



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.



**Rational Drug Design and Biochemical analysis in the
Development of Targeted Therapies against
Malignant Pleural Mesothelioma**

**Thesis submitted to the University of Dublin for the
Degree of Doctor of Philosophy**

Trevor T. Price B.A. (Mod)

Supervisor Prof. DC Williams

School of Biochemistry and Immunology,
Trinity College Dublin,
Ireland

January 2012



Thesis 10436

DECLARATION

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Signed:



[Faint signature]

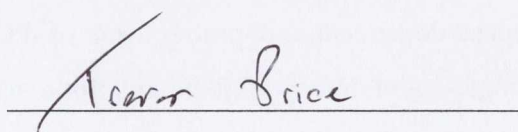
Thomas R. Fildes

Declaration

This thesis has not been submitted as an exercise for a degree at this, or any other University.

The work herein is entirely my own with the exception of;

- Figure 4.1.C: SMAC mimetic pharmacophore design, created by Dr. Darren Fayne (Molecular Design Group, School of Biochemistry and Immunology, Trinity College Dublin)
- Figure 5.14: CLL analysis using MDG 44, experiment performed by Dr. Sandra Bright (Williams Lab, School of Biochemistry and Immunology, Trinity College Dublin)
- Figure 6.3: MDG 44 TR-FRET PPAR- γ competitive binding assay performed by Daniel Nevin (Molecular Design Group, School of Biochemistry and Immunology, Trinity College Dublin)

A handwritten signature in black ink, reading "Trevor Price", is written over a horizontal line.

Trevor T. Price

Abstract

Malignant Pleural Mesothelioma (MPM) is a fatal neoplasm arising from cells of the mesothelial lining of the pleural cavity and associated with prolonged exposure to asbestos. This highly resistant malignancy is largely unresponsive to conventional chemotherapy and while promising novel treatment strategies are under investigation, to date no truly effective clinical solutions have been discovered. In this study, a rational drug design approach was undertaken to develop and biochemically evaluate novel targeted agents for the treatment of MPM.

The primary development strategy investigated in this study proposed the targeting of the Inhibitor of Apoptosis protein (IAP) family, which has been clinically associated with poor prognosis and chemotherapeutic resistance in MPM. An *in vitro* MPM model was used to validate the proposal that IAP overexpression communicated apoptotic resistance in MPM and this mechanism was then challenged using conventional IAP inhibitor therapies (SMAC mimetics) in combination with the chemotherapeutic, Cisplatin. Overexpression of IAPs in MPM cells was shown to associate with apoptotic resistance and this effect could be alleviated through the use of SMAC mimetic therapy. Specific targeting of X-linked IAP (XIAP) was shown to synergistically enhance Cisplatin induced apoptosis in XIAP expressing MPM cells. Computational design, modelled on the SMAC-AVPI / XIAP-BIR3 binding interaction, was used to identify a series of novel, computationally-predicted SMAC mimetic compounds. Biochemical validation refuted the predicted "On Target" affinity of these compounds for XIAP and a secondary putative target, cIAP-1/2. However, a lead compound (MDG 44) with potent synergistic apoptotic activity in combination with Cisplatin was identified.

Biochemical evaluation of the cellular activities and molecular target of this compound were performed. Programmed cell death (PCD) analysis revealed MDG 44 specifically induced a caspase-dependent, apoptotic form of PCD through the intrinsic apoptotic pathway. Both single-agent and synergistic apoptotic activity was quantified. At sub lethal concentrations (15 - 30 μ M), MDG 44 was observed to synergistically enhance the apoptotic activity of Cisplatin. Numerous putative molecular targets for MDG 44 were evaluated. Targets were proposed via identification of compounds with similar structure to the 2-indolinone basic scaffold of MDG 44 and with known cellular target; or evaluation of the cellular effects induced by MDG 44 and proposal of rational putative targets for

investigation. The observed formation of large cytoplasmic vesicles in MPM with MDG 44 treatment prompted the evaluation of autophagic flux and autophagosome formation in response to MDG 44. The complementary protein degradation program, the ubiquitin-proteasome system (UPS) was also evaluated as a target for MDG 44. Formation of autophagosomes was detected by increased expression of the autophagosome marker LC3-II and confocal detection of LC3 positive structures in LC3-GFP stably transfected macrophages. Analysis of the UPS revealed MDG 44 induced dose-dependent inhibition of NF κ B activation via inhibiting proteasomal degradation of I κ B. MDG 44 had no effect on accumulation of poly-ubiquitinated proteins, suggesting the ubiquitin ligase pathway, as opposed to the 26S proteasome, as a potential target for MDG 44 for future biochemical evaluation.

As a continuation of the drug discovery program, second generation analogues of MDG 44 were designed and tested in order to identify more potent analogues of this class of compound. A number of analogues with increased cytotoxic activities were observed, but marginal increases in single-agent and synergistic apoptotic activities were detected even in the most potent analogues. The analogue screen however did provide a large amount of structural-activity-relationship (SAR) data, informing the future design of successive generations of MDG 44 analogues.

A second drug development project investigated the “Warburg effect” and the displacement of mitochondrial-associated Hexokinase as a target in MPM. Potentially novel Mito-HK dissociating compounds were evaluated for anti-proliferative and apoptotic activities in Burkitt’s Lymphoma and MPM cell lines. Compounds were shown to induce potent and potentially selective apoptotic activities; however “On Target” evaluation of these classes of compounds is still under investigation.

In conclusion, this study reports on the promising therapeutic strategy of IAP targeting / SMAC mimetic therapy in the treatment of MPM. Overexpression of IAPs was shown to mediate apoptotic resistance. This could be relieved with SMAC mimetic therapy and synergistically enhance apoptotic induction in combination with Cisplatin. We also report on the identification of a novel compound under continuing target evaluation, which is a potent single-agent apoptotic and a synergistic partner with the chemotherapeutic Cisplatin in MPM. The capacity to synergise with Cisplatin is a promising drug

development strategy as Cisplatin remains today the primary combinational treatment option for MPM in the clinic.

Acknowledgements

There are many, many people to thank for all the help and support over the last three plus years. First and foremost thanks must go to Prof. Clive Williams for all the support and guidance given, for taking a chance on a rag-tag Immunologist to work in your biotech and then keeping him on for a PhD. It really has been a pleasure working in the lab. To the rest of the DCW crew past and present many, many thanks are warranted. To Suzanne, for showing me the ropes when I was a green research assistant back in the day, barely able to detect a repeatable, significant difference between my arse and my elbow. To the lab captain herself, Sandra Bright, there I admit it, you win, but you sure have been fun to argue and laugh with the last year. Big thanks also to the other current and prospective PhD students in the lab, Luisa, Kim and Anna it was a pleasure to come in and work with all you guys every morning (...afternoon, I suppose). Thanks finally to Nick Jagoe, you beautiful bastard! The ad hoc member of the DCW lab. Those were some times back in Codex, testing compounds all week, but always good for few pints and smokes in Kennedys on a Friday (Smokes is a science term mam, don't worry about it....).

Many thanks to all in MDG for their help throughout and in particular Darren, Georgio, Andy and Tim for their computational work in the design and analysis of the compounds tested in this thesis and the many others we have worked on throughout. Special thanks go to Dr. David Lloyd for his guidance on the computational side of the project and for his considerate time and effort in building a multi-million euro biomedical sciences institute with my own massive office in which to write up this thesis. That was an absolute godsend and very much appreciated.

Thanks must go to the lads, obviously – Chris, Dan, Nial, Fernando and McKay. There is not many of us around the department, but certainly a few good ones. To the honorary lads – Laaauuuura, Kate and Sinead, it's been a pleasure. Special thanks to Sinead who was always good for some advice and sometimes even a little antibody and reagent which were really vital in completing some of this work. Thanks also to all the new members of Mega-Lab and the rest of the 6th floor of the TBSI, the department as a whole, Alex Ferguson and the family back home – The aulde one and the old man, Kim, Dave and Jay. It's been a tough and long process, but through three whole years pipetted away in the lab, four extra months cramming in every experiment under the sun and the last few weeks

of restless nights and relentless writing, I'm proud to say, thanks to all those above, not a single day ever felt like work.

P.s: Some jokesters I know quite well (two eyes looking at you Clive) may question the last statement as not an appreciate gesture to friends and colleagues but rather that arriving into the lab at quarter past eleven some mornings does not exactly a day's work make! While admittedly a fair point, let your attention be drawn to the words and figures on pages 1 through 192. Vinny your full of the same kind of sass, have a flick through some of those pages too!.....and Thanks again!

Dedicated to:

The aulde one and the old man:

Colette Elizabeth Mary & James Edward Jude Price

I'm sure you probably hate this bloody thesis, it being the main preoccupation of thoughts and efforts and concerns on both ends and the reason I rarely made it out of the lab and home these last three years, but of anyone who deserves to be honorary co-authors of this beast it's you guys. This really would be little more than leather-bound sound and fury without the unseeing help from home. Over the years there has been many a question asked about labs and experiments and Kingston (I keep telling you its Clive but....), the one most often ventured is about letters and dots after names and a name on a book in the Trinity library. Well I can't really do anything about letters or dots, but names.....Consider your own on the shelf.

Table of Contents

<i>Declaration</i>	<i>i</i>
<i>Abstract</i>	<i>ii</i>
<i>Acknowledgements</i>	<i>v</i>
<i>Dedication</i>	<i>vii</i>
<i>Table of contents</i>	<i>ix</i>
<i>Abbreviations</i>	<i>xvii</i>

Chapter 1: General Introduction

Rational drug design and biochemical analysis in the development of targeted therapies against Malignant Pleural Mesothelioma

1.1	Cancer	1
1.2	Malignant Pleural Mesothelioma	2
1.3	Programmed cell death	5
1.4	Autophagy	5
1.5	Apoptosis	8
1.6	Caspases	10
1.7	Bcl-2 family proteins	12
1.8	Inhibitor of Apoptosis Proteins	13
1.8.1	XIAP	14
1.8.2	cIAP-1/2	16
1.8.3	Survivin	18
1.8.4	Livin	19
1.8.5	NAIP, IP2 and Bruce	19
1.9	SMAC	19
1.10	Apoptotic signalling pathways	21
1.10.1	Intrinsic / Mitochondrial apoptotic pathway	21
1.10.2	Extrinsic / Death-receptor apoptotic pathway	23
1.11	IAP regulation of Intrinsic and Extrinsic apoptotic pathways	24
1.12	SMAC mimetic therapy	25
1.12.1	SMAC mimetic therapy and MPM	29
1.13	The Warburg Effect: Aerobic glycolysis and resistance in cancer	30
1.13.1	Advantages of an aerobic glycolysis metabolic phenotype in cancer	30
1.13.2	Mitochondrial-associated Hexokinase-I/II is essential for aerobic glycolysis and mediates a metabolic advantage in cancer	31

1.14	Association of Hexokinase-I/II with the Voltage Dependant Anion Channel -1 and apoptotic resistance in cancer	32
1.14.1	Regulation of Hexokinase-I/II binding to VDAC	35
1.15	Dissociation of Hexokinase-I/II from the mitochondrion induces apoptosis in cancer	36
1.15.1	Mitochondrial Hexokinase-Dissociating compounds	37
1.16	The need for new targeted anti-cancer therapies	39
1.17	Aims	40

Chapter 2

Materials and Methods

2.1	Materials	42
2.1.1	Source of materials	42
2.1.2	Source of cell Lines	46
2.2	Cell culture	46
2.2.1	Malignant Pleural Mesothelioma cell lines	46
2.2.2	Burkitt's Lymphoma cell lines	47
2.2.3	HeLa cell line	47
2.2.4	Chronic Lymphocytic Leukemia cell lines	48
2.2.5	LC3-GFP Macrophages	48
2.2.6	Hek-Blue TM -hTLR4 cells	49
2.3	Cell maintenance, experimental set up and treatments	49
2.4	Cell cryo-storage	50
2.5	Western Blots	50
2.5.1	Preparation of whole cell lysates	50
2.5.2	BCA protein assay	50
2.5.3	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	51
2.5.4	Polyvinylidene Difluoride (PVDF) wet transfer	52
2.5.5	Blocking, Immuno-Blotting and protein detection	52
2.5.6	Stripping blots and loading control re-probing	53
2.6	Alamar Blue cell viability assay	53
2.7	Propidium Iodide cell cycle Flow Cytometry analysis	54
2.8	Caspase assays	56
2.8.1	Apoptotic inhibition using pan-caspase inhibitor, z-VAD-FMK	56
2.8.2	Apoptotic inhibition using Caspase-9 specific inhibitor, z-LEHD-FMK	58
2.8.3	Fluorogenic caspase – 3, -8, & -9 activation assay	58
2.9	Annexin V / PI staining apoptosis assay	59
2.10	Comet assay	60

2.10.1	Comet assay: Cell treatment	62
2.10.2	Comet assay: Agarose slide preparation, cell lysis and Single Cell Gel Electrophoresis	62
2.10.3	Comet assay: Fixation of cells, Propidium iodide DNA staining and fluorescent imaging of “Comet Tails”	62
2.11	Cytospin and RapiDiff staining of cells	63
2.12	XIAP-BIR3 fluorescence polarisation competitive binding assay	63
2.13	TR-FRET PPAR-γ Competitive Binding assay	66
2.14	Catalase Spectrophotometric Enzyme assay	68
2.14.1	Catalase assay: Km Calculations	69
2.14.2	Catalase assay: Sodium azide inhibition assay	69
2.15	Autophagic flux assay	70
2.15.1	Nutrient Starvation assay	70
2.15.2	LC3-II and p62 Western Blot analysis	70
2.16	Confocal Microscopy: LC3-GFP Macrophage imaging	71
2.17	Hek-Blue NFκB Reporter assay	72
2.18	IκB Degradation assay	73
2.19	Subcellular fractionation and mitochondrial hexokinase assay	74
2.19.1	Subcellular fractionation and mitochondrial isolation	74
2.19.2	Assessment of purity of fraction: Lactate Dehydrogenase and Citrate Synthase Assay	74
2.19.3	Mitochondrial-Hexokinase dissociation assay: Spectrophotometric enzyme assay	77
2.20	Mitochondrial-Hexokinase dissociation assay: Western Blot analysis	77

Chapter 3

Investigating the potential of SMAC mimetic therapy to overcome IAP-mediated chemotherapeutic resistance in Malignant Pleural Mesothelioma

	Introduction	79
	Results	82
3.1	IAP expression levels in Mesothelioma cell lines	82
3.1.1	Mesothelioma cell lines have high basal expression levels of c-IAP-1	82
3.1.2	CRL 5820 cells have high basal expression levels of c-IAP-2, XIAP and Survivin	82
3.1.3	One 58 cells have negligible protein expression levels of XIAP	84
3.2	High IAP expression mediates chemoresistance in Mesothelioma cell lines	84
3.2.1	High IAP expressing CRL 5820 cells are more resistant than Ju77 cells to cell death from Etoposide challenge	87
3.2.2	CRL 5820 cells are more resistant to apoptotic induction from DNA damaging agents	89
3.2.3	CRL 5820 cells are more resistant to apoptotic induction from Microtubule Targeting Agents (MTA)	89

3.2.4	CRL 5820 and Ju77 cells show similar susceptibility to apoptosis from anti-folate therapy	91
3.3	SMAC mimetic therapy relieves IAP mediated resistance in Mesothelioma	91
3.3.1	Embelin treatment sensitised XIAP expressing cells to Cisplatin induced apoptosis	94
3.3.2	Embelin induces single agent cell cycle arrest, cytotoxicity and apoptosis in all Mesothelioma cell lines.	99
3.4	Cell permeable AVPI did not sensitise Mesothelioma cells to cisplatin or TNF-α induced cell death	99
	Discussion	101

Chapter 4

Biochemical analysis of a series of novel computationally-designed SMAC mimetics in Malignant Pleural Mesothelioma

	Introduction	106
	Results	108
4.1	Computational Ligand Based Design of a series of compounds based on the SMAC N-terminal tetra-peptide, AVPI	108
4.2	Optimisation and validation of an in vitro fluorescence polarisation assay for competitive binding analysis of computational “Hit” compounds to XIAP-BIR3 domain	110
4.3	Computationally designed MDG compounds did not bind to the XIAP-BIR3 domain	114
4.4	Evaluation of the cytotoxic and apoptotic potential of MDG compounds in Mesothelioma cell lines	114
4.4.1	MDG 39, 42 and 44 induced cell death in selected Mesothelioma cell lines	116
4.4.2	MDG 42 and MDG 44 synergistically increased cisplatin induced apoptosis in CRL 5915 and One 58 cell lines, but not Ju77 or CRL 5820 cell lines	116
4.5	MDG compounds do not bind to cIAP-1 or cIAP-2, a secondary putative IAP binding partner	122
4.5.1	MDG 44 does not induce proteosomal degradation of cIAPs: MDG 44 induces an increase in expression of cIAP-1 and a consequential decrease in cIAP-2.	125
4.5.2	All Mesothelioma cell lines express abundant levels of TNFR1	127
4.5.3	TNF- α and TRAIL treatment did not affect cell viability in Mesothelioma cell lines	127
4.5.4	MDG compounds in combination with death-receptor signalling did not synergistically induce cell death in One 58, Ju77 and CRL 5915 cell lines	127
4.5.5	Embelin did not synergistically induce cell death in combination with TNF- α in CRL 5915, Ju77 and One 58 cells	130
4.6	TNF-α induced a protective effect against cytotoxicity in CRL 5820 cells	130
4.6.1	TNF- α induced a protective effect against Etoposide and MDG SMAC mimetic induced cell death in CRL 5820 cells	133

4.6.2	TNF- α induced a protective effect against Embelin induced cell death in CRL 5820 cells.	136
4.6.3	Western blot analysis of IAP expression levels in response to TNF- α treatment in MPM cell lines	136
	Discussion	139

Chapter 5

Investigating the mechanism of MDG 44 induced cell death in Malignant Pleural Mesothelioma

	Introduction	143
	Results	147
5.1	MPM cell lines expressed abundant levels of pro-apoptotic Bcl-2 family proteins	147
5.2	Characterisation of apoptotic response induced by MDG 44 in MPM cell lines	149
5.2.1	MDG 44 single agent treatment induced pre -G1 apoptosis in MPM cells in a dose dependent manner	149
5.2.2	MDG 44 single agent treatment induced PARP cleavage in MPM cells in a dose dependent manner	149
5.3	Investigation into the time-dependence and potency of MDG 44 treatment in the CRL 5915 MPM cell line	151
5.3.1	MDG 44 induced dose and time dependent cell death in CRL 5915 cells	151
5.3.2	MDG 44 induced a time dependent increase in apoptosis in CRL 5915 cells	154
5.4	MDG 44 induced a caspase dependent form of apoptosis in the CRL 5915 cell line	154
5.4.1	MDG 44 induced cell death is inhibited by pan-caspase inhibitor, z-VAD-FMK	155
5.4.2	MDG 44 induced cell death occurs through the intrinsic apoptotic pathway	155
5.4.3	MDG 44 induced cell death was inhibited by the caspase-9 specific inhibitor, z-LEHD-FMK	159
5.4.4	MDG 44 treatment of CRL 5915 cells induced the proteolytic cleavage of pro-caspase-9	159
5.5	MDG 44 treatment induced significant increases in Annexin V + / PI + staining in CRL 5915 cells	162
5.6	MDG 44 induced DNA damage indicative of apoptosis, determined by Single-Cell Gel Electrophoresis analysis (Comet assay)	162
5.7	Examination of synergistic induction of Programmed Cell Death with MDG 44 and Cisplatin combinational treatments in CRL 5915 cells	165
5.7.1	MDG 44 synergistically increased cisplatin induced PARP cleavage in CRL 5915 cells	165
5.7.2	MDG 44 synergistically increased Cisplatin induced apoptosis in a dose dependent manner	167
5.7.3	MDG 44 synergistically increased Cisplatin induced Caspase-3 activation in CRL 5915 cells	169
5.7.4	MDG 44 in combination with Cisplatin induced a synergistic increase in Annexin V + / PI + apoptotic cells	169
5.8	MDG 44 induces single agent apoptosis in Chronic Lymphocytic Leukaemia (CLL) in	169

	vitro cell models	
5.9	Investigation into the mechanism of cell death induced by MDG 44 treatments in the CRL 5915 cell line	171
5.9.1	MDG SMAC mimetics did not induce S phase arrest in Mesothelioma cell lines	171
5.9.2	MDG 44 induced G1 arrest in CRL 5915 cells at sub-lethal concentrations	174
5.9.3	Reactive oxygen species (ROS) / Oxidative stress was not involved in MDG 44 mediated cell death	174
5.9.4	MDG 44 treatment induced an increase in cellular vesicles and morphological changes indicative of apoptosis in CRL 5915 cells	175
	Discussion	177

