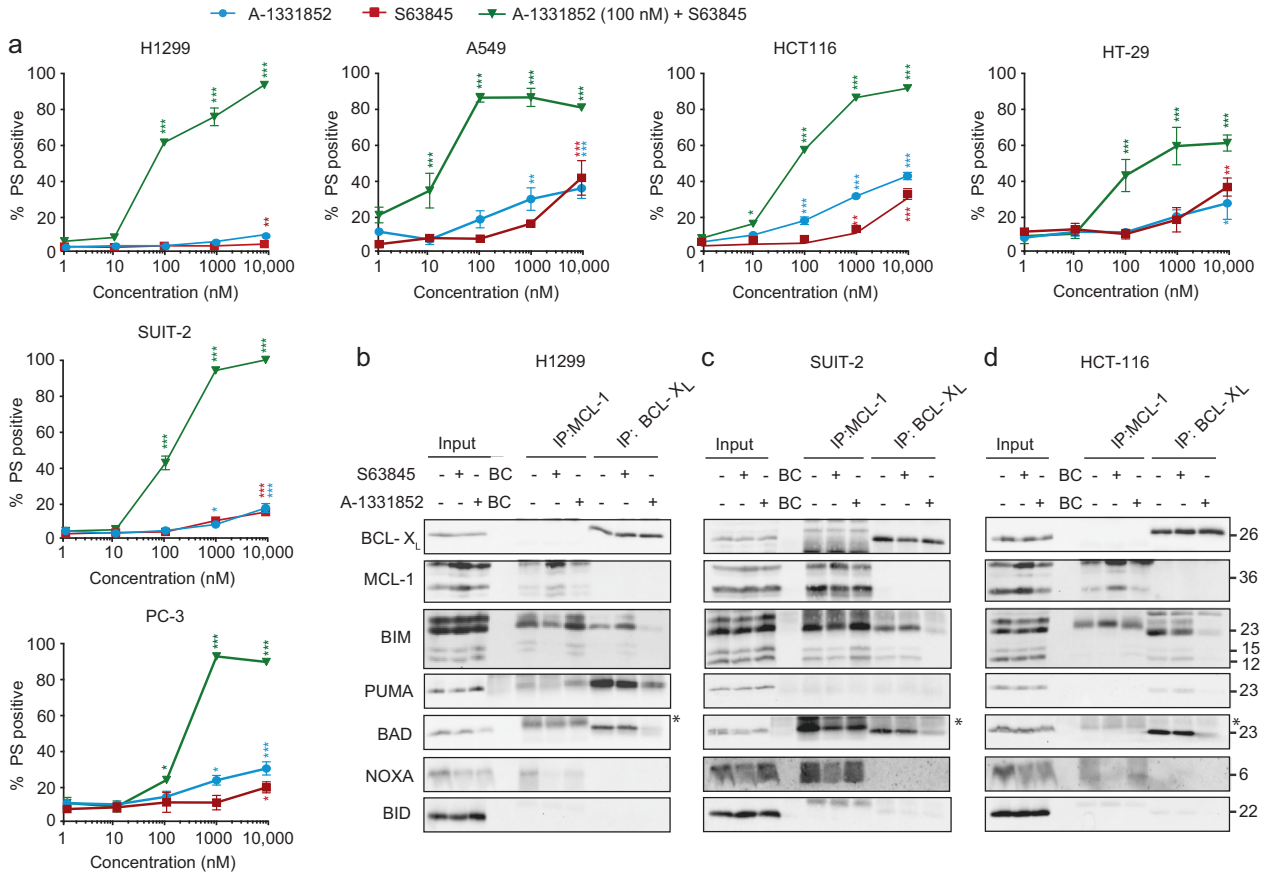

Fig. 3 BH3-only proteins have different binding preferences in various solid tumor cell lines despite similar sensitivities to BH3 mimetics. **a** Solid tumor cell lines were treated with a concentration range of S63845 and a constant concentration of 100 nM of A-1331852 for 4 h and assessed for PS externalization. Immunoprecipitation of MCL-1 and BCL-X_L was carried out in H1299 **b**, SUIT-2 **c**, and HCT116 **d** cells exposed to S63845 (100 nM) or A-1331852 (100 nM) for 2 h, and the eluted complexes were immunoblotted for the indicated proteins. * in the blots denotes non-specific bands. Error bars = Mean ± SEM (standard error of the mean); *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$

profile, despite similar expression levels of the different proteins and cell death responsiveness to the combination of A-1331852 and S63845. This was even more evident in HCT116 cells, in which most of the BH3-only proteins, with the exception of NOXA, interacted exclusively with BCL-X_L, and could be readily displaced from their binding partners with A-1331852 (Fig. 3d). Collectively, these results suggest that the preferential sequestration of different BH3-only proteins to distinct anti-apoptotic counterparts does not solely dictate dependency of a cell line on an individual anti-apoptotic protein.