Chapter 6

Examination of alternate molecular targets and mechanisms of action for MDG 44 induced apoptosis in Malignant Pleural Mesothelioma

	Introduction	185
	Results	192
6.1	A compound patent search for 2-Indolinone / MDG 44 scaffold compounds did not identify any molecular targets for investigation with MDG 44	192
6.2	MDG 44 did not show affinity for PPAR-γ in a TR-FRET PPAR-γ competitive binding assay	195
6.3	Known PPAR-γ ligands possessed anti-proliferative activity in MPM cells	196
6.4	PPAR-γ antagonism did not inhibit MDG 44 induced cellular responses in MPM cells	199
6.4.1	PPAR- γ antagonist, GW9662, did not inhibit MDG 44 induced cell death in CRL 5915 cells	199
6.4.2	PPAR- γ antagonist, GW9662, did not inhibit MDG 44 induced formation of cytoplasmic vesicles in CRL 5915 cells	200
6.5	Examination of cytoplasmic vesicles as being Peroxisomes: Catalase Spectrophotometric enzyme assay	200
6.5.1	Investigation of Catalase activity in CRL 5915 cells	204
6.5.2	MDG 44 treatment did not induce increased Catalase activity in CRL 5915 cells	207
6.6	Examination of the accumulation of autophagosomes in MPM cells in response to MDG 44 and inhibitors of autophagy	207
6.6.1	Chloroquine induced accumulation of autophagosomes in MPM cells; detected by LC3 Western Blot analysis	210
6.6.2	Chloroquine induced accumulation of autophagosomes in MPM cells; detected by Cytospin analysis and RapiDiff staining	212
6.7	Examination of the effect of MDG 44 treatment on Autophagic Flux in MPM cell	215
6.7.1	Nutrient starvation induced an increase in Autophagic flux in MPM cells	216

6.7.2	MDG 44 induced increased expression of the autophagosome marker, LC3-II in CRL 5915 cells	218
6.8	MDG 44 induced the formation of LC3-GFP positive structure in LC3-GFP stably transfected macrophages	221
6.9	PI3-Kinase inhibitor, 3-Methyl Adenine, did not inhibit MDG 44 induced cell death in CRL 5915 cells	223
6.10	Examination of MDG 44 as a proteasome inhibitor	227
6.10.1	MDG 44 inhibited NF κ B activation in the Hek-Blue-hTLR4 / NF κ B reporter assay	229
6.10.2	MDG 44 inhibited TNF- α induced I κ B degradation in a dose dependant manner in CRL 5915 cells	231
6.10.3	MDG 44 did not induce accumulation of Poly-Ubiquitinated proteins in CRL 5915 cells	234
	Discussion	236

Chapter 7

Design and biochemical evaluation of second generation MDG 44 analogues

	Introduction	242
	Results	247
7.1	Cell viability screen of 86 analogues of MDG 44 and MDG 42	247
7.2	Cell viability dose response analysis of MDG 44 analogues CDX 45, 25, 24, 66, 13, 78 & 68	251
7.3	Single-agent apoptotic analysis of MDG 44 analogues CDX 45, 25, 24, 66, 13, 78 & 68	251
7.4	Investigation into the mechanistic activity of lead analogues CDX 45 and CDX 78	255
7.4.1	CDX 45 and CDX 78 synergistically increased Cisplatin induced apoptosis in CRL 5915 cells	257
7.4.2	CDX 45 and CDX 78 induce the formation of large Cytoplasmic vesicles in CRL 5915 cells	257
7.5	Investigation of a second series of MDG 44 analogues with increased predicted solubility	260
7.5.1	MDG 44 analogues CDX 87 – 92 were more potent inducers of cell death than lead compound MDG 44	260
7.5.2	CDX 91 and 92 did not induce significant levels of single-agent or synergistic apoptosis in CRL 5915 cells	262
	Discussion	262

Chapter 8

Biochemical analysis of putative Mitochondrial-Hexokinase dissociating compounds in Burkitt's Lymphoma and MPM cell lines

	Introduction	269
--	--------------	-----

	Results	272
8.1	Mutu-1, but not DG-75 BL cell lines express pro-apoptotic BCL-2 family proteins Bax and Bak	272
8.2	Examination of the effect of anti-fungal azole compounds on induction of cell death and apoptosis in BL and MPM cell lines	272
8.2.1	Azole compounds induced dose dependent cell death in Mutu-1 and DG-75 cell lines	275
8.2.2	Mutu-1 cells were more susceptible to apoptosis induced by anti-fungal azole compounds than DG-75 cells	278
8.2.3	Azole compounds induced dose and time dependent apoptosis in Mutu-1 and DG-75 cell lines	278
8.2.4	Azole compounds induced PARP cleavage in Mutu-1, but not DG-75 cell lines	281
8.2.5	Azole compounds induce Caspase-3 activation in Mutu-1, but not DG-75 cell lines	281
8.2.6	Sub-lethal concentrations of azole compounds induced G1 cell cycle arrest in the DG-75 cell line	283
8.3	Azole compounds effect in MPM cell lines	283
8.3.1	Azole compounds induced dose and time dependent apoptosis in MPM cell lines	286
8.3.2	Azole compounds induced PARP cleavage in all MPM cell lines	289
8.3.3	Sub-lethal concentrations of azole compounds induced G1 cell cycle arrest in MPM cell lines	289
8.4	Mito-HK-dissociation compounds were screened for apoptotic efficacy in BL and MPM cell lines	291
8.4.1	A4 and A5 induced dose dependent cell death in Mutu-1 and DG-75 BL cell lines	291
8.4.2	A4 induced potent cell death in all four MPM cell lines	294
	Discussion	298
	Concluding Remarks	304
	Appendix I – Analogue structure CDX 1 - 92	308
	Bibliography	318

Abbreviations

3-MA	3-Methyl adenine
4-MTA	4-Methylthioamphetamine
AA	Amino acid
AB	Alamar Blue
ADP	Adenosine diphosphate
AML	Acute myeloid leukaemia
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide complex
APAF-1	Apoptotic protein activating factor-1
ATG	Autophagy related gene protein
ATP	Adenosine 5'-triphosphate
AVPI	Alanine; Valine; Proline; Isoleucine tetra peptide
AVPI-R(8)	Cell-permeable AVPI peptide
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology domain
BIR	Baculovirus IAP Repeat
BL	Burkitt's Lymphoma
BRUCE	BIR-containing ubiquitin conjugating enzyme
BSA	Bovine Serum Albumin
CAD	Caspase activated DNase
CARD	Caspase recruitment domain
CC	Coiled coil
CDK	Cyclin dependant kinase
cIAP	Cellular Inhibitor of Apoptosis Protein
CK	Creatine kinase
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic myelogenous leukaemia
CO ₂	Carbon dioxide
CSH	Citrate synthase
Cyto c	Cytochrome c
DAPI	4, 6-dimidino-2-phenylindole
DD	Death domain
DED	Death effector domain
DFF40/CAD	DNA fragmentation factor 40 / caspase-activated DNase
DISC	Death inducing signalling complex
DKO	Double knockout
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTNB	Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid))
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
ER	Endoplasmic reticulum

ESFT	Ewing's Sarcoma family tumours
EtOH	Ethanol
EWSR	Ewing's Sarcoma breakpoint region
FACS	Fluorescence associated cell sorter
FADD	Fas associated protein with death domain
FBS	Foetal bovine serum
FI	Fluorescence intensity
FLI1	Friend Leukaemia integration 1
Flour	FP fluorescently labelled peptide ligand
FP	Fluorescence polarisation
G6P	Glucose-6-phosphate
GFP	Green Fluorescent Protein
HBSS	Hank's buffered salt solution
HBXIP	Hepatitis B X-interacting protein
Hek	Human embryonic kidney cells
HK	Hexokinase
HM	Human pleural mesothelium
HPV	Human Papilloma virus
hXIAP-BIR3	Human recombinant XIAP BIR3 domain
IAP	Inhibitor of Apoptosis Proteins
IBM	IAP binding motif
IGF	Insulin-like growth factor
IMS	Inter-membrane space
K_m	Michaelis-Menten constant
LBD	Ligand binding domain
LC3	Micotubule-associated Protein Light Chain 3
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAP	Mitogen activated protein
MCP-1	Monocyte chemotactic protein-1
MDG	Molecular Design Group
MDM2	Murine double minute 2
MEF	Mouse embryonic fibroblasts
MIM	Mitochondrial inner membrane
Mito	Mitochondria
MM	Malignant Mesothelioma
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilisation
mP	Milli-polarisation
MPM	Malignant pleural mesothelioma
MPTP	Mitochondrial permeability transition pore
MTA	Microtubule targeting agents
mTORC	Mammalian target of rapamycin complex
MTS	Mitochondrial targeting sequence
NAC	N-acetyl cysteine
NAD^+	β -Nicotinamide Adenine Dinucleotide

NADH	β -Nicotinamide Adenine Dinucleotide, reduced
NADP	β -Nicotinamide Adenine Dinucleotide Phosphate
NADPH	β -Nicotinamide Adenine Dinucleotide Phosphate, reduced
NAIP	Neuronal apoptosis inhibitory protein
NF κ B	Nuclear factor kappa B
NIK	NF κ B inducing kinase
NK	Natural killer
NSCLC	Non-small cell lung carcinoma
Oligos	Antisense oligonucleotides
Ox Phos	Oxidative Phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly-ADP ribose polymerase
PBR	Peripheral benzodiazepine receptor
PBS	Phosphate buffered saline
PCD	Programmed cell death
PDGDR	Platelet-derived growth factor receptor
PDT	Photo dynamic therapy
PE	Phosphatidylethanolamine
Pen-Strep	Penicillin-Streptomycin
PET	Positron emission tomography
PI	Propidium iodide
PI3K	Phosphoinositide-3-kinase
PI α	Proteasome inhibitors
PMSF	PhenylMethylSulfonyl Fluoride
PPAR	Peroxisome proliferator-associated receptor
PS	Phosphatidylserine
PVDF	Polyvinylidene Difluoride
QSAR	Quantitative Structure-Activity Relationship
RHA	RNA helicase
RING	Really interesting new gene
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute medium 1640
RXR	Retinoid X receptor
SAR	Structure-activity relationship
SDS	Sodium dodecyl sulphate
SEAP	Secreted embryonic alkaline phosphatase
SERT	Serotonin re-uptake transporter
SMAC/DIABLO	Second mitochondrial-derived activator of caspase / Direct IAP-binding protein with low PI
Syk	Spleen tyrosine kinase
TAB-1	TAK-1 binding protein
TAK-1	TGF- β -activated kinase-1
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TLR	Toll-Like Receptor

TNF	Tumour necrosis factor
TNFRSF	TNF receptor super family
TRAF	TNF receptor associated factor
TRAIL	TNF related apoptosis inducing ligand
TR-FRET	Time Resolved Fluorescence Resonance Energy Transfer
TS-IAP	Testis specific IAP
ULK	UNC-51 like kinase
UPR	Unfolded protein response
UPS	Ubiquitin-Proteasome system
VDAC	Voltage dependent anion channel
VS	Virtual screening
XIAP	X-linked Inhibitor of Apoptosis Protein

Chapter 1: General Introduction

*Rational drug design and biochemical analysis in the development of
targeted therapies against Malignant Pleural Mesothelioma*

General Introduction

1.1 Cancer

Cancer is a malignant neoplasm resulting from genetic alterations to homeostasis, growth and cell death regulation of somatic cells in vertebrates. Such malignancies cause uncontrolled cell growth, invasion and metastasis resulting in death without surgical or therapeutic intervention. In Ireland, 20,000 new cases of cancer are diagnosed each year with 7,500 deaths, a quarter of the annual death toll (Cancer-research-Ireland, 2010). Worldwide, cancer incidence and deaths are increasing with the projected death toll to be upwards of 12 million by the year 2030 (WHO-statistics, 2010).

A century worth of research and epidemiology studies have discovered a large number of physical, chemical and environmental carcinogens and genetic factors relating to tumorigenesis. Personal exposure to radiation, cigarette smoke and asbestos fibres; genetic predisposition for expression of cancerous oncogenes (e.g. BRCA1); and even diet and pollution have been implicated as causative factors for the development of different tissue type cancers. In a biochemical context, cancers initially develop when carcinogens induce genetic mutations to protein loci regulating cell growth and cell death machinery. For example, mutations to p53, the “Guardian of the Genome” or Bcl-2 family proteins, both essential players in detection of cellular damage and induction of cell death pathways, disrupt the capacity of cells to die through conserved cell death programs (Apoptosis, Autophagy). Resistance to cell death prevents the removal of damaged / mutated cells, resulting in accumulation of genetic insults contributing to the cancer phenotype – limitless replication, autocrine growth factor production, cell death resistance, aerobic glycolysis (Warburg effect), angiogenesis and metastasis.

In a biochemical sense, cancer can be thought of as not a single disease or a tissue specific condition, but rather as hundreds of related disorders, defined by the collection of genetic alterations present in each malignancy. For example, Chronic Myelogenous Leukaemia (CML) is defined by the occurrence of a particular chromosomal translocation (“Philadelphia Chromosome”) forming the oncogenic fusion protein BCR-abl, which drives tumour growth (Guo and Ding, 2003). Numerous cancer subtypes can be better classified by the occurrence of specific mutations or epigenetic alterations to the expression of specific oncogenes (E.g.: p53, Ras, Her2 and nuclear receptors, etc.), the overexpression of anti-

apoptotic regulators or the presence of altered metabolic phenotypes within tumours, rather than simply tissue location. This stratification of cancer subtypes into groups defined by the characteristic genetic and biochemical alterations within tumours aids clinical evaluation and treatment. While these specific “driver oncogenes” promote cancer resistance, survival and growth, the dependency of tumours on such mutations means these are prime targets for a new strategy of “Rational Drug Design” in anti-cancer drug development. The molecular characterisation of tumours, the identification of clinical biomarkers and the development of targeted therapies against cancer are all steps towards a personalised approach to cancer medicine and away from the use of broadly toxic, adjuvant chemotherapy.

1.2 Malignant Pleural Mesothelioma

Malignant Mesothelioma (MM) is a fatal, chemoresistant neoplasm arising from cells of the mesothelial lining of the pleural, peritoneal and pericardial cavities. Malignant Pleural Mesothelioma (MPM) is the most common form of the disease, accounting for approximately 75% of diagnosed cases. Normal human pleural mesothelial (HM) cells make up a protective covering of the lung cavity, which is involved in secretion of a lubricating fluid facilitating the free movement of lungs and permits transport of fluid and cells across the pleural cavity (Mutsaers, 2004). Prolonged asbestos exposure is believed to be the primary cause of MPM carcinogenesis with over 80% of MPM patients having previous exposure to asbestos. However other factors, including simian virus 40 (SV40) infection and genetic predisposition, have been associated with individual susceptibility to asbestos tumorigenesis (Belli et al., 2009). The time between exposure and onset of the disease is characterised by a long latency period of between 30-40 years and while in the Developed World asbestos has been widely outlawed, incidence and death rates of MPM are not expected to peak in Britain until 2020 – reports currently stand at approximately 1,000 deaths per year (Hodgson et al., 2005). Prospects are worse in areas of naturally occurring asbestos particles, including Eastern Australia and South Africa (Hendrickx, 2009), and the Developing World where industrial asbestos use still commonly occurs and rates are expected to continue to rise.

The disease occurs in three distinct morphological tumour types – Epithelioid, Sarcomatoid or a combination of the two (Biphasic) – with specific variance in severity and

resistance to treatment between each MPM sub type (Corson, 2004). Out of four disease stages, 85 – 90% of patients are diagnosed presenting with advanced stage III or IV metastatic disease, exhibiting chest pain, dyspnea, weight loss and repeat pleural effusion (Belli et al., 2009). Advanced stage tumours are unresectable, with broad metastasis to lymph nodes and distal organs, and chemotherapeutic treatment at this point of the disease is merely palliative. MPM shows very poor response to conventional chemotherapeutic options with a median survival time of less than 1 year. The current treatment option of choice employs an aggressive co-treatment regime using the anti-folate Pemetrexed and platinum-based genotoxic agent Cisplatin; however this prescribed option provides only a 2.9 month increase in median survival time and response rate of just 41.3 % of patients (Vogelzang et al., 2003). In the 1 – 5% of diagnosed cases that are good candidates for surgery – i.e. patients healthy enough for invasive surgery, presenting with localised disease, limited tumour size and the epithelioid tumour variant – the procedure is not curative and even in combination with radiation therapy there is only a median survival time of 14.2 months and a 3 year survival rate of just 20% (Rice et al., 2007). Due to the current absence of any curative procedure and lack of therapeutic options with any significant increase in mean survival time, even screening for early stage MPM is considered controversial due to the lack of treatment options for patients presenting with the disease (Bertino et al., 2009).

The biochemical aetiology of MPM tumorigenesis has not been fully elucidated to date. However theories on the mechanism by which asbestos induces HM cell transformation have been reported. Long form asbestos fibres are believed to penetrate deep within the bronchioles of the lung, into the pleural mesothelium and persist. Asbestos can penetrate the cell membrane of HM cells and cause DNA damage and mutagenesis by disrupting replication, chromosomal assembly and mitotic spindle formation (Hesterberg et al., 1986). Monocyte chemotactic protein-1 (MCP-1) release from HM cells recruits alveolar macrophages (Mo) to the site of asbestos damage. Frustrated phagocytosis of long asbestos fibres by Mo results in persistent secretion of inflammatory cytokines and Reactive Oxygen Species (ROS), contributing to an inflammatory tumour microenvironment causing further mutagenesis in HM cells (Xie et al., 2000, Tanaka et al., 2000). Paracrine and autocrine Tumour Necrosis Factor alpha (TNF- α) signalling activates survival pathways within HM cells allowing DNA damage and mutations to persist in altered cells and results in MPM tumorigenesis (Fig 1.1). Consistent with this theory, TNF- α has been shown to

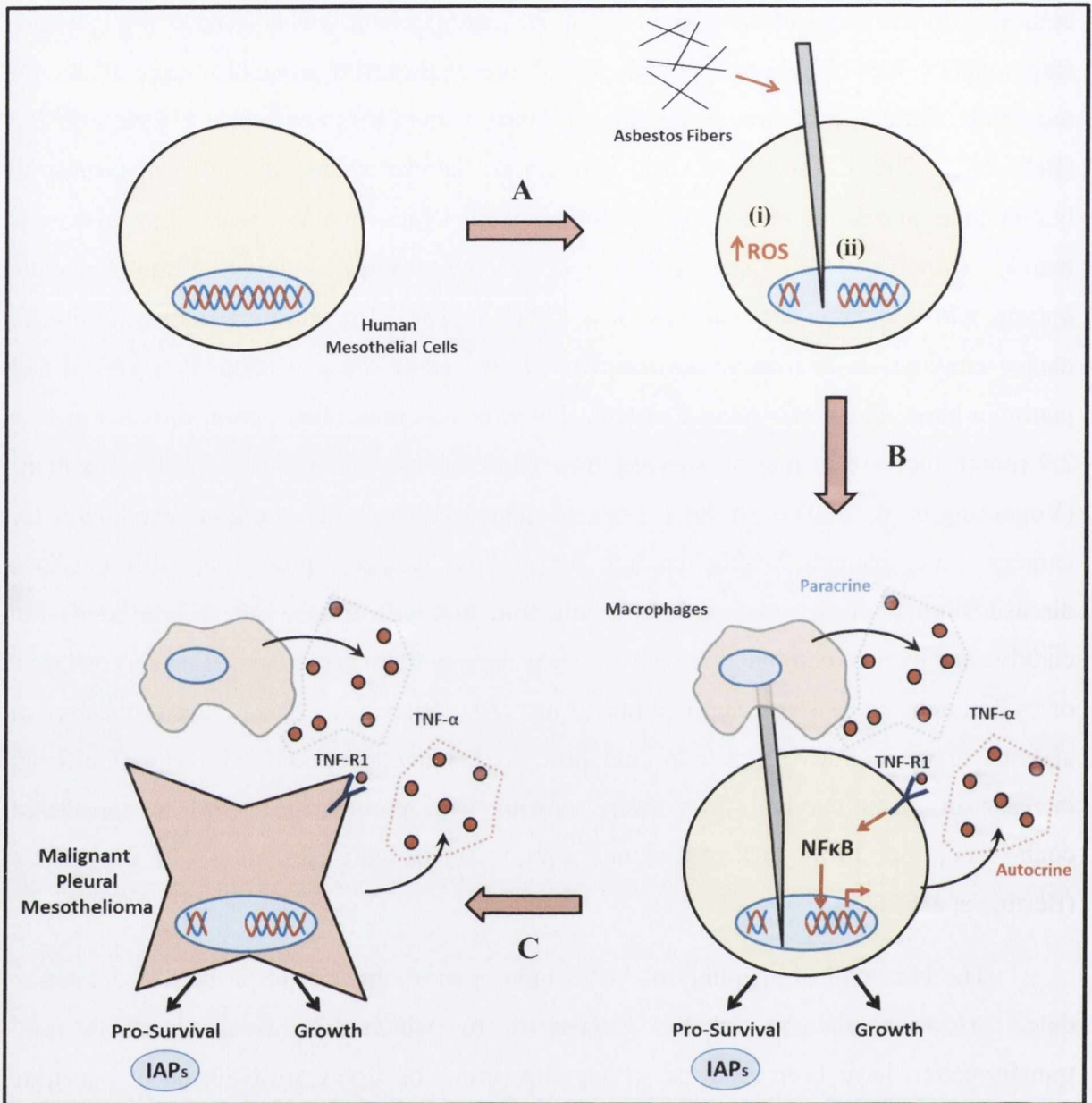


Figure 1.1: Prolonged Asbestos exposure induces malignant transformation of Mesothelial cells into Malignant Pleural Mesothelioma.

(A) Long and thin Asbestos (Crocidolite) fibers penetrate deep within the lung and into the pleural mesothelial lining (HM cells), inducing an increase in the generation of reactive oxygen species (i) and inducing chromosomal damage within the cell (ii). While being toxic to mesothelial cells, asbestos contributes to the creation of an inflammatory environment that allows the persistence of these DNA damaged cells. (B) Frustrated phagocytosis of asbestos fibers by macrophages causes paracrine $\text{TNF-}\alpha$ signalling. This is coupled with autocrine $\text{TNF-}\alpha$ signalling from HM cells and induces the expression of pro-survival and growth signals within the cell, through TNF-R1 ligation and $\text{NF}\kappa\text{B}$ signalling pathways. These pro-survival mediators include the Inhibitor of Apoptosis Protein (IAPs) family. (C) Successive cycles of asbestos induced chromosomal damage and inflammation results in the persistence of mutated cells and the eventual malignant transformation of HM cells into Malignant Pleural Mesothelioma (MPM).

have protective effects on cytotoxicity in MPM cells (Yang et al., 2006) and may potentially mediate this through regulation of Inhibitor of Apoptosis protein (IAP) family protein expression – TNF- α treatment has been shown to increase the expression of apoptotic regulators cIAP and XIAP in MPM and induce resistance to cisplatin challenge (Gordon et al., 2007b).

1.3 Programmed cell death

Programmed Cell Death (PCD) encompasses a number of distinct cellular processes concerned with the controlled destruction of cells and recycling of cellular products. These processes include Type I PCD or Apoptosis (Caspase-dependant (Elmore, 2007) and caspase-independent (Ravagnan et al., 2002)), Type II PCD or Autophagy (Gozuacik and Kimchi, 2004) and the newly defined necrotic-like form of regulated cell death, Necroptosis (Galluzzi and Kroemer, 2008). PCD is necessary for development, homeostasis and removal of infected, damaged or malignant cells. Contrary to uncontrolled mechanisms of cell death, like classical Necrosis, in which the cell membrane ruptures and the cellular content is released, inducing an inflammatory response, PCD allows for the ordered destruction of the cells and catabolism of cellular components. Induction, regulation and execution of PCD processes are facilitated by large networks of receptors, signalling kinases, transcription factors and proteases interacting primarily with the mitochondria, nucleus, endoplasmic reticulum and lysosomes (Merino and Cordero-Campana, 1998).

In cancer, all strategies of chemotherapeutic development are currently concerned with specific engagement of a PCD response. The precise nature of cancer, being merely a genetically altered version of healthy native tissue, necessitates this. Triggering wide spread cell death in a malignant organ would induce severe side effects, including cytotoxicity of healthy cells, inflammation and tissue damage. Specifically inducing PCD in cancer cells and not in healthy tissue is the *modus operandi* of an effective chemotherapeutic drug.

1.4 Autophagy

The process of autophagy, literally meaning “cellular self-digestion”, is a highly regulated pathway encompassing the selective or non-selective uptake and trafficking of

cytoplasmic organelles and cytosolic components for lysosomal degradation. Autophagy is an evolutionarily conserved adaptation upregulated within cells during times of cellular stress and energy / nutrient deprivation (Kroemer et al., 2010). Autophagy is triggered as a cytoprotective response to nutrient starvation or environmental stresses (hypoxia, oxidative stress, infection, chemotherapeutic / radiation treatment) where cytosolic components are catabolised in order to maintain cellular homeostasis. During autophagy, cellular components are sequestered into forming vesicles, termed phagophores, which eventually mature into complete double-membraned vesicles called autophagosomes. These vesicles traffic the cellular material to late endosomes / lysosomes, where they fuse to form an autolysosome. Lysosomal proteases degrade and recycle the cytoplasmic material sequestered and trafficked within the autophagosome to replenish depleted nutrient and energy reserves within the cell (Mizushima et al., 2008).

Basal levels of autophagic flux – autophagic trafficking and lysosomal degradation of components – also occurs within cells and comprises one half of the complementary protein degradation pathways of the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system, both pathways being regulators of protein quality and cellular homeostasis (Goldberg, 2003). The UPS involves the ubiquitin targeted, proteasomal degradation of “short-lived” proteins involved in transient cellular processes – cell cycle transition (Cyclins; p21); apoptosis (Bax; p53) (Blagosklonny et al., 1996, Wu et al., 2010). In comparison, autophagy encompasses the sequestration of “long-lived” proteins, ubiquitinated-protein aggregates and defective organelles for lysosomal degradation (Mizushima et al., 2008). While autophagy is an essential regulator of protein homeostasis and a cytoprotective response to cellular stresses, defective autophagy or prolonged stress and nutrient deprivation can result in excessive autophagic self-digestion and eventual induction of cell death (Gozuacik and Kimchi, 2004). Induction of PCD under these conditions occurs via the morphologically distinct, Type II / autophagic PCD pathway, or in combination with cellular crosstalk with the Type I / apoptotic PCD pathway (Gonzalez-Polo et al., 2005).

Initiation of autophagy occurs downstream of cellular sensors of energy stress (AMPK), genotoxic damage (p53) and nutrient deprivation (Growth factor receptor tyrosine kinase), resulting in inhibition of a key regulator of autophagic flux, the mammalian target of Rapamycin protein (mTor) (illustrated in Figure 1.2). Downstream of mTOR inhibition, components of the Atg (autophagy-related genes) protein family, referred to as the core

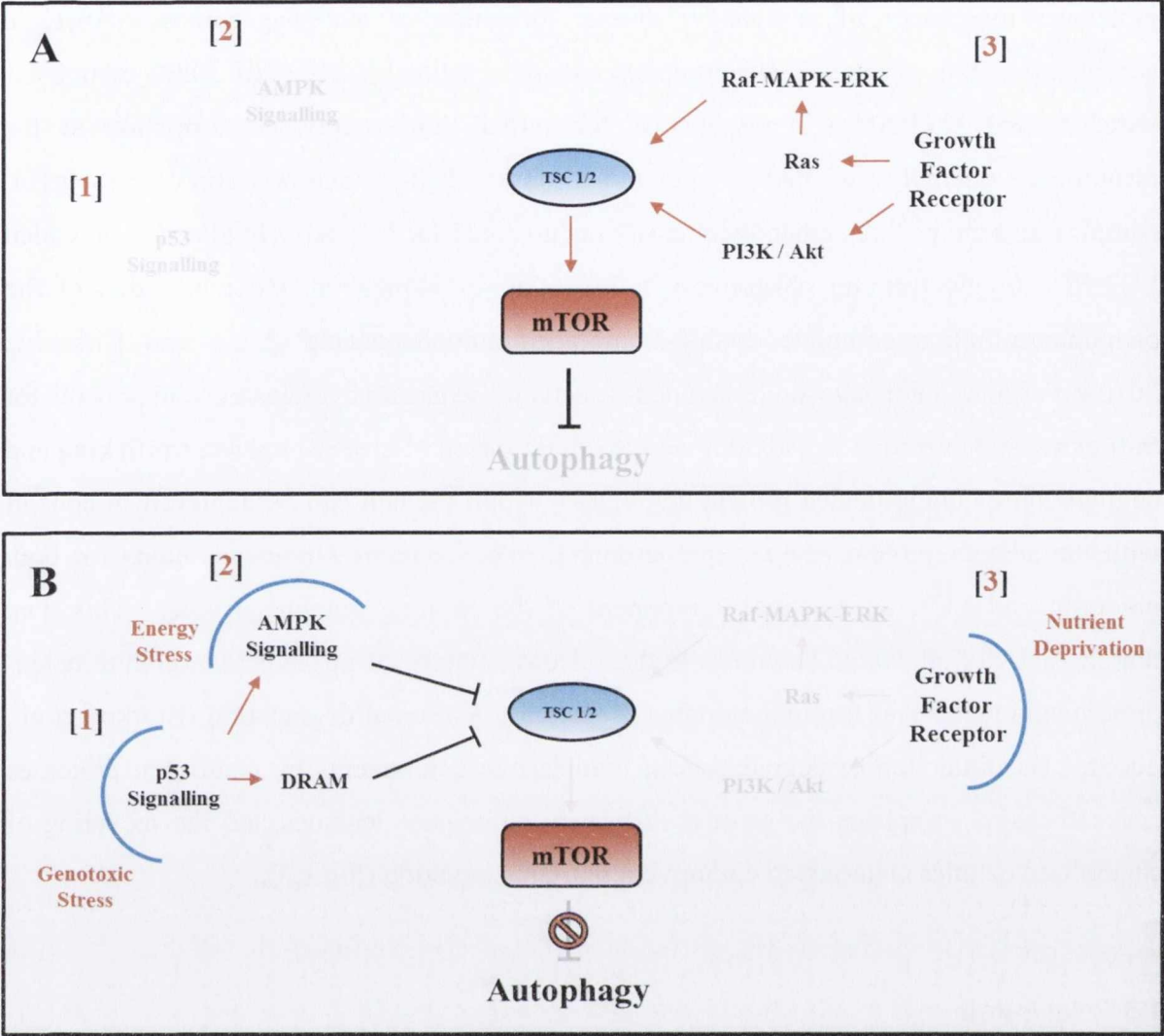


Figure 1.2: Cellular stress and nutrient deprivation induce autophagy within cells.

The Mammalian Target of Rapamycin (mTOR) is a key regulator of inhibition or initiation of increased autophagic flux in response to perturbations to cellular homeostasis. (A) Under nutrient and energy competent conditions, upregulation of autophagic flux is inhibited by the activity of mTOR. Growth factor ligation (3) and receptor tyrosine kinase activation results in downstream signalling via the PI3K / Akt pathway and the Ras GTPase / Raf-MAPK-ERK pathway, resulting in activation of TSC 1/2 and mTor, inhibiting increased autophagic flux. (B) Under conditions of cellular stress – Genotoxic damage (1); Energy deprivation (2); Nutrient deprivation (3) – mTor activity is inhibited and increased autophagic flux is initiated. The lack of growth factor signalling diminishes PI3K / Akt and Raf-MAPK-ERK activation of mTor, while energy deprivation and genotoxic stress sensors, AMPK and p53 respectively, inhibit TSC 1/2 and mTor activity. Inhibition of mTor results in increased autophagic flux in response to cellular stress.

molecular machinery of autophagy, initiate formation of autophagosomes. Atg1, in combination with the ULK 1/2 complex and the Beclin-1 / class III PI3K complexes (Atg14L and UVRAG), is responsible for initial lipid membrane formation at the phagophore assembly site (PAS) (Yang and Klionsky, 2010). Incorporation of the Atg16L complex and the phosphoethanolamine (PE)-conjugated LC3 protein (lipidated form called LC3-II) into the forming phagophore is essential for elongation and maturation of the phagophore into a complete double-membraned autophagosome (Yang and Klionsky, 2010). Forming autophagosomes can non-selectively sequester cytoplasmic components for trafficking and eventual degradation via lysosomal fusion. Selective uptake, trafficking and degradation of ubiquitinated-protein aggregates within the cell can be achieved in consort with the adapter protein p62 / sequesterome-1. P62 contains binding domains for both ubiquitin and LC3, an essential component of the forming autophagosome. This dual binding activity allows p62 to mediate the selective uptake of p62-positive, ubiquitinated-protein aggregates into forming autophagosomes for lysosomal degradation (Bjorkoy et al., 2005). The final step of degradation of cytoplasmic components by lysosomal proteases and cathepsins completes the process of protein aggregate clearance and the recycling of catabolised cellular materials to circumvent nutrient starvation (Fig 1.3).

1.5 Apoptosis

Apoptosis is a highly regulated, energy-dependent mechanism of PCD, characterised by the biochemical events which regulate its induction and the morphological features underlining its execution (Elmore, 2007). Apoptosis functions as an important physiological process, contributing to homeostatic cell turnover, embryonic development, adaptive immune response and elimination of old, damaged, mutated or infected cells. However, perturbation to this highly regulated mechanism has pathological consequences. Diminished apoptotic control can lead to autoimmune and neurodegenerative disorders, while increased regulation or mutated apoptotic machinery is indicative of cancer.

Apoptotic execution is mediated in a caspase-dependent or caspase-independent manner, with its induction occurring via intrinsic or extrinsic stimuli in each case. Intrinsic stimuli like telomere-shortening, DNA damage, Reactive Oxygen Species (ROS) or ER stress / Unfolded Protein Response (UPR) coalesce at the mitochondria, causing release of

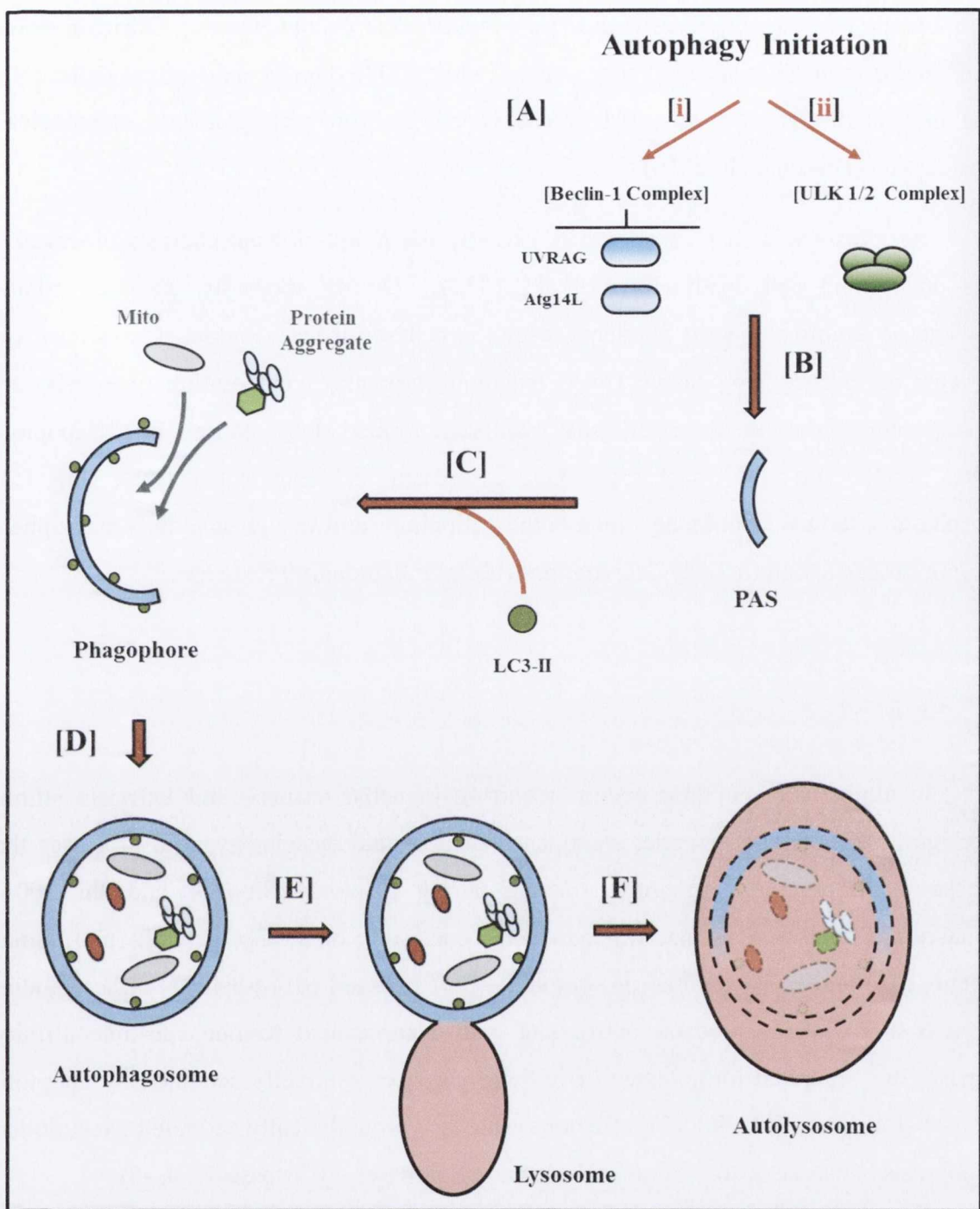


Figure 1.3: Schematic diagram of the Autophagy – Lysosome System.

(A) Downstream of mTor inhibition and initiation of autophagy, Beclin-1 containing protein complexes (i), Atg14L and UVRAG, in combination with the activated ULK 1/2 complex (ii), initiate formation of autophagosomes. (B) Autophagosome maturation occurs through a series of steps, starting with initiation at the Phagophore Assembly Site (PAS) and elongation of the double membraned vesicle with the addition of lipidated LC3 protein (LC3-II). (C) The elongated Phagophore sequesters cytoplasmic components, organelles and p62 tagged protein aggregates. (D) Autophagosome maturation occurs as the vesicle closes and sequestered material is trafficked for lysosomal degradation. (E) A late Endosome / Lysosome will fuse with the autophagosome (via Lamp2A). (F) Once fully engulfed, forming an Autolysosome, lysosomal proteases degrade and recycle cellular components sequestered within the autophagosome, replenishing nutrient and energy reserves.

pro-apoptotic caspase activators and caspase-independent endonucleases. Extrinsic stimuli from death receptor ligands activates caspase, while CD8+ T-cells and Natural Killer (NK) cells induce extrinsically activated apoptosis via perforin and granzyme endonuclease translocation (Hoves et al., 2010).