BH3-only proteins are dispensable for BH3 mimetic-induced apoptosis in HCT116 cells

Since BH3-only proteins have recently been demonstrated to be dispensable for cell death, [18, 26] we evaluated whether selective inhibitors of BCL-X_L and MCL-1 could induce apoptosis in the absence of all known BH3-only proteins. Remarkably, in HCT116 8KO cells, which lack the 8 key BH3-only proteins, BIM, BID, PUMA, NOXA, HRK, BMF, BIK and BAD, [18] a combination of S63845 and A-1331852 induced apoptosis to the same extent as that of wild-type (WT) HCT116 cells (Fig. 4a). Furthermore, this combination of BH3 mimetics resulted in a BAX-dependent but BAK-independent apoptosis in these cells (Fig. 4b), emphasizing a crucial role for BAX in BH3 mimetic-mediated apoptosis. This raised the possibility of BAX directly interacting with BCL-X_L and/or MCL-1 to antagonize apoptosis. To test this hypothesis, we immunoprecipitated MCL-1 and BCL-X_L complexes from both HCT116 WT and 8KO cells but failed to detect BAX interaction with either anti-apoptotic protein (Fig. 4c). As a positive control, we immunoprecipitated active-BAX in these lysates, which pulled down BAX only following treatment with a combination of S63845 and A-1331852, indicative of BAX activation during apoptosis (Fig. 4c). These findings suggested that the BH3 mimetics could

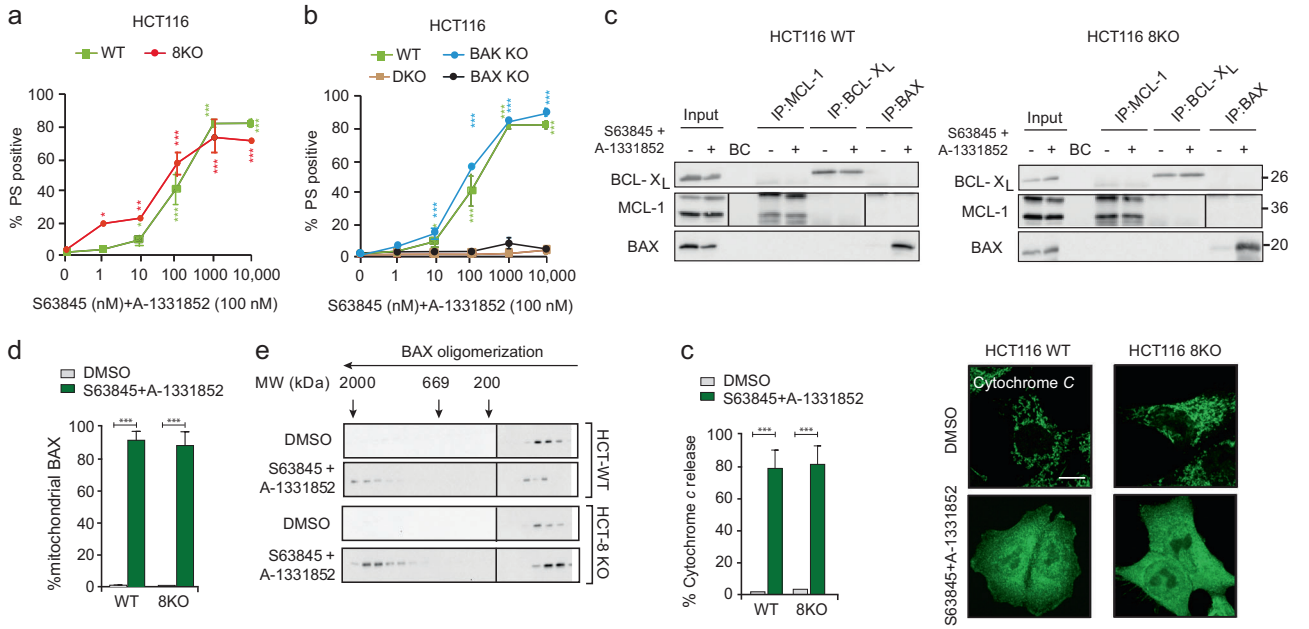

Fig. 4 BH3 mimetics induce apoptosis even in the absence of BH3-only proteins in HCT116 cells. **a** HCT116 cells, wild-type (WT) and deficient in 8 different pro-apoptotic BH3-only proteins (8KO), were exposed to A-1331852 (100 nM) in combination with increasing concentrations of S63845 for 4 h and assessed for PS externalization. **b** Same as (**a**) but the experiments were carried out with HCT116 WT cells or cells deficient in BAX, BAK or both (DKO). **c** Immunoprecipitation of MCL-1, BCL-X_L and active-BAX in the indicated cells exposed to a combination of S63845 (100 nM) and A-1331852 (100 nM), following a 0.5 h pre-treatment with z-VAD.FMK (30 µM), were performed to assess BAX interaction. **d** The level of mitochondrial BAX in WT and 8KO cells following S63845 and A-1331852 treatment (both 100 nM) for 4 h was assessed by immunocytochemistry using an anti-BAX antibody. **e** Western blots of different molecular weight fractions from FPLC showing BAX oligomerization in HCT116 WT and 8KO cells upon exposure to S63845 (100 nM) and A-1331852 (100 nM) for 2 h. **f** Quantification and representative images of cytochrome *c* release in the indicated cell lines, from three independent experiments, following exposure to S63845 (100 nM) and A-1331852 (100 nM) for 4 h in the indicated cells. Error bars = Mean ± SEM (standard error of the mean); $*p \le 0.05$; $**p \le 0.01$; $***p \le 0.001$

have displaced other protein(s) distinct from the 8 BH3-only proteins from BCL-X_L and/or MCL-1, which in turn activated BAX to facilitate mitochondrial outer membrane permeabilization (MOMP) and apoptosis. Apoptosis induction in the 8KO cells was accompanied by translocation of BAX from the cytosol to the mitochondrial membrane (Fig. 4d), BAX oligomerization into high molecular mass complexes as assessed by gel filtration (Fig. 4e), and MOMP, measured by the release of mitochondrial cytochrome *c* (Fig. 4f). Together, this negates a role for BH3-only proteins in several critical steps of the intrinsic apoptotic pathway induced by BH3 mimetics.

Inhibition of BCL-X_L and MCL-1 results in BH3-independent apoptosis

Although many cell lines derived from solid tumors depend on both BCL-X_L and MCL-1 for survival, our studies revealed that inhibition of BCL-X_L following exposure to A-1331852 was sufficient to induce significant apoptosis in a panel of cell lines (Fig. 5a). To assess whether A-1331852-mediated apoptosis also occurred in the absence of all known BH3-only proteins, we employed HCT116 WT and 8KO cells to assess several hallmarks of apoptosis following BCL-X_L inhibition. Strikingly, exposure to A-1331852, but not S63845, resulted in enhanced apoptosis, as assessed by phosphatidylserine (PS) externalization, MOMP and mitochondrial translocation of BAX in HCT116 WT but not 8KO cells (Fig. 5b–d) suggesting that A-1331852-mediated apoptosis requires the presence of BH3-only proteins. Support for this suggestion was also provided by the enhanced apoptosis observed in WT, but not 8KO, cells following siRNA knockdown of BCL-X_L (Fig. 5e). However, this protective effect in 8KO cells was abolished when both BCL-X_L and MCL-1 were downregulated, suggesting a requirement of BH3-only proteins in BCL-X_L but not when both BCL-X_L and MCL-1 were downregulated. Similarly, exposure to A-1331852 significantly reduced the surviving fraction of WT, but not 8KO, cell populations (Fig. 5f). As expected, S63845 had no effect on clonogenic survival in either of the cell types, while a combination of the two BH3 mimetics obliterated clonogenicity in both cell types (Fig. 5f). Taken together, our data convincingly argue against the requirement of