Apoptosis was first described in 1972 by the morphological changes observed in cells undergoing cell death (Kerr et al., 1972). During apoptosis, caspase-mediated cleavage of lamins and actin filaments results in cell shrinkage. Endonucleases (caspase-activated or independent) cleave DNA, inducing chromatin condensation (pyknosis) and nuclear collapse (karyorrhexis). Plasma membrane forms “blebs” and enclosed “apoptotic bodies” containing still intact organelles, cytoplasm and fragmented DNA (Fig 1.4). Phosphatidyl-serine containing membrane flipping allows phagocytic macrophage recognition and apoptotic body degradation without inflammatory response.

1.6 Caspases

In almost all apoptotic events occurring in cells, intrinsic and extrinsic stimuli converge at the caspase cascade, a sequence of activated cysteine-aspartic proteases that orchestrate the ordered disassembly of cells during apoptosis (Shiozaki and Shi, 2004). Caspases are expressed as inactive zymogens, consisting of a large subunit (p20), small subunit (p10) and an N-terminal pro-domain. Four p20 and p10 α -helical surface binding loops form a cysteine protease active site with aspartic acid residue substrate affinity. Caspases are separated into classes of initiator caspases – directly activated by apoptotic stimulus (Caspase-2, -8, -9, -10); effector caspases – proteolytically activated executioners of apoptosis (Caspase-3, -6, -7); and inflammatory caspases – (Caspase-1, -4, -5).

Initiator pro-caspases exist as monomers until death receptor ligation or mitochondrial pro-apoptotic protein release occurs, recruiting pro-caspase proximal localisation and autocatalytic intrachain cleavage of the p20 / p10 domains and the N-terminal pro-domain (Shi, 2004). These cleavage events allow formation of a functional caspase homodimer with enhanced proteolytic activity. Active initiator caspases bind specific aspartic acid residues of downstream effector pro-caspases, inducing proteolytic

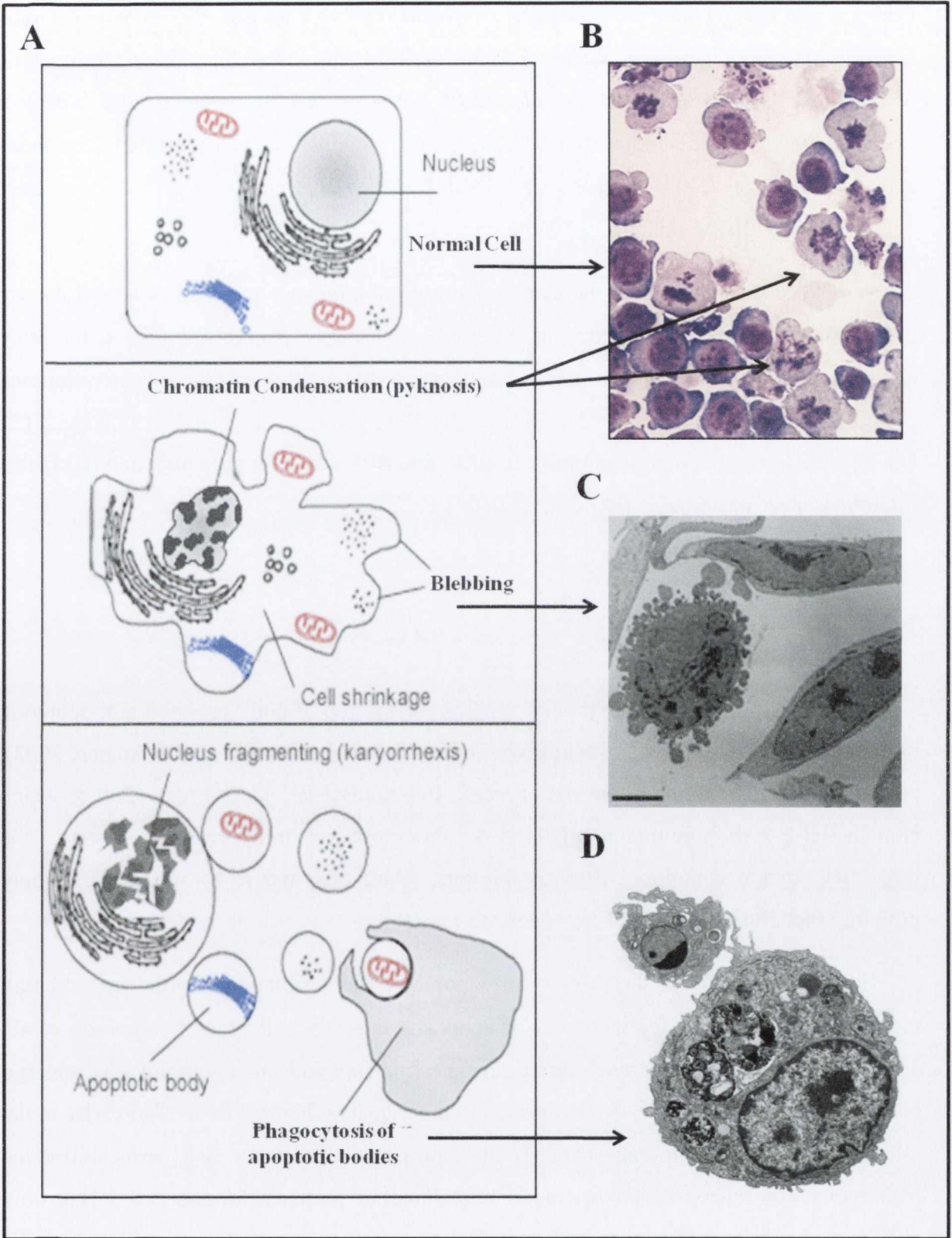


Figure 1.4: Morphological features of apoptosis.

(A) Graphical features of apoptotic induction. (B) Nuclear staining of cell cytopins – normal cells and cells undergoing pyknosis (Dvorakova et al., 2002). (C) Transition electron microscopy (TEM) image of cellular “blebbing”. (D) TEM depiction of macrophage phagocytosis of neutrophil apoptotic bodies.

cleavage and loss of their pro-domains. Activated effector caspases also exist as catalytic homodimers, binding to specific tetrapeptide motifs of proteins and inducing proteolytic degradation. The catalytic activity of effector caspases execute the molecular events of apoptosis, including the degradation of cytoskeletal proteins, cytokeratins, nuclear proteins and activation of CAD (Caspase Activated DNase) endonuclease to induce nuclear degradation (Widlak and Garrard, 2005).

The caspase cascade is the central pathway of apoptosis and as such a vital control point in its induction. Activation at the level of caspases is highly regulated to insure against either overactive or ineffective employment. Regulation of caspase activation and proteolytic activity occurs at three levels: (A) Expression as an inactive zymogen; (B) Upstream control of apoptotic stimulus (c-FLIP and BCL-2 family proteins); and (C) Direct inhibition of active caspases (IAPs).

1.7 Bcl-2 family proteins

Bcl-2 (B-cell lymphoma 2) family proteins are a group of both pro- and anti-apoptotic proteins which regulate apoptosis upstream of caspase activation (Cory and Adams, 2002). Anti-apoptotic Bcl-2 proteins consist of Bcl-2, Bcl-X_L, Bcl-W, A1 and Mcl-1, all of which contain Bcl-2 homology domains (BH) 1-4. Pro-apoptotic family proteins consist of the three BH domain containing Bax-like proteins (Bax, Bak and Bok) and the BH3-only proteins (Bid, Bim, Bad, Bmf, Hrk, Noxa and Puma).

Bax-like proteins are the primary pro-apoptotic Bcl-2 subfamily, with Bax and Bak reported to be essential for induction of apoptosis in many cell types (Antonsson et al., 2001). Upon intrinsic apoptotic stimuli, cytoplasmic Bax and mitochondrial Bak undergo conformational changes, Bax is translocated to the mitochondria and both oligomerise in the mitochondrial outer membrane (MOM). It is proposed that Bax / Bak permeabilise the MOM to allow release of pro-apoptotic mitochondrial proteins through Bax / Bak only oligomer channels (Antonsson et al., 1997) or via association with a component of the MPTP (Mitochondrial Permeability Transition Pore), VDAC (Voltage Dependant Anion Channel) (Shimizu et al., 1999).

The anti-apoptotic Bcl-2 sub-family are essential regulators of mitochondrial homeostasis and in particular the apoptotic activities of Bax and Bak. BH1, BH2 and BH3 domains of Bcl-2 proteins form a hydrophobic groove that bind to BH3 and form heterodimers with Bax and Bak, inhibiting their ability to oligomerise and form a MPTP (Cory and Adams, 2002). In another level of pathway control, BH3-only sub-family proteins function as regulators of Bcl-2. BH3-only proteins bind and neutralise the anti-apoptotic activity of Bcl-2 subfamily proteins, preventing their antagonism of Bax and Bak. As well as being negative regulators of Bcl-2, the BH3-only sub-family protein BID can induce Bax / Bak oligomerisation. Caspase-8 proteolytically cleaved BID (tBID) acts as a nucleation site for oligomer formation in the MOM (Chou et al., 1999). Caspase-8 cleavage of BID to tBID is a positive feedback loop from the extrinsic apoptotic pathway, induced by ligation of death receptors with their native ligands. The formation of tBID promotes apoptosis induction through the Intrinsic / Mitochondrial apoptotic pathway, further amplifying induction of apoptosis in cells.

1.8 Inhibitor of Apoptosis Proteins

IAP family members are a cellular network of anti-apoptotic proteins which act as central regulators of caspase-mediated cell death. IAPs facilitate this through direct binding or indirectly inhibiting caspase activation and proteolytic activity (Shiozaki et al., 2003, Yang and Du, 2004), abrogating cell death stimuli at the execution phase of apoptosis. This caspase regulation occurs at both the initiator and effector level and IAPs are the only endogenous cellular proteins known to inhibit at both stages of the caspase pathway, making IAPs a robust road block to a wide array of apoptotic stimuli. Beyond this role in caspase inhibition, IAP family members also have been shown to participate in diverse cellular functions, including regulation of NF κ B signalling, mitotic control and protein ubiquitination (Galban and Duckett, 2010).

IAPs are characterised by the presence of one to three Baculovirus IAP Repeat (BIR) domains, an approximate 80 residue Zinc-finger tertiary fold of highly conserved structure and sequence from *Drosophila* to mammals (Mace et al., 2010). Within each BIR domain there contains an IBM (IAP Binding Motif) binding groove, a protein pocket facilitating association of IAPs to binding partners which contain a conserved four residue N-terminal

IBM, and it is this motif which participates in direct inhibition of caspase by IAPs (Huang et al., 2001). BIR containing proteins constitute an eight member protein family in humans (LaCasse et al., 2008) (Illustrated in Figure 1.5). Of these eight IAP family members, X-linked IAP (XIAP / hILP) (Duckett et al., 1996), cellular IAP1 (cIAP1 / HIAP2), cellular IAP2 (cIAP2 / HIAP1) (Rothe et al., 1995), Livin (ML-IAP) (Vucic et al., 2000) and Survivin (TIAP) (Ambrosini et al., 1997) have been implicated as potent inhibitors of cell death and, in numerous cancerous malignancies, are frequently overexpressed, promoting cell survival and conferring apoptotic resistance to chemotherapeutic challenge.

1.8.1 XIAP

XIAP is a 57 kDa member of the IAP protein family, consisting of three sequential amino terminal BIR domains and a carboxy terminal Really Interesting New Gene (RING) finger domain, containing E3 ubiquitin ligase activity (Fig. 1.5). XIAP was the first IAP family member studied for its control over caspase signalling and is thought to be the only mammalian IAP able to directly bind and potently antagonise both initiator and effector caspase proteolytic activity (Deveraux et al., 1998, Deveraux et al., 1997). The third BIR domain (BIR3) of XIAP binds and inhibits caspase-9 (Sun et al., 2000, Deveraux et al., 1999), an initiator caspase central to induction of the caspase cascade downstream of intrinsic apoptotic stimulus. X-Ray crystallographic studies into XIAP-BIR3 and caspase-9 association revealed two sites essential for binding (Shiozaki et al., 2003). The N-terminal sequence of the small subunit of proteolytically processed caspase-9 contains an IBM motif which binds to the IBM binding groove of XIAP-BIR3. A secondary association occurs between the 3 α , 4 α , & 5 α helices of BIR3 and the hydrophobic, homodimerisation region of caspase-9 (Shiozaki et al., 2003). This association sequesters caspase-9 in an inactive, monomeric state and prevents caspase-9 forming an active homodimer and subsequent downstream signalling.

XIAP also directly regulates caspase activity downstream of caspase-9 via antagonism of active caspase-3 and caspase-7 (Scott et al., 2005). The linker peptide region between BIR1 and BIR2 of XIAP and BIR2 itself both participate in direct inhibition of active caspase-3 and caspase-7. The BIR1 and BIR2 linker peptide region resembles a conserved

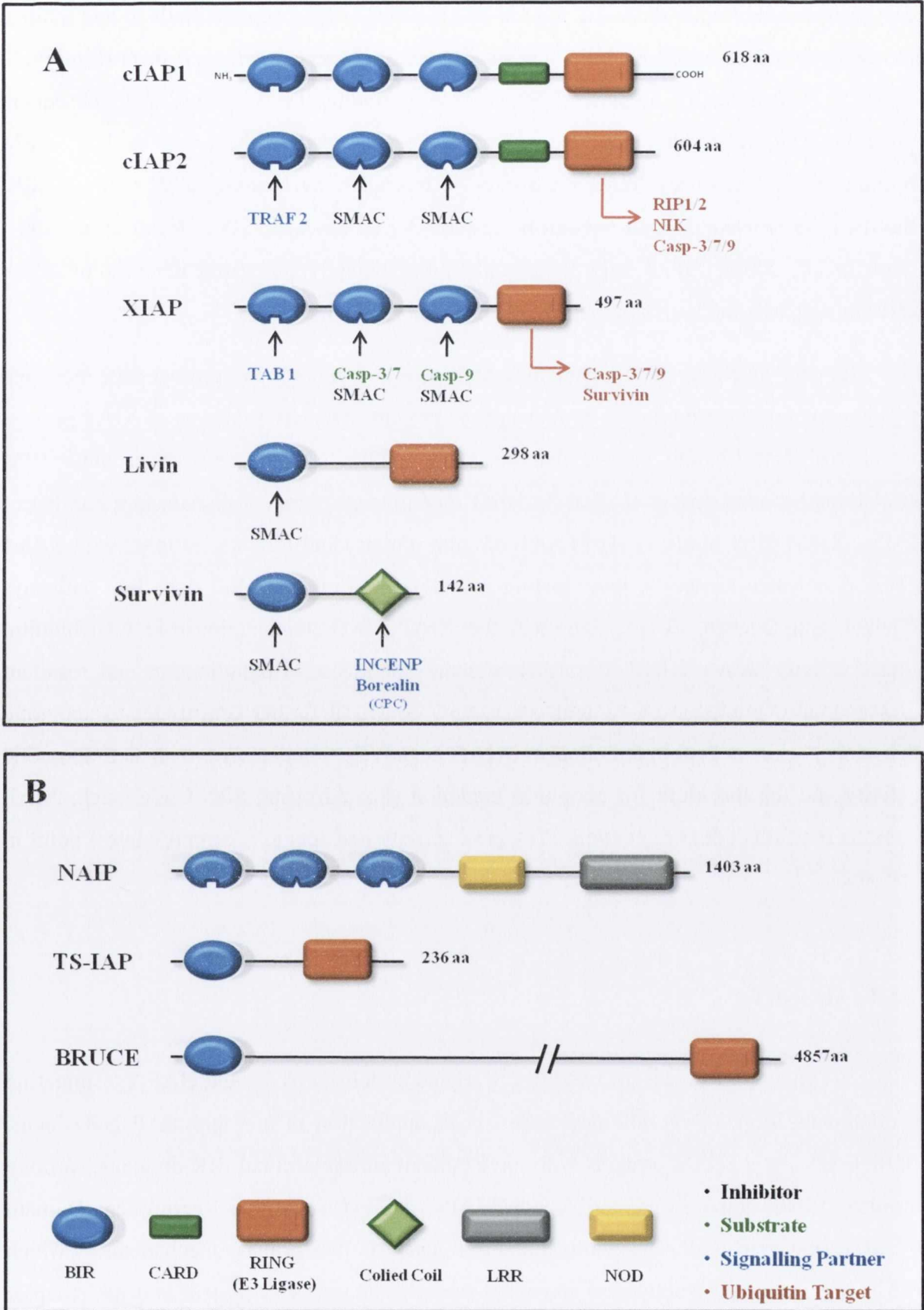


Figure 1.5: Inhibitor of Apoptosis proteins: Domain structure and binding partner.

caspase substrate / inhibitor motif and the XIAP-BIR1/2 linker peptide binds to and inhibits the catalytic site of active caspase-3/7, but not the unprocessed zymogen form (Sun et al., 1999). This sterically hinders effector caspase binding to substrates and subsequent proteolytic cleavage, disrupting execution of the cell death pathway. The BIR2 IBM binding groove stabilises XIAP / caspase-3/7 inhibition by binding an N-terminal IBM motif of the processed small subunit of caspase-3/7 (Chai et al., 2001, Riedl et al., 2001, Scott et al., 2005). Both sites target only proteolytically processed residues of active effector caspases and both associations are necessary for inhibition.

Alternate functions of XIAP include both pro- and anti-apoptotic processes, mediated by the two non-caspase binding domains of the protein. The BIR1 domain of XIAP possess an altered IBM binding groove that lacks the ability to bind caspases or other IBM containing proteins (Lin et al., 2007). BIR1 functions as a binding domain in a number of IAPs. XIAP-BIR1 binds TAB1 (TAK1 Binding protein) and forms a complex with TAK1 (TGF- β -activated kinase-1), participating in TGF- β , NF κ B and JNK signalling pathways (Galban and Duckett, 2010). Through the XIAP-RING domain, intrinsic E3-Ubiquitin ligase activity allows XIAP to further regulate apoptosis. Ubiquitination and resultant proteosomal degradation of effector caspase-3/7 by XIAP further contributes to apoptotic resistance, while autoubiquitination of XIAP negatively regulates its own anti-apoptotic activity and the threshold for apoptotic induction (Suzuki et al., 2001). As such, XIAP exhibits multifunctional regulation of caspase activity and acts as a central control point in apoptosis.

1.8.2 cIAP-1/2

Cellular IAPs consist of two closely related proteins, cIAP-1 and cIAP-2, originating in mammals from an evolutionary recent gene duplication of a single cIAP gene locus. Both cIAPs are ~ 72kDa proteins with three tandem amino terminal BIR domains, carboxy terminal RING domain and, unlike other IAPs, possess a Caspase Recruitment Domain (CARD) (LaCasse et al., 2008), illustrated in figure 1.5. The primary mechanism by which cIAPs regulate caspase activation and apoptosis is as an accessory protein in death receptor signalling, specifically to the Tumour Necrosis Factor alpha Receptor Super Family (TNFRSF) (Silke and Brink, 2010, Rothe et al., 1995). Upon TNF- α ligand binding, cIAP

is recruited to the receptor complex by TNF receptor associated factor-2 (TRAF-2), by association of the TRAF-N domain to BIR1 of cIAP (Samuel et al., 2006). E3-Ubiquitin ligase activity of the cIAP RING domain promotes K63 polyubiquitination of another TNFR accessory protein, Receptor Interacting Protein-1 (RIP1). This RIP1 modification promotes RIP1 TNFR recruitment, NF κ B signalling and prevents RIP1 dependant effector caspase-8/10 activation (Bertrand et al., 2008).

cIAP E3-Ubiquitin ligase activity can also mediate other diverse pro-apoptotic and signalling events. Direct binding and ubiquitination of caspases promotes their proteasomal degradation (Suzuki et al., 2001). cIAPs participate in innate immune signalling in a similar way to RIP1 regulation. Pattern recognition NOD like receptors, NOD1 and NOD2, bind their cognate bacterial ligand and recruit cIAP1/2 to induce polyubiquitination of RIP2, allowing accessory protein association and downstream NF κ B and MAP Kinase signalling (Bertrand et al., 2009).