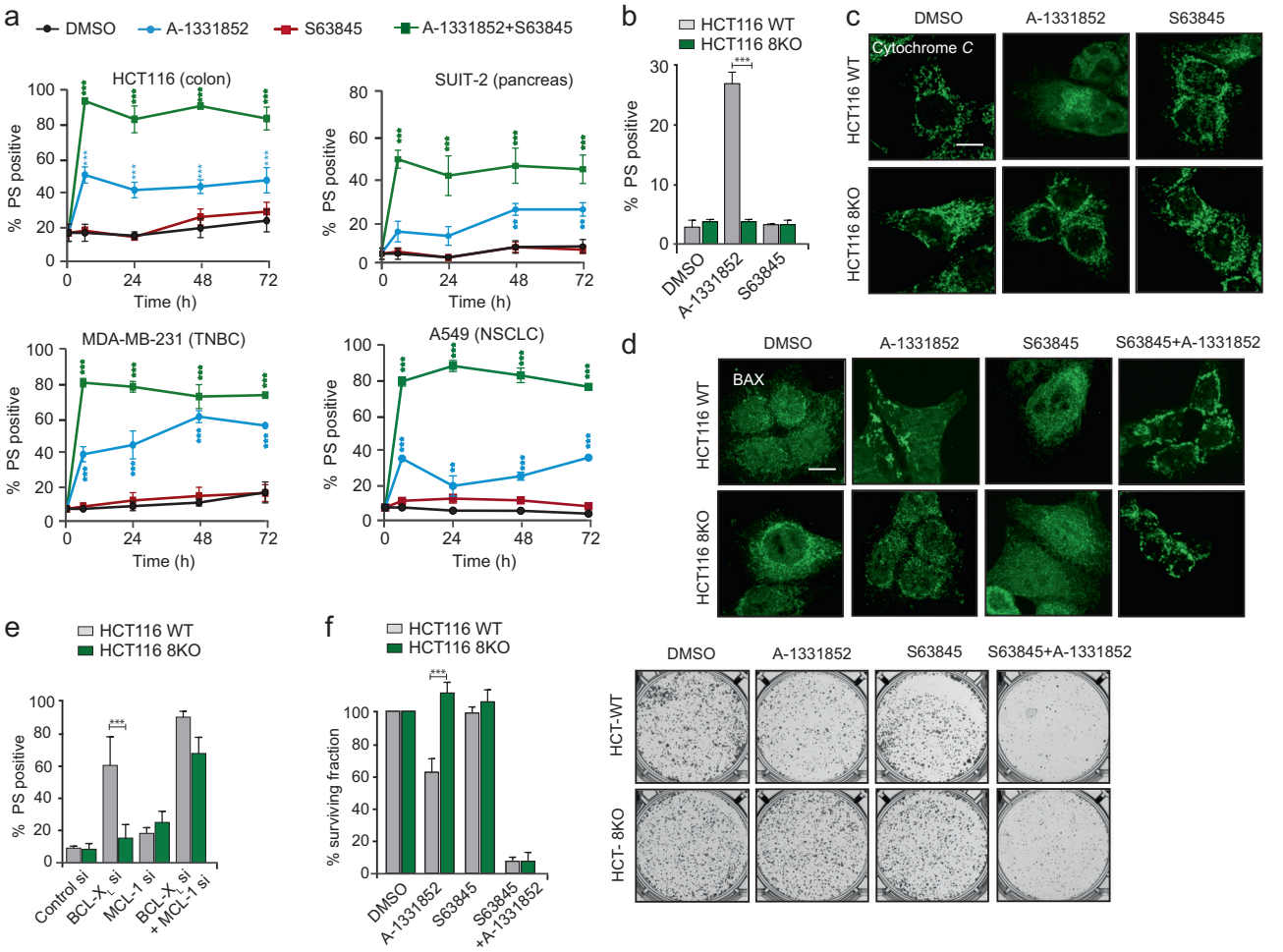

Fig. 5 Apoptosis regulated by BCL-X_L requires BH3-only proteins. **a** Apoptosis was assessed by PS externalization in the indicated cell lines following exposure to A-1331852 (100 nM) and/or S63845 (100 nM) for the indicated times. HCT116 WT and 8KO cells were exposed to S63845 or A-1331852 (100 nM) for 24 h and assessed for **b** PS externalization, **c** cytochrome *c* release and **d** BAX translocation. For (**c**) and (**d**), cells were pre-treated with z-VAD.FMK (30 μM) for 0.5 h before exposure to BH3 mimetics. HCT116 WT and 8KO cells were **e** transfected with siRNA against BCL-X_L and/or MCL-1 for 72 h and assessed for PS externalization or **f** exposed to A-1331852 (100 nM) and/or S63845 (100 nM) and the number of colonies formed after 7 d was counted and images taken using GelCount. Representative images from the clonogenic assay are shown in the right panel. Error bars = Mean ± SEM (standard error of the mean); *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001

BH3-only proteins in apoptosis regulated by a combination of both BCL-X_L and MCL-1 (Fig. 6).