While cIAPs, like XIAP, possess IBM binding grooves and bind to caspases, unlike XIAP, their ability to act directly as potent caspase inhibitors is not fully established (Eckelman and Salvesen, 2006). Experiments performed in simplified Yeast *in vitro* systems indicate that cIAPs can inhibit caspase-9 activity, however, whether this is achieved via direct antagonism of caspase-9, upstream signalling partner ubiquitination control or ubiquitin tagging and proteosomal degradation of caspase, by the cIAP-RING domain, is still to be answered (Wright et al., 2000). Protein alignment studies show that while the BIR2 and BIR3 IBM binding groove of XIAP, cIAP1 and cIAP2 are highly conserved, the secondary inhibitory sites of caspase-9 (BIR3 α -helices) and caspase-3/7 (BIR1-BIR2 linker peptide) on cIAPs lack essential residues present in XIAP necessary for effective inhibition (Eckelman et al., 2006). This indicates that cIAPs may bind caspases via their IBM binding groove, but lack the inhibitory capacity (and protein stability (Eckelman and Salvesen, 2006)) of XIAP to act as potent direct inhibitors of caspase. In two more recent studies, it was reported that cIAP-1 was able to directly inhibit effector caspase activation through two distinct alternate mechanisms. *Burke et al* report that the BIR2 and BIR3 domains of cIAP-1 interact with oligomerised active caspase-9 of the apoptosome and block downstream caspase-3/7 activation (Burke et al., 2010), while a study by *Choi et al* showed that cIAP-1 binds and ubiquitin tags for degradation active caspase-3 and caspase-7 when present in proteolytically activated intermediate states (Choi et al., 2009). While a conclusive

definition on the subject of cIAP / caspase binding has not been reached, it is believed that cIAPs predominantly mediate caspase control at the extrinsic pathway of apoptosis.

1.8.3 Survivin

Survivin, the smallest of the IAPs at 16.5 kDa, is a unique family member in terms of its cellular functions and differential expression. Survivin expression is absent in normal tissue, except during embryonic development and in adult fast proliferating tissues, like cells of the liver and gastrointestinal mucosa (Pennati et al., 2007). However, in numerous cancers, Survivin is overexpressed (Ambrosini et al., 1997), contributing to apoptotic resistance (Islam et al., 2000, Zaffaroni et al., 2002) and if inhibited or downregulated sensitises cells to chemotherapy (Ambrosini et al., 1998). While other IAPs are known to have similar significance in cancer, Survivins specificity is of particular note. Survivin is also unique by way of its novel mechanism of caspase inhibition and its secondary function in mitotic regulation (Mita et al., 2008).

The Survivin gene expresses three splice variants of the protein –Survivin-wild type, Survivin-2B and Survivin- Δ Ex3 – and forms functional homodimers in cells. Each Survivin monomer contains a single BIR and coiled coil (CC) domain which mediate the caspase inhibition and mitotic stabilisation of Survivin, respectively (Altieri, 2003). Survivin-BIR is most structurally similar to XIAP-BIR3 and binds caspase-9, but lacks the conserved residues necessary for inhibition (Eckelman et al., 2006). However, Survivin can inhibit caspase-9 when associated with cofactor Hepatitis B X-interacting protein (HBXIP) or when bound to XIAP, forming a more active and stable caspase inhibiting IAP – IAP complex (Dohi et al., 2004).

As well as tissue specific expression, Survivin expression is highly regulated within cell cycle stages (increased metaphase expression) and localised to cellular compartments (“mitochondrial pools” in cancer to inhibit caspase upon intrinsic apoptotic stimulus (Pennati et al., 2007)). Transcriptional and post-translation modifications by Cyclin dependant kinase-1 (CDK1) insure Survivin expression and protein stability in metaphase, facilitating Survivin regulation of mitotic assembly (Altieri, 2003). Survivin localises to microtubules of the mitotic spindle and the kinetochore chromosomal passenger complex, via the CC domain, and helps regulate spindle binding and chromosome segregation (Skoufias et al., 2000).

1.8.4 Livin

Livin, another IAP of significance in cancer, is encoded as two splice variants in mammals, Livin- α and Livin- β , both consisting of a single BIR and RING domain. Livin- α displays potent anti-apoptotic activity, however, similar to cIAP and Survivin, Livin is a weak binder and inhibitor of caspases due to alterations in secondary binding residues of the BIR domain, indicating Livin anti-apoptotic activity is not mediated via direct caspase inhibition (Vucic et al., 2000). Livin does bind IAP antagonising protein SMAC / DIABLO (Second Mitochondrial-derived Activator of Caspase / Direct IAP-binding protein with Low PI) with nanomolar affinity and one proposed mechanism of its anti-apoptotic activity is by Livin sequestration of SMAC, preventing SMAC-XIAP interaction and release of XIAP bound caspase (Vucic et al., 2005).

Conversely, Livin- β has pro-apoptotic function in cells. Truncated forms of Livin- β reduce tumour growth and induce cell death (Abd-Elrahman et al., 2009). This duality in Livin activity explains how in a clinical context, overexpression of Livin can confer resistance in melanoma (Gong et al., 2005) and colon cancers (Wang et al., 2010), but high Livin expression is a positive prognostic factor in other forms of cancer (Haferkamp et al., 2008).

1.8.5 NAIP, IP2 and BRUCE

Other IAP members, neuronal apoptosis inhibitory protein (NAIP), testis specific IAP (TS-IAP / ILP2) and BIR-containing ubiquitin conjugating enzyme (BRUCE / Apollon), possess at least one BIR domain and, as such, caspase binding potential – BIR3 of NAIP has recently been reported to bind and inhibit proteolytic activation of caspase-9 (Davoodi et al., 2010) - however, they are believed to be predominantly involved in alternate cellular functions. These include innate immune signalling and neuroprotection (NAIP), cytokinesis (BRUCE) and spermatogenesis (TS-IAP) (LaCasse et al., 2008).

1.9 SMAC

SMAC is head of a class of IBM-containing proteins which act as antagonists of IAPs – pro-apoptotic agents, inhibiting the inhibitors of caspase activation. The 25 kDa

protein is encoded at a nuclear locus and localised to the mitochondrial inter-membrane space (IMS) by an N-terminal mitochondrial targeting sequence (MTS) (Du et al., 2000). Cleavage of the MTS allows exposure of the N-terminal IBM four peptide sequence – Alanine, Valine, Proline and Isoleucine (AVPI) – and homodimerisation of SMAC occurs, forming a highly stable and active conformation able to bind IAPs (Chai et al., 2000). Apoptotic stimuli inducing mitochondrial outer membrane permeabilisation (MOMP) results in release of mitochondrial stores of SMAC. Released active SMAC is able to antagonise IAP function by three separate mechanisms; (A) Competitive inhibition of the caspase / XIAP interaction (Chai et al., 2000), (B) Induced autoubiquitination and degradation of cIAPs (Yang and Du, 2004) (C) Binding and antagonism of Survivin and Livin anti-apoptotic activity (Oikawa et al., 2010, Vucic et al., 2002).

Caspase sequestration by XIAP is facilitated by IBM binding to the IBM binding groove of BIR domains. The caspase-9 IBM is the tetrapeptide sequence of Ala-Thr-Pro-Phe (ATPF) (Shiozaki et al., 2003), highly analogous to the AVPI IBM sequence of SMAC. Detailed structural studies have shown SMAC-AVPI to be a potent agonist for the IBM binding groove of BIR2 (Chai et al., 2000) and BIR 3 (Liu et al., 2000) of XIAP, with similar affinities for each. Mechanistic experiments also showed that SMAC-AVPI relieves XIAP inhibition of active caspase-9 and effector caspase-3/7 through competitive inhibition of caspase binding to their associate BIR domain. One SMAC homodimer (two SMAC proteins; two AVPI sequences) can compete for binding to both BIR2 and BIR3 domains of a single XIAP molecule (Huang et al., 2003). This releases active initiator and effector caspases from IAP sequestration, which are free to implement the execution stage of apoptosis.

SMAC also regulates cIAP's indirect inhibition of caspase-8 activity through binding of SMAC-AVPI to the BIR2 and BIR3 domain binding grooves of cIAP-1/2. A SMAC homodimer binds BIR2 and BIR3 of the same cIAP, inducing a conformational change that triggers activation of the RING E3-Ubiquitin ligase and autoubiquitination of cIAPs, tagging them for rapid proteosomal degradation (Yang and Du, 2004). Loss of cIAPs disrupts the negative regulation of RIP1 dependant caspase-8 activation. SMAC does not induce autoubiquitination of RING domain containing Livin or RING absent Survivin, but splice variant SMAC3, not wild type SMAC, induces autoubiquitination and degradation of XIAP (Fu et al., 2003).

As a regulator of IAPs, factors participatory in tumorigenesis and apoptotic resistance in cancer, SMAC expression levels can be a prognostic factor in some cancers. Its activity has a significant effect on IAP function and is a potent inducer of apoptosis. An altered or a mutated expression profile of SMAC can exacerbate IAP mediated tumour resistance and promote cancer persistence and progression. Studies on Colon (Endo et al., 2009), Renal (Mizutani et al., 2005) and Non-Small Cell Lung Carcinomas (NSCLC) (Zhao et al., 2006) report alterations and reduction in SMAC expression is prognostic for increased tumour progress and resistance. This relates both to these cancers having reliance on IAPs survival and reduced ability of SMAC to antagonise IAP function, diminishing the apoptotic potential within the tumour.

Other IBM containing IAP antagonists' include OMI/HtrA2, GSPTq/eRF3 and XIAP associated factor 1 (XAF1) (LaCasse et al., 2008). OMI/HtrA2, present in the IMS, can antagonise XIAP – caspase binding, GSPTq/eRF3 displays anti-IAP activity localised to the endoplasmic reticulum (ER), while XAF1 can bind XIAP and induce E3 ligase ubiquitination and subsequent degradation of Survivin (Arora et al., 2007).

1.10 Apoptotic signalling pathways

As outlined above, apoptosis is a complex cellular process of PCD, whose induction, regulation and execution is controlled by the elaborate interplay of caspase, Bcl-2, IAP and IBM protein networks. This intricate patchwork of signalling and inhibitory cascades, however, can be delineated into two primary pathways of apoptotic induction – (A) The Intrinsic / Mitochondrial apoptotic pathway and (B) The Extrinsic / Death Receptor apoptotic pathway.

1.10.1 Intrinsic / Mitochondrial apoptotic pathway

The Intrinsic / Mitochondrial apoptotic pathway is one arm of the apoptotic machinery which responds to intracellular apoptotic insults and modulates a cell death response through the mitochondria (illustrated in Fig. 1.6). This organelle is a key player in detection and response to cellular perturbation or induction of forms of cellular stress. A wide array of intrinsic apoptotic stimuli converges at the point of Bax / Bak activation.

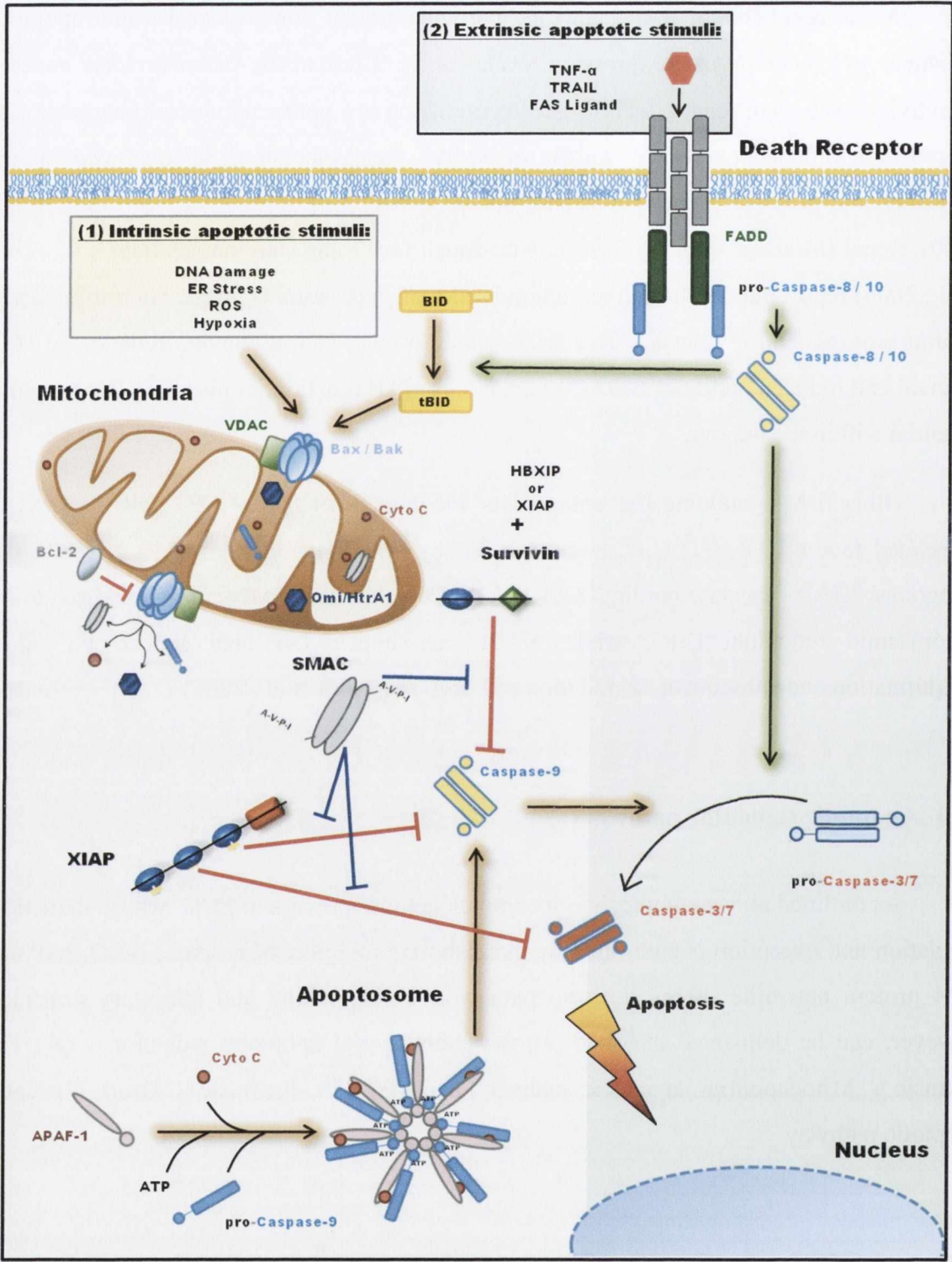


Figure 1.6: Extrinsic and Intrinsic pathways of apoptosis.

Oligomerisation and MPTP formation allows for MOMP and release of pro-apoptotic proteins sequestered to the mitochondrial IMS. These include pro-caspases, IBM-containing proteins (SMAC, OMI/HtrA2), endonucleases and cytochrome c (cyto c), which functions as the continuation of the apoptotic signal (Jourdain and Martinou, 2009).

Released cyto c interacts with a cytoplasmic protein APAF-1 (Apoptotic Protein Activating Factor – 1) and ATP (Adenosine 5'-triphosphate) to form a multi-domain apoptotic signalling platform called the apoptosome (Riedl and Salvesen, 2007). ATP binds the NOD domain of APAF-1 and with sufficient apoptotic induction and cyto c present in the cytoplasm, cyto c binds the WD40 domain of APAF-1 and causes cyto c / APAF-1 / ATP complex oligomerisation into a seven member wheel-shaped structure (Yu et al., 2005). The apoptosome acts as a platform for pro-caspase-9 monomer binding, autocatalytic activation converting the pro-caspase-9 zymogen to proteolytically active caspase-9 and formation of an active caspase-9 protein homodimer. Active caspase-9 can proteolytically activate effector caspases-3/7 and signal intrinsic apoptotic stimulus to its execution.

1.10.2 Extrinsic / Death-receptor apoptotic pathway

As well as induction of apoptosis occurring in response to cellular damage or stress, cells can be induced to undergo apoptosis by ligation of cell surface death receptors by their cognate extracellular death ligands, the second signalling cascade of apoptosis entitled the extrinsic / death-receptor pathway (illustrated in Fig. 1.6). This second mechanism of apoptotic induction is employed in the adaptive immune response for destruction of infected or mutated cells and prevention of auto-reactive T cell generation (Brunner et al., 2003) and is also an apoptotic strategy exploited in anti-cancer therapies (Leong et al., 2009).

Death receptors, or the tumour necrosis factor receptor super family (TNFRSF), consist of six distinct death domain (DD) containing transmembrane structures which bind extracellular death ligands, such as FAS ligand, TNF- α and TRAIL / Apo2 (TNF related apoptosis inducing ligand) (Mahalingam et al., 2009). Binding and trimerisation of death-receptor / ligand complexes forms an intracellular signalling platform which recruits DD containing accessory protein FADD (Fas associated protein with death domain). FADD in turn recruits initiator pro-caspases-8 / 10 through death effector domain (DED) dimerisation,

forming the death inducing signalling complex (DISC). Proximal localisation of pro-caspase-8 / 10 via this death receptor signalling scaffold allows auto-catalytic activation of caspase and dissociation from DISC (pro-domain contains the DED). Active caspase-8 / 10 directly trigger apoptosis through proteolytic activation of effector caspases and also modulate intrinsic pathway signalling by cleaving Bid to active tBid, amplifying apoptotic stimulus via this intrinsic signalling loop.

1.11 IAP regulation of Intrinsic and Extrinsic apoptotic pathways

IAP family proteins, in particular XIAP and cIAP-1/2, play an important role in the regulation of apoptotic induction via the Intrinsic / Mitochondrial apoptotic pathway and the Extrinsic / TNF- α receptor apoptotic signalling pathway. Control and induction of the Intrinsic / Mitochondrial apoptotic pathway is a multi-faceted process in the cell, with a multitude of proteins participating in ensuring appropriate activation in response to stimuli. IAP proteins function as negative regulators of caspase-dependant execution of the pathway. Once Bcl-2 family signalling has committed to mitochondrial release of cyto c, signalling continues to apoptosome-mediated initiator and subsequent effector-caspase activation. IAPs Survivin and, in particular, XIAP are able to control the caspase cascade, inhibiting at both the initiator and effector level. XIAP can completely negate cell death signalling at the very apical edge of apoptotic execution, inhibiting initiator caspase-9 and effector caspase-3/7, silencing the caspase cascade induced in response apoptotic stimuli. IAP negative regulation of the intrinsic apoptotic pathway occurs with IBM-containing proteins released from the mitochondrial inter-membrane space. Pro-apoptotic IMS proteins like SMAC, are able to antagonise XIAP control on the intrinsic / mitochondrial pathway and conserve signalling commitment to apoptosis.

The Extrinsic / Death Receptor (TNFR) signalling pathway has pleiotropic functions in cells, participating in immunity and survival signalling, as well as being an inducer of apoptosis. IAP family members participate significantly in regulation of these signalling events. It has been discussed extensively already how XIAP, Survivin and Livin inhibit caspase activity and as such can impede extrinsic apoptosis downstream of stimulus. Also, cIAP1 and cIAP2, their initial discovery being as part of a TNFR signalling complex (Rothe et al., 1995), can prevent RIP1 dependant caspase-8/10 activation. Supplementary to

caspase control at the TNFR, cIAP1/2 plays an essential regulatory role in NFκB activation, a signalling cascade with both survival and apoptotic consequences for cells.

TNFR signalling can facilitate both canonical (RIP1 dependant) and non-cannonical (NFκB Inducing Kinase – NIK dependant) NFκB signalling pathways (Silke and Brink, 2010). As part of the TNFR signalling complex, cIAP1/2 positively regulates canonical and negatively regulates non-canonical signalling through RING-E3 Ligase K63 and K48 polyubiquitination of RIP1 and NIK, respectively (Ea et al., 2006, Vince et al., 2007), (illustrated in Fig. 1.7). IAP-1/2 control of TNFR / NFκB signalling is important in relation to regulation of caspase-8 activation and induced expression of tumour survival factors. K63 polyubiquitination chains of RIP1 function as a docking platform for signalling partners, IKK complex (IKK-α; IKK-β; IKK-γ) and TAK1, activation of which leads to phosphorylation of the p50/RelA NFκB heterodimer inhibitor, IκB. Phosphorylation of IκB leads to K48 Ubiquitin targeting for proteosomal degradation, liberating NFκB to bind IκB response elements and transcribe cell survival factors like c-FLIP, IL-8, IAPs and TNF-α. K48 polyubiquitination of NIK, similarly to IκB ubiquitination, targets NIK for proteosomal degradation, preventing non-cannonical NFκB signalling.

Release of SMAC from the IMS induces autoubiquitination and degradation of cIAPs (Yang and Du, 2004), which significantly modulates TNFR signalling and its functional outcome (illustrated in Fig. 1.8). A study by Vince *et al* using transient transfection of human SMAC and the *Drosophila* analogue Grim showed that loss of cIAPs caused activation of both canonical and non-cannonical NFκB pathways (Vince et al., 2007). Loss of cIAP negative regulation allows accumulation of NIK and activation of the RelB / p52 NFκB heterodimer. Both pathways are involved in *de novo* synthesis of TNF-α, which subsequently undergoes autocrine stimulation of TNFR (Vince et al., 2007). Loss of RIP1 ubiquitination and recruitment to the TNFR due to cIAP degradation, allows release and formation of the RIP1-FADD-pro-Caspase-8/10 signalling complex II (Micheau and Tschopp, 2003). This activates initiator caspases-8/10 and induces apoptosis.

1.12 SMAC mimetic therapy

A vast amount of genetic and molecular evidence over the last decade has confirmed the role of IAP family members in the apoptotic resistance phenotype of cancer.

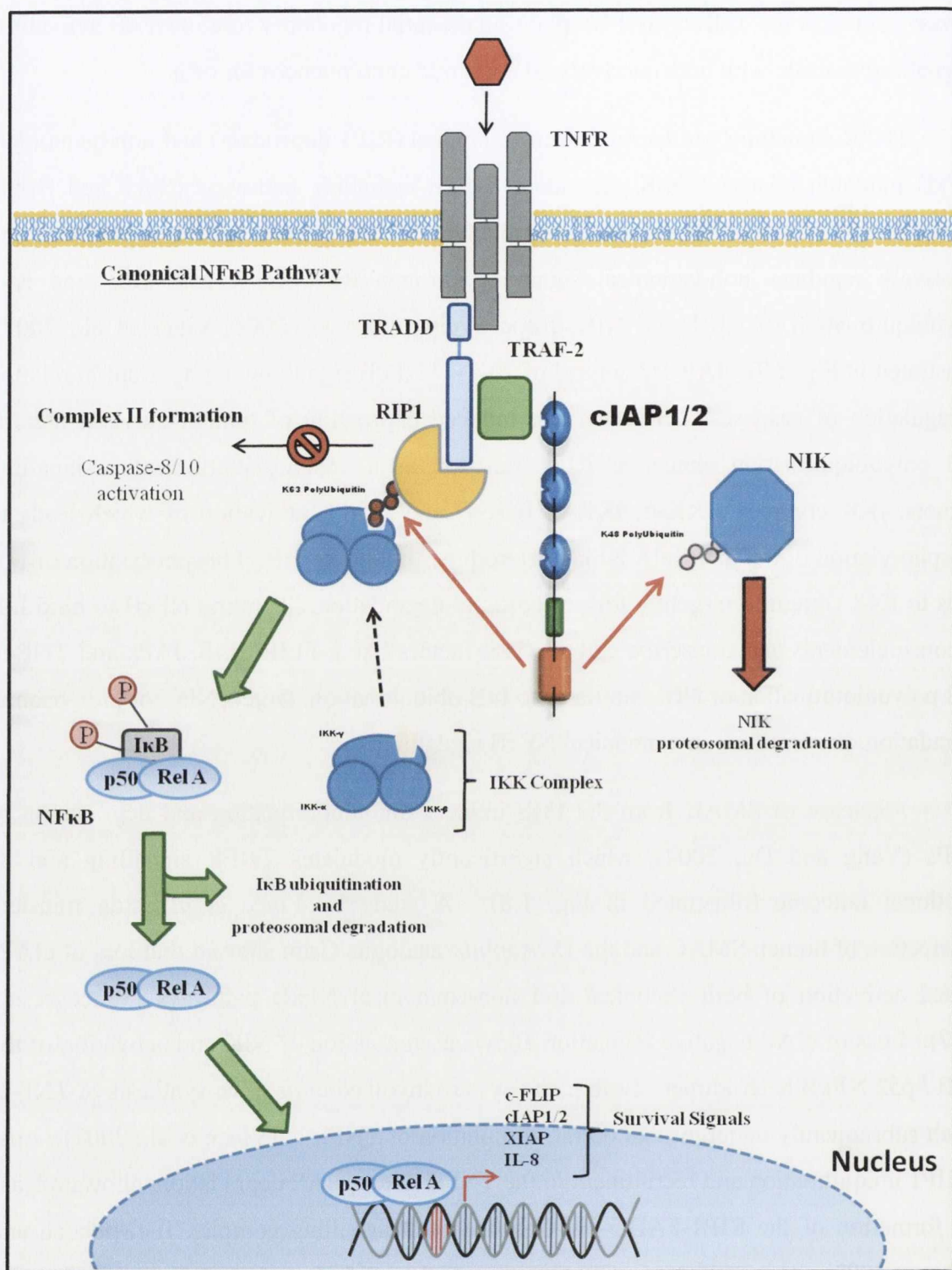


Figure 1.7: c-IAP regulation of TNF- α signalling.

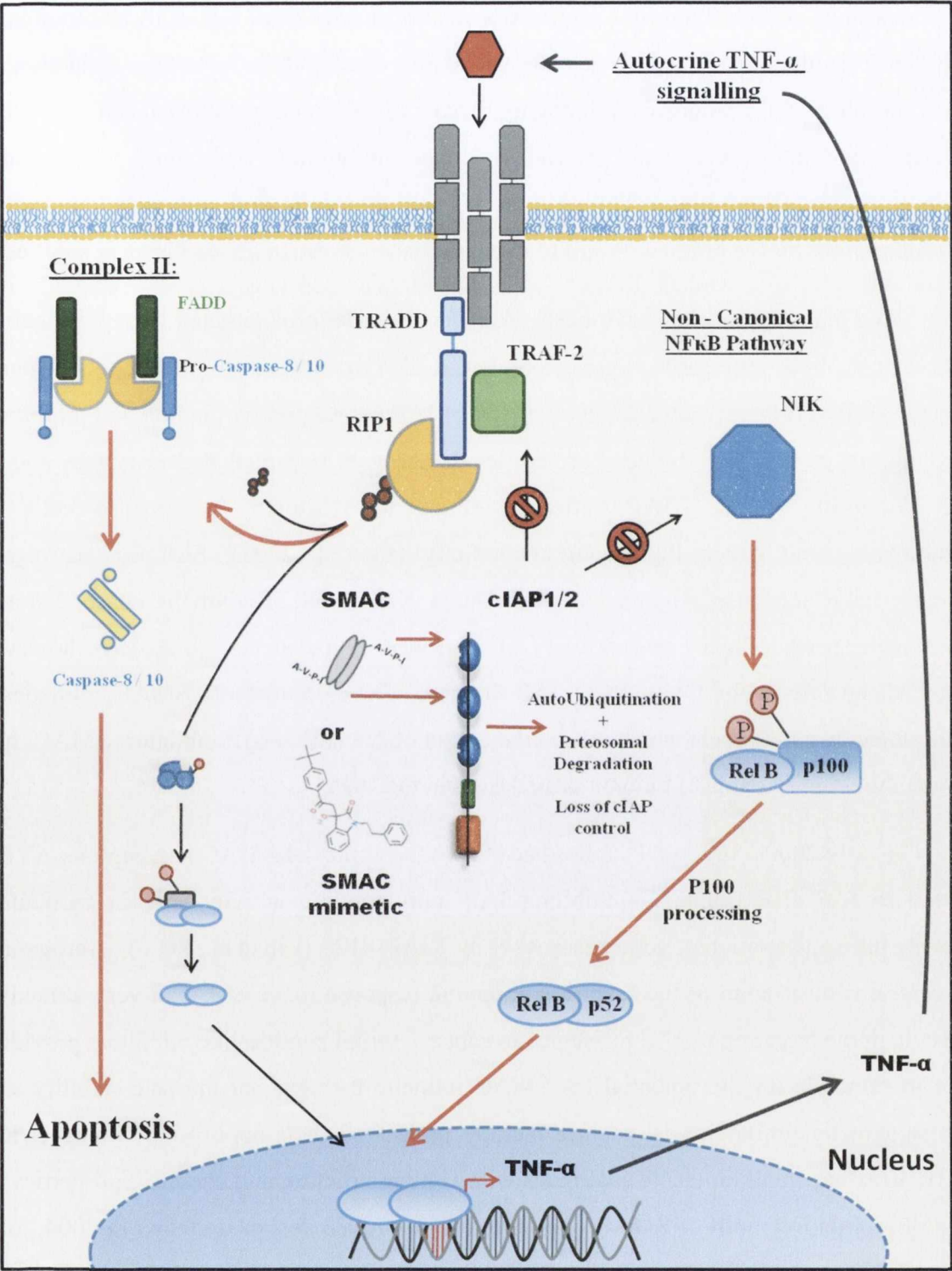


Figure 1.8: SMAC inhibition of c-IAPs and autocrine TNF-α signalling.

Overexpression of XIAP, Survivin and cIAPs and the fundamental role of these proteins in multi-level regulation of caspase activation and the threshold for apoptotic induction in cancer, promotes poor prognosis in many malignancies. As such, many cancers rely on this cellular alteration for survival and growth. However, inhibition of IAPs could preferentially kill these cancers by hitting their reliance on this cellular mechanism, making IAPs a prime molecular target for the rational design of chemotherapeutic drugs for such cancer subtypes.

Two main approaches have been taken for the design of targeted therapies against IAPs – (A) IAP antisense oligonucleotides; and (B) SMAC mimetics. Antisense oligonucleotides (oligos) cause direct silencing of IAP expression by binding and inhibiting translation of its mRNA. Reduced protein levels abrogate IAP-mediated resistance and in lung xenograft studies, XIAP antisense oligos were shown to sensitise cells to chemotherapy, and induce single agent cytotoxicity (Hu et al., 2003). Eli Lilly and Aegea currently have antisense oligonucleotides against XIAP and Survivin in Phase 2 trials. Issues with speed, efficacy and delivery of antisense oligos in the Clinic have however stilted advancements for this type of IAP therapy. Development of “SMAC mimetics”, small molecule compounds which mimic the action of the native IAP inhibitor, SMAC, has become the primary focus of rational drug design in this area.

In early 2000s, structural studies had shown the ability of SMAC tetrapeptide, AVPI, to bind to IAP BIR domains, inhibiting IAP anti-apoptotic activity and, in particular, relieving the sequestration of active caspase-9 by XIAP-BIR3 (Liu et al., 2000). Abrogating this central control point in the caspase / apoptotic response made SMAC a very attractive molecule in the targeting of IAP resistance in cancer. Initial peptide-like inhibitors provided proof in principle for the potential for SMAC mimetic therapy, but intrinsic stability and pharmacokinetic limitations in peptide therapy prohibited their application (Fulda et al., 2002). Creating small molecule analogues with similar structure and chemical properties to the AVPI inhibiting motif of SMAC constituted an improved design strategy. In 2004, four separate studies by Oost (Oost et al., 2004), Sun (Sun et al., 2004), Nikolovska-Coleska (Nikolovska-Coleska et al., 2004) and Li (Li et al., 2004), described the discovery of SMAC mimetic XIAP-BIR3 inhibitors based on the AVPI structure – employing structural-based design and virtual screening of compound databases onto an AVPI-BIR3 pharmacophore. Sun, Nikolovska-Coleska and Li showed enhanced apoptotic response to chemotherapy with SMAC mimetic co-treatment in cancer models. Oost and Nikolovska-Coleska showed single agent apoptotic induction by SMAC mimetics, with the Oost study showing no

correlation with XIAP burden, suggesting alternate targets than XIAP-BIR3 (Oost et al., 2004). Interestingly, Li *et al* SMAC mimetics displayed enhancement of extrinsic / death receptor mediated cell death. SMAC mimetic treatment sensitised cells to TRAIL and TNF- α mediated apoptosis, inducing a sequential caspase-8 and caspase-3 activation pathway. This response was independent of targeting of XIAP and suggested the potential for SMAC mimetics to work through cIAP targeting to enhance death receptor mediated apoptosis (Li et al., 2004).

In 2007, three simultaneous publications in *Cell* confirmed an alternate target for SMAC mimetics being cIAP-1/2 (Petersen et al., 2007, Varfolomeev et al., 2007, Vince et al., 2007). SMAC mimetics developed in each lab showed identical function to native SMAC in cultured cells. Mimetics disrupted cIAP-1/2 regulation of caspase-8/10 activation and NF κ B signalling through binding to BIR domains and inducing autoubiquitination and subsequent proteosomal degradation. Loss of c-IAPs allows apoptotic Complex II formation, in response to TNF- α ligation and downstream apoptotic induction via caspase-8/10. Single agent apoptotic induction by SMAC mimetics was also observed in some susceptible cell types. This was attributed to deregulation of NF κ B signalling due to loss of cIAPs. Activated canonical and non-canonical NF κ B signalling induced expression and autocrine signalling of TNF- α , which triggers caspase-8/10 mediated apoptosis in susceptible cells (Petersen et al., 2007). A later study by Wang *et al* in *Cell* (Wang et al., 2008), described SMAC mimetic mediated TNFR cell death as a distinct pathway to caspase-8/10 activation.

Numerous studies have also investigated the specific targeting of Survivin with small molecules to overcome its association with resistance and poor prognosis (Islam et al., 2000, Zaffaroni et al., 2002). As well as the potential of direct targeting of Survivin with SMAC mimetic, indirect inhibition of Survivin promoter sequence (Nakahara et al., 2007) and HSP90 chaperone proteins (Plescia et al., 2005) are under clinical study.

1.12.1 SMAC mimetic therapy and MPM

Recently there has been growing evidence for the role of IAPs mediating apoptotic resistance in MPM. IAP expression studies in MPM tumours have shown abundant expression of cIAPs, XIAP and Survivin (Gordon et al., 2007a, Kleinberg et al., 2007). In

particular, high expression levels of cIAP-1 and Survivin have been correlated with poor patient survival and tumour resistance in MPM (Gordon et al., 2002, Gordon et al., 2007a). This reliance on IAPs for tumour survival in MPM presents an interesting strategy for using SMAC mimetic therapy to overcome resistance in this highly aggressive and incurable cancer.

1.13 The Warburg Effect: Aerobic glycolysis and resistance in cancer

The Warburg effect is the phenomenon in cancer and normal proliferating cells in which ATP production occurs predominantly through Glycolysis (~80%) as opposed to Oxidative Phosphorylation (Ox Phos), even in the presence of abundant levels of O₂. The Warburg effect results in the fermentation of glucose to lactate, as opposed to the full catabolism of the carbon source into reduction intermediates for Ox Phos ATP generation. The phenomenon was first described by its discoverer, Otto Warburg, in 1930 after observing this altered metabolic framework in cancer cells and has subsequently been determined as a characteristic feature of cancer metabolism (Zhivotovsky and Orrenius, 2009). The effect is so ubiquitous, that increased glycolytic flux in cancer is used as a diagnostic tool to detect the presence, location and size of tumours using a fluorescent glucose derivative (F-deoxyglucose) and Positron Emission Tomography (PET) imaging (Lammering et al., 2010). The Warburg effect was first predicted to be due to defects occurring in the electron transport chain during tumorigenesis. However Ox Phos has been shown to still occur in cancer cells and its restricted process is still essential in maintaining the aerobic glycolytic phenotype. The shift from energy production via Ox Phos to glycolysis is an adaptation in cancer which provides a distinct advantage in energy metabolism, cellular proliferation and apoptotic resistance, even though glucose fermentation to lactate, instead of feeding into the citric acid cycle as pyruvate, is a far less efficient method of energy production (Glycolysis produces a net gain of 2 ATP compared to a possible 36 ATP with complete catabolism of glucose via Ox Phos).

1.13.1 Advantages of an aerobic glycolysis metabolic phenotype in cancer

The reasons and benefits for an aerobic glycolytic phenotype being so prevalent in cancer are as yet unconfirmed but, compelling theories have been reported for the existence

of this phenomenon. While aerobic glycolysis is a deficient mechanism of energy production in cells, the metabolic requirements of fast proliferating cancer cells are different to normal somatic tissue and extend beyond ATP demands. Fast proliferating cells quickly replicate all their cellular components through successive cycles of mitosis, placing a huge requirement on the biosynthesis of cellular intermediates like nucleotides, lipids, amino acids and reducing components (NADPH), to drive tumour growth. As such, the primary selective pressure in the metabolism of cancer cells is the availability of carbon source for the production of cellular components, as opposed to ATP energy demand. Glucose is the primary carbon and energy source in cells. Instead of fully catabolising glucose to 36 ATP and 6 CO₂, glycolytic flux is redirected allowing intermediates enter pathways of nucleotide, amino acid and lipid synthesis. Loss of carbon source by way of CO₂ excretion is a greater inefficiency in proliferating cancer cells than deficient energy production by aerobic glycolysis (Vander Heiden et al., 2009).

After catalysis of glucose to glucose-6-phosphate (G-6-P) by Hexokinase, G-6-P can be diverted into the Pentose Phosphate pathway, producing nucleotides and reduction components for cell proliferation. Citric acid cycle intermediates, citrate and acetyl CoA, are also trafficked into amino acid and fatty acid / lipid synthesis pathways. A large proportion of glucose uptake culminates in fermentation to lactate which produces 2 ATP for energy requirements, helps maintain NAD⁺/NADH cycling and is quickly excreted from the cell. Excretion of lactate losses three carbons per molecule, however it is essential to prevent glycolysis inhibition and maintain fast cell proliferation. It has been reported that excreted lactate can be taken up and catalysed in other heterogeneous parts of the tumour mass and excreted lactate can be toxic to surrounding tissue and immune cells.