Discussion

Prior to the discovery of S63845, putative MCL-1 inhibitors were poorly suited for clinical evaluation because they either lacked potency (high micromolar concentrations were required to inhibit MCL-1) or specificity resulting in undesirable toxicity. [3, 24, 27, 28] However, the development of S63845 potentially strengthens the use of a wide variety of BH3 mimetics in cancer therapy. In our hands, S63845 bound MCL-1 more extensively than A-1210477 and resulted in marked apoptosis in MCL-1-dependent cell lines (Fig. 1), and synergized with ABT-199 and A-1331852 to induce apoptosis in various cancer cell lines (Figs. 2 and 3), supporting the concept that a potent MCL-1 inhibitor, such as S63845, could be a valuable addition to the BH3 mimetic armamentarium.

Our finding that BH3-only proteins are dispensable for BH3 mimetic-mediated apoptosis in HCT116 cells is striking, particularly when the proposed mechanism of action of BH3 mimetics is taken into consideration (Fig. 4). Since apoptosis induction in these cells still required BAX and not BAK, [29] it is possible that BH3 mimetics could indirectly activate BAX by disrupting the interaction of BAX with BCL-X_L and/or MCL-1. However, we could not detect any BAX interaction with either of these anti-apoptotic proteins, despite efficient mitochondrial translocation and oligomerization of BAX, which resulted in MOMP and PS externalization (Fig. 4). Failure to detect BAX in complex with anti-apoptotic