1.13.2 Mitochondrial-associated Hexokinase-I/II is essential for aerobic glycolysis and mediates a metabolic advantage in cancer

Hexokinase has an important role in facilitating aerobic glycolysis in cancer and confers a number of metabolic and apoptotic resistance advantages in tumours. Hexokinase (HK) mediates the first committal step in glycolysis, catalysing the ATP-dependant phosphorylation of glucose to glucose-6-phosphate, and this process is rate determinant for total cellular glycolytic flux, energy production and biosynthesis of cellular components

(Wilson, 2003). HK exists as four enzyme isoforms (HK-I – HK-IV) with close structural and functional similarity, however alternate glucose affinity and tissue distribution allows for individual metabolic roles within cells (Wilson, 2003). HK-I and HK-II, high affinity glucose binding isoforms, are essential components in facilitating aerobic glycolysis in cancer cells and are overexpressed in many cancer sub-types. These HK isoforms uniquely express a 15 Amino Acid (AA) N-terminal sequence which facilitates localisation of HK to the mitochondria (Mito). HK-I/HK-II localisation to the Mito positions HK for preferential access to Ox Phos generated ATP, required for initiating glycolysis. Overexpression of Mito localised HK, continuous ATP exposure and abundant glucose levels facilitates an increased glycolytic rate, even in the presence of O₂. High glycolytic flux feeds the metabolic needs of fast proliferating cancer cells (i.e.: Biosynthesis) and provides a mechanism of energy production for insufficiently vascularised solid tumours under hypoxic conditions. As such, Mito localised HK is integral to aerobic glycolysis and cancer metabolism. It provides a distinct metabolic advantage in malignant neoplasms and it is reported that up to 75% of cellular HK is localised to the mitochondria in some tumours (Linden et al., 1982).

1.14 Association of Hexokinase-I/II with the Voltage Dependant Anion Channel -1 and apoptotic resistance in cancer

At the mitochondrion, HK associates with the Voltage Dependent Anion Channel - 1 (VDAC), an abundant mitochondrial outer membrane (MOM) protein and central component of the MPTP. The hydrophobic 15 AA N-terminal sequence of HK inserts into the MOM bilayer and interacts with transmembrane β -barrel domains of VDAC. Site directed mutagenesis studies have also revealed association points of glutamate, lysine and aspartic acid residues of the 1st and 4th cytoplasmic loops of VDAC essential for HK localisation to the mitochondria (Abu-Hamad et al., 2008) (illustrated in Figure 1.9.A). Binding of HK to VDAC culminates in the oligomerisation of the complex into tetrameric structures and also results in the association of VDAC with another member of the MPTP, the Adenine Nucleotide Transporter (ANT). The inner to outer mitochondrial membrane complex of HK, VDAC and ANT forms at dual mitochondrial membrane contact sites. While reports are conflicted (Azoulay-Zohar et al., 2004), a general consensus exists that

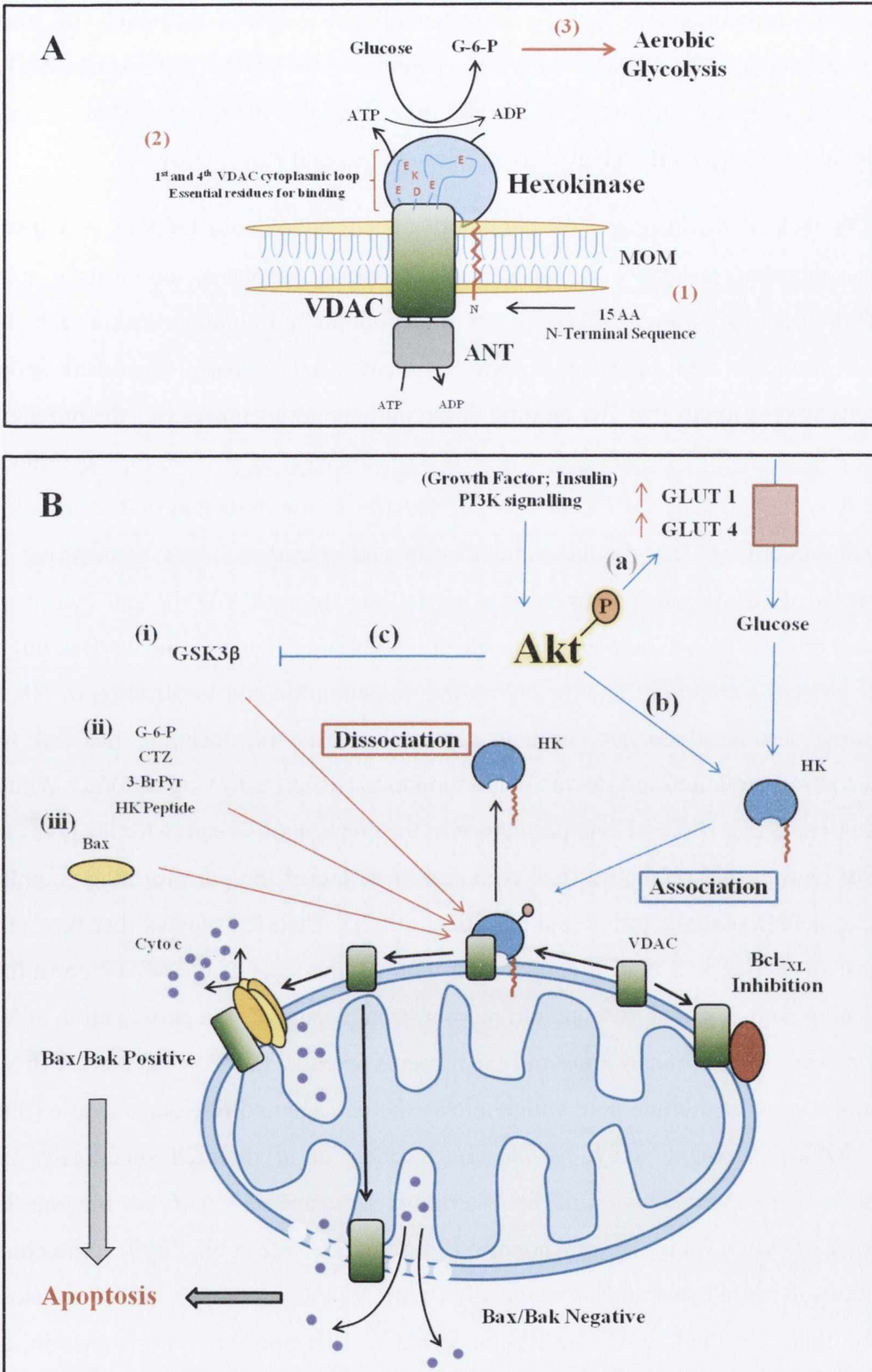


Figure 1.9: Regulation of Hexokinase association to the Mitochondrion.

(A) Hexokinase association to VDAC at the Mitochondrion – essential residues for binding. (B) Regulation of association (→) and dissociation (→) of hexokinase to the mitochondria.

HK binding promotes an “Open” conformation for VDAC trafficking of ions and metabolites across the MOM and when bound to the MIM transporter ANT, this VDAC/ANT complex facilitates ATP/ADP flux from the matrix to VDAC-bound HK, preferentially driving increased glycolytic rates (Robey and Hay, 2006).

As well as facilitating a metabolic advantage, hexokinase binding to VDAC also mediates apoptotic resistance in cancer cells. During apoptosis, an intrinsic apoptotic stimulus induces localisation of Bax to the mitochondria and oligomerisation with Bak, an essential initiation step in MOMP, cyto c release and caspase dependant apoptosis. Numerous studies report that HK binding to the mitochondria antagonises the pro-apoptotic effects of Bax and Bak (Majewski et al., 2004a, Majewski et al., 2004b, Pastorino et al., 2002). An original study by *Pastorino et al* described how mitochondrial localised HK-II prevented binding of Bax to the mitochondria and release of cyto c, however forced dissociation of HK-II sensitises mitochondria to Bax induced MOMP and cyto c release (Pastorino et al., 2002). These and subsequent results suggest that both Bax/Bak oligomers and HK possess a common binding site on the mitochondria and localisation of HK to the mito during aerobic glycolysis consequently inhibits Bax translocation, Bax/Bak hetero-oligomer pore formation and downstream apoptosis (Robey and Hay, 2006). While it is well established that Bax and Bak participate in the creation of selective MOM pores for the release of cyto c, the mechanism and complete structure of the pore forming complex are still under debate (Kumarswamy and Chandna, 2009). Theories suggest that Bax and Bak on their own or Bax and Bak in cooperation with components of the MPTP constitute the pore forming complex, and new and old reports strongly suggest the participation of VDAC in this process. Reconstituted liposome experiments revealed that Bax interacts with VDAC and forms a large membrane pore which allows the passage and release of cyto c (Shimizu et al., 1999). A recent study by *Shoshan-Barmatz et al* reported specifically on the importance of the internalisation of the N-terminal sequence of VDAC for oligomerisation and release of cyto c from the mitochondria (Shoshan-Barmatz et al., 2010). Mitochondrial localisation of hexokinase and its association with VDAC is able to inhibit apoptosis by sterically hindering binding of Bax/Bak to VDAC. This prevents pore formation, cyto c release and apoptosis and is further evidence for mitochondrial-localised HK being a highly beneficial adaptation in cancer.

1.14.1 Regulation of Hexokinase-I/II binding to VDAC

As a central component in sustaining aerobic glycolysis and apoptotic resistance in cancer cells, initiation and maintenance of HKs association to the mitochondria is a highly regulated process. An enzymatically active conformation of hexokinase is necessary for VDAC affinity and, as such, abundant levels of glucose are required for sustained binding to the mitochondria. Reduced glucose availability and a compensatory increase in levels of the G-6-P can cause HK dissociation via product negative feedback inhibition. Mechanisms exist to subvert this glycolytic checkpoint as HK-I possess reduced affinity for its product, G-6-P (Skaiff et al., 2005). Akt (Protein Kinase B) signalling is a central control point for promoting HK association to the Mito. Akt is activated by phosphorylation from the Phosphoinositide-3-kinase (PI3K) signalling pathway, downstream of Insulin-like Growth Factor (IGF) receptor ligation. Growth factor stimulus, via Akt, induces HK localisation to the Mito, promoting increased cellular proliferation by aerobic glycolysis (Robey and Hay, 2006). Overactive IGF and Akt signalling are common alterations promoting proliferation in cancer and reports by *Majewski et al* have implicated Akt as being essential in inhibition of apoptosis by promoting localisation of HK to the Mito and inhibiting dissociation (Majewski et al., 2004b). Akt achieves this via a number of control mechanisms including directly phosphorylating the threonine 473 residue of HK, which increases its affinity for the Mito, and stimulating increased protein expression of glucose transporters, Glut 1 and Glut 4 (Pastorino and Hoek, 2008). This helps maintain positive glucose levels, promoting an active HK conformation and glycolysis. Akt is also able to inhibit Glycogen Synthase Kinase 3 β (GSK3 β), a negative regulator of HK binding to VDAC. GSK3 β phosphorylation of VDAC causes HK dissociation and sensitisation to apoptosis (Fig 1.9.B).

Other components of the MPTP, besides VDAC and ANT, are able to regulate HK binding. The mitochondrial matrix protein and member of the MPTP, Cyclophilin D, is essential in stabilisation of HK association with VDAC and does so indirectly by interaction with ANT in the bound complex (Machida et al., 2006). The Peripheral-type Benzodiazepine Receptor (PBR), in consort with VDAC, facilitates uptake of cholesterol into the MOM which promotes HK association to the Mito. Anti-apoptotic Bcl-2 family protein, Bcl-XL, also can affect the HK, VDAC, Bax/Bak binding dynamic. Like HK, Bcl-XL, can directly interact with VDAC via insertion of its N-terminal into the

Mito and interaction of its BH4 domain with VDAC. The Bcl-XL / VDAC complex maintains a monomeric structure and ultimately prevents Bax and Bak binding to VDAC and initiation of apoptosis (Malia and Wagner, 2007).

1.15 Dissociation of Hexokinase-I/II from the mitochondrion induces apoptosis in cancer

As outlined above, HK association to the Mito is vital in maintaining aerobic glycolysis and participates in mediating apoptotic resistance in cancer cells. Increased glycolysis and HK localisation to the Mito is believed to be a common adaptation in cancer and as such, many cancers are characterised by overexpression of HK-I/II and VDAC and increased signalling and activity of regulators IGFR and Akt (Pastorino and Hoek, 2008). Consequently, dissociation of HK from the Mito has been shown to induce and sensitise cells to apoptosis, even in resistant cancer sub-types. Numerous reports have illustrated how HK dissociation sensitises cells to Bax/Bak dependant apoptosis or induces apoptosis on its own in a Bax/Bak dependant mechanism (Arzoiné et al., 2009, Majewski et al., 2004a, Shulga et al., 2009). The previously discussed study by *Pastorino et al* revealed that in functional isolated mitochondria experiments, HK dissociation allows binding of Bax to the Mito and release of cyto c. In whole cell treatments, HK dissociation in combination with Bax activation (50µM Indomethacin) promotes synergistic cyto c release and caspase dependant apoptosis (Pastorino et al., 2002). Alternate studies have shown that in some cases HK release from the Mito alone is sufficient to induce cell death in Bax/Bak expressing cancer cell lines (Chiara et al., 2008).

HK dissociation has also been shown to induce cyto c release and apoptosis in resistant Bax/Bak null cell lines, occurring through a mechanism independent of Bax/Bak pore formation with VDAC (Majewski et al., 2004a). *Majewski et al* reported that dissociation of HK from the Mito and subsequent cyto c release and apoptosis could occur in Bax/Bak double knock out (DKO) cells but effects were less potent compared to Bax/Bak competent wild type (WT) cells. A lower magnitude of HK release was observed in Bax/Bak DKO cells due to the lack of competition for VDAC binding. Artificial Akt overexpression attenuated cyto c release and apoptosis, indicating that HK dissociation from VDAC is specifically responsible for loss of mitochondrial integrity and MOMP, independent of Bax and Bak pore formation. HK dissociation and subsequent binding of

creatine kinase (CK), an IMS component of the MPTP, in between VDAC and ANT is suspected to contribute to the creation of a “Closed” VDAC conformation preventing adenine nucleotide and other metabolite exchange between the matrix and cytoplasm (Robey and Hay, 2006). Prolonged HK loss, via growth factor withdrawal or forced HK dissociation, in Bax / Bak deficient cells is believed to adversely affect mitochondrial integrity due to the “Closed” VDAC conformation. Ultimately this phenomenon results in MIM remodelling, matrix swelling and rupture of the outer membrane (MOM), culminating in cyto c release and cell death in Bax/Bak null cells (Robey and Hay, 2006).

1.15.1 Mitochondrial Hexokinase-Dissociating compounds

A number of distinct classes of compounds have been shown to act as mitochondrial HK dissociating compounds and are currently at various stages of development as anti-cancer agents. Anti-fungal azole derivatives, including clotrimazole (Fig 1.10.A), have been reported to induce detachment of HK from the mitochondria (Penso and Beitner, 1998). The mechanism of action for clotrimazole is as yet unclear, however it is predicted that the hydrophobic imidazole compound is able to disrupt the hydrophobic interaction between mitochondrial localised HK-I / HK-II isoforms and VDAC, inducing forced dissociation for hexokinase from the mitochondria (Majewski et al., 2004a). Clotrimazole-driven hexokinase dissociation occurs prior to induction of cell death, characterised by binding of Bax to VDAC, mitochondrial membrane permeabilisation, cyto c release, G1 cell cycle arrest and an apoptotic form of PCD (Pastorino et al., 2002, Chiara et al., 2008, Machida et al., 2006). Dissociation of mitochondrial hexokinase and apoptosis induced by clotrimazole has been reported in numerous cancer cells (Liu et al., 2010, Majewski et al., 2004a, Penso and Beitner, 1998), is used as a model reagent in studies investigating the Warburg effect, MPTP dynamics and apoptosis and is in development as a selective anti-cancer agent. Clotrimazole also has affinity for alternate molecular targets, including other enzymes of the glycolytic pathway, and has been reported to elicit cell death through alternate mechanisms. The triphenylmethyl-like motif present in clotrimazole has been implicated in affinity for a number of distinct anti-cancer targets (Palchaudhuri et al., 2008). Clotrimazole has been shown to be a calmodulin antagonist (Penso and Beitner, 1998), an inhibitor of cytochrome P-450 superfamily enzymes (Turan et al., 2001) and disrupts

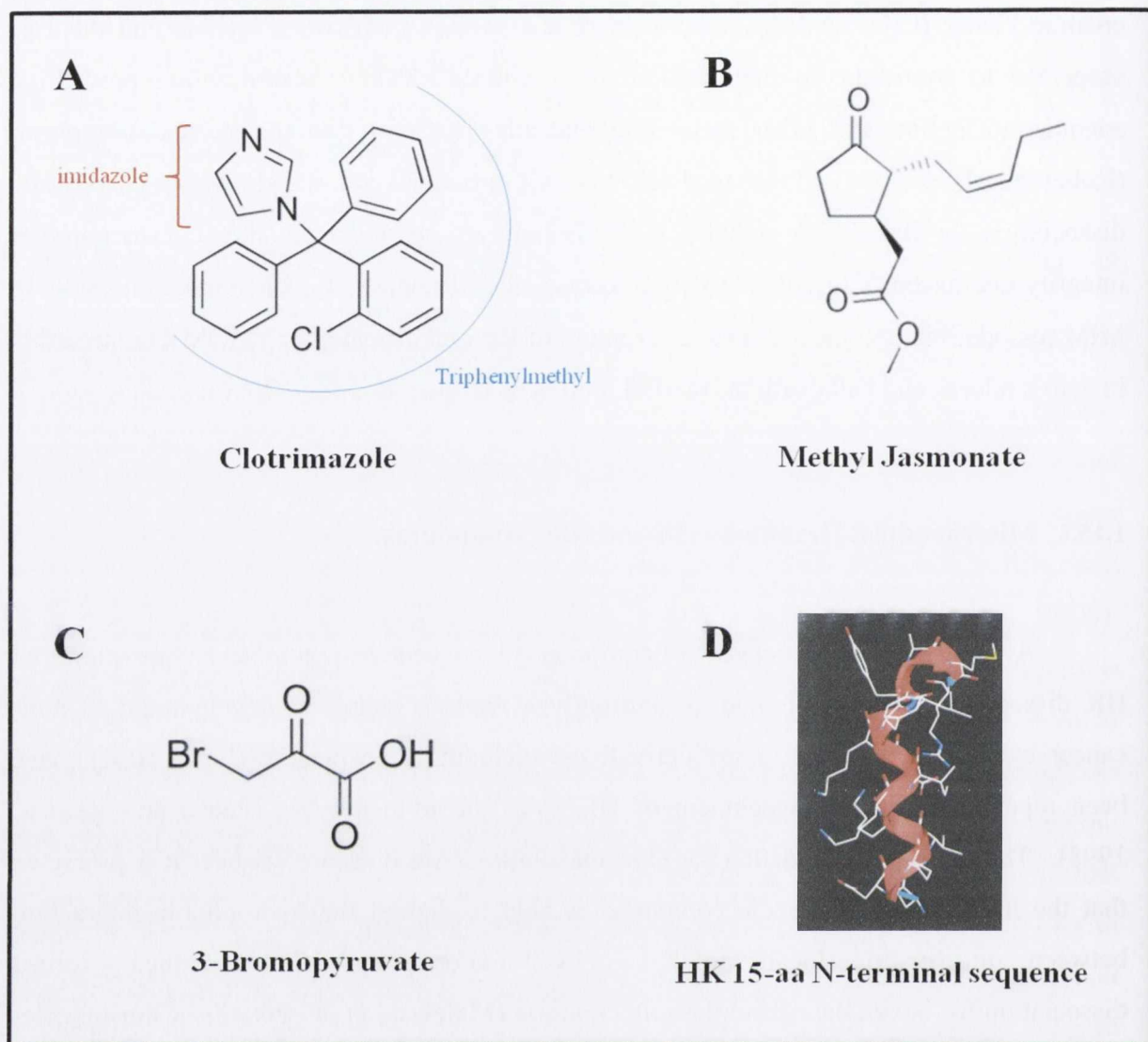


Figure 1.10: Hexokinase dissociating compounds.

Hexokinase dissociating compounds are able to release Hexokinase from the Mito and induce cyto c release and apoptosis in cancer. (A) Clotrimazole – anti fungal azole compound. (B) Methyl Jasmonate – Class of Jasmonate plant stress hormones. (C) 3-bromopyruvate – brominated pyruvate derivative. (D) 15 amino acid N-terminal sequence of hexokinase – synthetic peptides containing the HK 15 AA N-terminal sequence are able to compete for binding to VDAC and dissociate native hexokinase (Sequence diagram from PBD crystal structure, visualised using MOE (Aleshin et al., 1998).