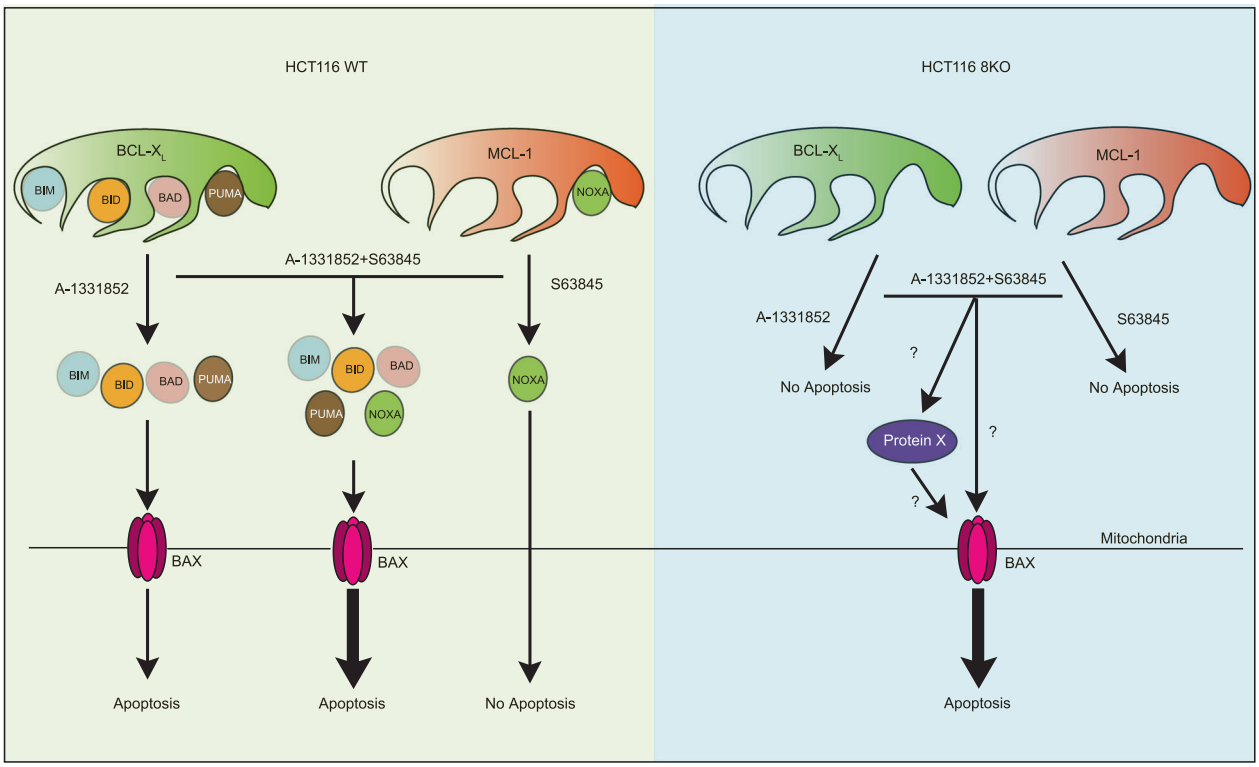

Fig. 6 BCL-2 family members differ in their dependence on pro-apoptotic BH3-only members to exert their anti-apoptotic functions. In HCT116 cells, multiple BH3-only proteins, such as BIM, BID, BAD and PUMA are sequestered by BCL-X_L, whereas NOXA is the only BH3-only protein bound to MCL-1. While the displacement of BH3-only proteins from BCL-X_L following A-1331852 is sufficient to induce apoptosis in these cells, displacement of NOXA from MCL-1 following S63845 failed to induce apoptosis. However, the combination of A-1331852 and S63845 released all the BH3-only proteins from the anti-apoptotic counterparts and resulted in pronounced apoptosis (bold arrow). In the absence of all eight BH3-only proteins, neither A-1331852 nor S63845 alone resulted in apoptosis. However, a combination of A-1331852 and S63845 still resulted in pronounced apoptosis, even in the absence of all BH3-only proteins. This could be due to the release of another pro-apoptotic BH3 domain-containing 'protein x' from MCL-1 and/or BCL-X_L, which in turn could activate BAX in the 8KO cells. Alternatively, BH3 mimetics could indirectly result in the accumulation of BAX (either by inhibition of retro-translocation or by passive diffusion, following the neutralization of the anti-apoptotic members) in the outer mitochondrial membrane, which in turn could result in BAX activation and apoptosis in the 8KO cells

proteins could possibly be due to the dynamic nature of this interaction. Although BAX is generally considered to translocate from the cytosol to the outer mitochondrial membrane following apoptotic stimuli, several reports suggest a role for the anti-apoptotic BCL-2 family members in the constitutive retrotranslocation of BAX from the mitochondrial outer membrane to the cytosol even under non-apoptotic conditions. [30–35] Neutralization of the anti-apoptotic BCL-2 family members with BH3 mimetics could markedly reduce BAX retrotranslocation to the cytosol, [33, 36] thus retaining it in the outer mitochondrial membrane, which may facilitate direct activation of BAX (Fig. 6). [18] This could explain the apoptosis observed following BH3 mimetics in HCT116 cells, even in the absence of all known BH3-only proteins (Fig. 4).

Our data reveal that the requirement for BH3-only proteins in BCL-X_L-regulated apoptosis can clearly be overcome when both MCL-1 and BCL-X_L are inhibited simultaneously (Figs. 4 and 5). Recent reports have demonstrated novel interacting partners of MCL-1, such as the BH3 domain-containing SUFU [37] and VLCAD, [38] which regulate distinct cellular functions. Therefore, other BH3-domain containing proteins distinct from the 8 key BH3-only members could interact with MCL-1 and/or BCL-X_L and play a role in these events (Fig. 6). In summary, our results demonstrate that BH3 mimetics induce apoptosis even in the absence of the eight best characterized BH3-only proteins, while also identifying differences in the regulation of cell survival by the different anti-apoptotic BCL-2 family members. This highlights the need to understand the

fundamental mechanisms of apoptosis in order to improve therapeutic approaches using BH3 mimetics.

Acknowledgements We thank AbbVie for providing A-1331852 and A-1210477. We thank Drs. Youle, Meier, Mielgo, Blenis and Borst for the different cells used in this study. This work was supported by North West Cancer Research Grants CR1040 and CR1127 (to SV and GMC), Science Without Borders, CNPq 233624/2014-7, Ministry of Education, Brazil (to MM) and NIH Grants R03CA205496 and R01GM118437 (to XL).

Funding This work was supported by North West Cancer Research Grant CR1040, CR1127 (to SV and GMC), Science Without Borders, CNPq 233624/2014-7, Ministry of Education, Brazil (to MM) and NIH Grants R03CA205496 and R01GM118437 (to XL).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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