6-phosphofructokinase (PFK) activity (Marcondes et al., 2010). In an alternate mechanism of glycolytic control, clotrimazole dissociates and inhibits the tetrameric state of active PFK, inducing detachment of the enzyme from the cytoskeleton, disrupting the rate of glycolytic flux, and cellular ATP / Homeostasis. Despite being a promiscuous inhibitor, its dissociation of HK is proposed to be a direct effect, independent of its alternate mechanisms of metabolic control.

Other classes of HK dissociating compounds include Methyl Jasmonate, 3-bromopyruvate and synthetic hexokinase 15-AA N-terminal peptides. The Jasmonate family of plant stress hormones are a class of hydrophobic small molecules reported to induce cell death in drug resistant forms of cancer. Methyl Jasmonate (Fig 1.10.B) and its synthetic derivatives have been shown to selectively induce apoptosis in cancer and methyl Jasmonate mediates this effect through direct binding to mitochondrial localised hexokinase and interfering with the hydrophobic interaction between HK and its anchor protein VDAC, inducing HK dissociation from the mitochondria and cellular events leading to cell death (Goldin et al., 2008, Park et al., 2010). 3-bromopyruvate (Fig 1.10.C) is a brominated pyruvate derivative which inhibits aerobic glycolysis by covalent binding to HK and inducing dissociation from the mitochondria (Chen et al., 2009). Dissociation of HK and glycolytic inhibition causes depletion of cellular ATP and apoptosis in cancer cells. Initial *in vivo* experimentation with 3-bromopyruvate in hepatocellular carcinoma produced startling results with complete eradication of cancers with no recurrence or significant toxicity to normal liver tissue (Ko et al., 2004). A final inducer of dissociation involves the 15 amino acid N-terminal sequence of HK-I and HK-II (Fig 1.10.D), the hydrophobic tail specific to these HK isoforms which allows association to the mitochondria via insertion into the outer mitochondrial membrane and interaction with VDAC (Robey and Hay, 2006). Numerous studies have employed synthetic forms of cell permeable peptide containing this N-terminal sequence to induce potent dissociation of HK from the mitochondria (Majewski et al., 2004a, Pastorino et al., 2002). These HK N-terminal derivative peptides displace endogenous full length HK from the mitochondria by competing for insertion into the mitochondria and association with VDAC.

1.16 The need for new targeted anti-cancer therapies

The goal for all anti-cancer therapies is to selectively tip the balance towards PCD in cancer cells, but leave normal tissue unaffected. Ideal therapies employ the conserved

cellular mechanisms of apoptosis to orchestrate the controlled destruction of aberrant cells through radiological or chemical challenge, specifically in cancer cells. However, cancer alterations in conserved cellular mechanisms, like apoptosis, and limitations in classical drug design make such a prospect difficult.

Since the inception of using pharmacological agents in the treatment of cancer, approximately seventy years ago, the issues of severe side effects and acquired resistance of cancers to adjuvant chemotherapies have persisted. In the 1940's, the initial breakthroughs in Oncology by Goodman and Farber into nitrogen mustard and anti-folate therapies were revolutionary in the treatment of lymphoma and other cancers (Goodman et al., 1984, Farber and Diamond, 1948). The growth of the field produced similar serendipitous discoveries including vinca alkaloid, taxane and platinum-based agents with apoptotic activity, but all presented severe off-target cytotoxicity and development of resistance by many cancers. In Cancer Biology today, the vast expansion in our understanding of the mechanisms and phenotypes of cancer has revolutionised drug discovery. Thinking of cancer by way of the collection of mutations facilitating its growth, survival and resistance opens up an alternate strategy for developing improved chemotherapies. Advanced molecular understanding, genomic analysis and computational techniques allows identification and selective targeting of these biochemical "weaknesses" in cancer subtypes with new "rationally designed" chemotherapeutic drugs, negating the reliance on serendipitous discoveries and reducing the scope for resistance and side effects.

Successful rational design programmes include targeting nuclear receptor signalling (antagonism of Estrogen receptor in ER dependant breast cancer), molecular chaperone function (HSP90 inhibitors in numerous cancer types, including melanoma (Porter et al., 2010)) and tyrosine kinase inhibitors (fusion oncogene Bcr-abl targeting with Gleevec in CML (Guo and Ding, 2003)). Other promising molecular targets in cancer, and ones which are the focus of this study, are relieving IAP mediated resistance through SMAC mimetic therapies and "the Warburg Effect" – a unique metabolic niche promoting both growth and apoptotic resistance in cancer.

1.17 Aims

The aims of this project were to employ a rational drug design approach for the development of treatments for resistant forms of cancer, with particular interest in the

development of therapeutic options for the highly resistant neoplasm, Malignant Pleural Mesothelioma. Attempts were made to identify mechanisms of apoptosis or drug resistance in this malignancy and specifically target the dependence of MPM on these cellular alterations for survival and growth. Commercially available and novel compounds developed through computational design were used to develop a rational and effective treatment strategy for MPM.

The Inhibitor of Apoptosis Protein family was a rational drug design target of interest in MPM. IAP family proteins are part of a control network that blocks execution of apoptosis and have been shown to be overactive and facilitate resistance in many cancer types. Previous reports have strongly implicated high IAP expression as mediating resistance specifically in MPM and IAP inhibition was thought worthy of investigation as a rational drug design target for treatment in this form of cancer. Initial experimentation looked to confirm a correlation between high IAP expression and apoptotic resistance in MPM. Commercially available SMAC mimetics and novel, computationally designed small molecule mimetics were tested as apoptotic inducers as single agents or in combination with a conventional MPM chemotherapy, cisplatin. This work aimed to firstly identify novel SMAC mimetic compounds with effective and potent activity in MPM and secondly determine whether MPM was a useful test model for the development and computational design of SMAC mimetic compounds.

A second molecular target under investigation in this study was the metabolic alteration of aerobic glycolysis, or “The Warburg Effect”, occurring in cancer and other fast proliferating cells. The overactive glycolytic flux of aerobic glycolysis has been shown to be a ubiquitous alteration in cancer which facilitates a metabolic advantage for fast proliferation of cells and helps inhibit intrinsic apoptotic activation through increased localisation of hexokinase to the mitochondria. Dissociation of HK causes apoptosis even in resistant Bax/Bak deficient cancers and was chosen as a target for rational drug design. The project aimed to identify ligands which act as HK dissociating compounds and develop novel, computationally designed compounds based on a pharmacophore from a known HK dissociating drug. Compounds were initially tested in “Bax/Bak null” and “Bax/Bak positive” Burkitt Lymphoma cell lines and a panel of MPM cell lines. The study aimed to develop “on target” HK dissociating drugs effective in the treatment of resistant MPM.

Chapter 2

Materials and Methods

2. Materials and Methods

Materials

2.1.1 Source of materials

Materials

96-Well plates (Sterile)
Ac-DEVD-AMC
Acetyl Coenzyme A
Ac-IETD-AMC
Ac-LEHD-AMC
Acrylamide : bis-Acrylamide (30:0.8)
Acrylamide : Methylenebisacrylamide (29:1)
Agarose
Alamar Blue
Alpha-thioglycerol
Amersham Molecular Weight Marker
Ammonium persulphate
Annexin-V-Fitc
Anti-Bak Rabbit mAb
Anti-Bax (n-20) Rabbit pAb
Anti-Bcl-2 (ab-1) Mouse mAb
Anti-cIAP-1 Mouse mAb
Anti-cIAP-2 Mouse mAb
Anti-Hexokinase-II (c64g5) Rabbit mAb
Anti-HSP60 Rabbit pAb
Anti-IκB moue mAb
Anti-LC3 rabbit mAb
Anti-Mouse IgG HRP
Anti-p62 rabbit mAb
Anti-Parp-1 (Ab-2) Mouse mAb
Anti-Rabbit IgG HRP
Anti-TNF-R1 (c25c1) Rabbit mAb
Anti-Ubiquitin mouse mAb
Anti-XIAP (3B6) Rabbit mAb
Anti-β-Actin Mouse mAb
Aprotinin

Suppliers

Greiner Bio-One
Enzo Life Sciences
Sigma-Aldrich
Enzo life sciences
Enzo life sciences
National Diagnostics
National Diagnostics
Sigma-Aldrich
Invitrogen
Sigma-Aldrich
GE Healthcare
Sigma-Aldrich
IQ Products
Cell Signalling
Santa Cruz Biotechnology
Merck Biosciences
BD Pharmingen
BD Pharmingen
Cell Signalling
AbCAM
Santa Cruz Biotechnology
Sigma-Aldrich
Promega
Sigma-Aldrich
Calbiochem
Promega
Cell Signalling
Santa Cruz Biotechnology
Cell Signalling
AbCAM
Sigma-Aldrich

ARPFAQ-[K-5-FAM]	Genscript Corporation
ARPFAQK	Genscript Corporation
AVPI-RRRRRRRR	Genscript Corporation
Bafilomycin A1	Sigma-Aldrich
Bathocuprione disulphonic acid	Sigma-Aldrich
BCA Protein Assay Kit	Pierce
Bovine Liver Catalase	Sigma-Aldrich
Bovine Serum Albumen (BSA)	Sigma-Aldrich
Bovine γ -globulins	Sigma-Aldrich
Bromophenol Blue	Sigma-Aldrich
CHAPS	Fluka Biochemocal
Chloroquine	Sigma-Aldrich
Ciglitizone	Tocris
cis-Diammineplatinum(II) dichloride (Cisplatin)	Sigma-Aldrich
Clotrimazole	Sigma-Aldrich
Complete Protease Inhibitor Cocktail	Roche Diagnostics
DAPI	Invitrogen
D-Glucose-6-Phosphate disodium salt hydrate	Sigma-Aldrich
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Dithiothreitol (DTT)	Sigma-Aldrich
Doxorubicin	Enzo life sciences
Dried Milk	Marvel
DTNB	Sigma-Aldrich
Dulbecco's Modified Eagles Medium (DMEM)	Invitrogen
E64d	Sigma-Aldrich
Econazole	Sigma-Aldrich
Embelin	Enzo life sciences
Ethanol	TCD Stores
Ethylene glycol tetraacetic acid (EGTA)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Etoposide	Sigma-Aldrich
Fetal Bovine Serum	Invitrogen
Glucose-6-phosphate Dehydrogenase (<i>S. cerevisiae</i>)	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
GW1929	Tocris

GW9662	Tocris
Hank's Balanced Salt Solution (HBSS)	Invitrogen
Hepes	Sigma-Aldrich
Hepes (cell culture)	Sigma-Aldrich
Human Recombinant TRAIL	Autogen Bioclear
Hydrogen Peroxide	Sigma-Aldrich
Immobilon Western Chemiluminescent HRP Substrate (ECL)	Millipore
Lantascree TM TR-FRET PPAR- γ assay	Invitrogen
Leupeptin	Sigma-Aldrich
Lipopolysccharide (LPS)	Sigma-Aldrich
Low Melting Point Agarose (LMPA)	Sigma-Aldrich
Methanol	TCD Stores
Methotrexate	Fluka Analytical
Miconazole	Sigma-Aldrich
Microscope Slides	Thermo Scientific
N-Acetyl-L-cysteine	Sigma-Aldrich
Nocodazole	Sigma-Aldrich
Nonidet P-40	Sigma-Aldrich
Oxaloacetic acid	Sigma-Aldrich
Penicillian Streptomycin (Pen-Strep)	Biosciences
Pepstatin A	Sigma-Aldrich
PhenylMethylSulfonyl Fluoride (PMSF)	Sigma-Aldrich
Phosphate buffered saline (PBS)	Oxoid chemicals
Potassium chloride (KCl)	Sigma-Aldrich
Potassium phosphate	Sigma-Aldrich
Propidium Iodide	Sigma-Aldrich
PVDF	Sigma-Aldrich
Quanti-Blue	Invitrogen
Raltitrexed monohydrate	Enzo life sciences
RapiDiff Nuclear and Cytoplasmic staining kit	Sigma-Aldrich
Recombinant Human Tumour Necrosis Factor α (TNF- α)	Invitrogen
Recombinant Human XIAP-BIR3	R&D Systems
RNAse A	Sigma-Aldrich
Rosiglitzone	Tocris
RPMI 1640 Medium	Invitrogen
Sodium azide	Sigma-Aldrich

Sodium chloride	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Fluka Biochemocal
Sodium Pyruvate	Sigma-Aldrich
Sodium Pyruvate (Cell Culture)	Sigma-Aldrich
Stripping buffer (10x)	Chemicon
Sucrose	Sigma-Aldrich
TEMED	Sigma-Aldrich
Tissue Culture Flasks	Greiner Bio-One
Triton-X-100	Sigma-Aldrich
Trizma base (Tris)	Sigma-Aldrich
Trypsin-EDTA (10x)	Sigma-Aldrich
Tween-20	Sigma-Aldrich
Vinblastine	Enzo life sciences
X-Omat LS Film	Kodak
Z-LEHD-FMK	MBL International
Z-VAD-FMK	MBL International
α -thioglycerol	Sigma-Aldrich
β -Nicotinamide Adenine Dinucleotide (NAD)	Sigma-Aldrich
β -Nicotinamide Adenine Dinucleotide Phosphate (NADP)	Sigma-Aldrich
β -Nicotinamide Adenine Dinucleotide Phosphate, reduced (NADPH)	Sigma-Aldrich
β -Nicotinamide Adenine Dinucleotide, reduced (NADH)	Sigma-Aldrich

2.1.2 Source of cell Lines

Mesothelioma cell lines CRL 5915 (NCI-H2052), CRL-5820 (NCI-H28), One 58 and Ju77 were obtained from Prof. Ken O'Byrne (Institute of Molecular Medicine, St. James Hospital, Trinity College Dublin, Ireland). Burkitt's lymphoma cell lines Mutu-1 (c179) and DG-75 were provided by Dr. Dermot Walls (School of Biotechnology, Dublin City University, Ireland). HeLa cells were provided by Prof. Mary Meegan from the School of Pharmaceutical Sciences, Trinity College Dublin, Ireland. Chronic Lymphocytic Leukaemia (CLL) cell lines, PGA and HG3, were obtained from Prof. Anders Rosén of Linköping University, Sweden. LC3-GFP stably transfected macrophages were obtained from Dr. Jim Harris (School of Biochemistry and Immunology, Trinity College Dublin).

Methods

2.2. Cell culture

2.2.1 Malignant Pleural Mesothelioma cell lines

The CRL 5915 cell line was established from pleural effusion of a 65 year old Caucasian male diagnosed with advanced stage 4 Malignant Pleural Mesothelioma (MPM). The CRL 5820 cell line was also established from pleural effusion of advanced stage 4 Malignant Pleural Mesothelioma of a 48 year old Caucasian male. Both JU77 and ON58 MPM cell lines were obtained from pleural effusion of patients with known industrial crocidolite asbestos exposure, positively diagnosed for Malignant Pleural Mesothelioma, but had not received treatment for the malignancy. Establishment and characterisation of these two MPM cell lines was fully described in an *International Journal of Cancer* publication by Manning *et al* (Manning et al., 1991).

In culture, all Mesothelioma cell lines were grown using RPMI-1640 + Glutamax Medium containing 10% (v/v) Foetal Bovine Serum (FBS) and 100µg/ml Penicillin and Streptomycin (Pen-Strep). Cells were maintained in 75cm² tissue culture flasks, containing 18ml supplemented media, at a temperature of 37°C in a 5% CO₂ / 95% O₂ humidified atmosphere. Cells were split at a ratio of 1 in 5 three times a week and used in experimentation up until a cell line exceeded 35 passages.

2.2.2 Burkitt's Lymphoma cell lines

The MUTU-1-(c179) cell line is a Burkitt's lymphoma (BL) cell line model derived from patient biopsy. These cells are an isogenic stable group-I BL cell line, positive for BL-type Epstein-Barr Virus (EBV) genomic expression (Gregory et al., 1990). Mutu-1 cells were cultured in RPMI 1640 + Glutamax medium, supplemented with 10% (v/v) FBS, 100µg/ml Pen-Strep, 5mM alpha-thioglycerol and 20µM bathocuprione disulphonic acid in phosphate buffered saline (PBS), 100mM sodium pyruvate and 1mM Hepes. All media supplements were sterile and of cell culture grade.

The DG-75 cell line was established by Ben-Basset et al in 1977 from pleural effusion of a diagnosed paediatric case of Burkitt's Lymphoma, negative for the presence of the EBV genome or EBV receptor expression (Ben-Bassat et al., 1977). Recent publications have classified the DG-75 cell line as being highly resistant to apoptotic challenge and it is proposed that the lack of expression of pro-apoptotic Bcl-2 family proteins, Bax and Bak, in DG-75 cells can potentiate compromised apoptotic induction and subsequent resistance to apoptotic stimuli (Sun et al., 2007, Cloonan and Williams, 2010). DG-75 cells were cultured in RPMI 1640 + Glutamax medium, supplemented with 10% (v/v) FBS and 100µg/ml Pen-Strep. Both BL suspension cell lines were maintained in 75cm² tissue culture flasks, containing 15ml supplemented media, at a temperature of 37°C in a 5% CO₂ / 95% O₂ humidified atmosphere. Cells were split at a ratio of 1 in 7 for Mutu-1 and 1 in 10 for DG-75, three times a week and used in experimentation until a passage number of no greater than 35 passages.

2.2.3 HeLa cell line

The HeLa cell line is a Human Papilloma Virus (HPV) positive cervical adenocarcinoma cell model. The cell line was derived from pre-treatment biopsy of Henrietta Lacks, a 31 year old, African American, female cervical cancer patient, in 1951. HeLa cells represent the first immortalised *in vitro* cancer cell model and have been extensively used in cancer cell biology research (Jones, 1997). HeLa cells were cultured in Dulbeccos Modified Eagles Medium (DMEM) + Glutamax supplemented with 10% (v/v) FBS and 100µg/ml Pen-Strep. Cells were maintained in 75cm² T75 tissue culture flasks, containing 18ml supplemented media, at a temperature of 37°C in a 5% CO₂ / 95% O₂

humidified atmosphere. Cells were split at a ratio of 1 in 6, three times a week and used in experimentation until a passage number of no greater than 35 passages.

2.2.4 Chronic Lymphocytic Leukemia cell lines

Chronic Lymphocytic Leukaemia (CLL) is a malignancy of B-cell lymphocytes characterised by massive clonal expansion of malignant CD5+, CD23+ monoclonal B-cells and accumulation of these cells in peripheral lymphatic tissue, bone marrow and blood (Masood et al., 2011). PGA1 and HG3 are Epstein-Barr virus transformed CLL cell lines representing disease models which are responsive and unresponsive to treatment, respectively. PGA1 expresses mutated Immunoglobulin heavy chain genes (M-IgV_H), a good prognostic factor in CLL, while the HG3 cell line expresses unmutated Immunoglobulin heavy chain genes (U-IgV_H), a poor prognostic factor in patients with CLL (Hamblin et al., 1999). PGA1 and HG3 cell lines were cultured in RPMI-1640 + Glutamax Medium containing 10% (v/v) Foetal Bovine Serum (FBS) and 100µg/ml Penicillin and Streptomycin (Pen-Strep). Cells were maintained in 75cm² tissue culture flasks, containing 18ml supplemented media, at a temperature of 37°C in a 5% CO₂ / 95% O₂ humidified atmosphere. Cells were split at a ratio of 1 in 5, three times a week and used in experimentation up until a cell line exceeded 35 passages.

2.2.5 LC3-GFP Macrophages

LC3-GFP (Green Fluorescent Protein) stably expressing macrophages were generated by transfection of a Macrophage cell line with an expression plasmid containing the GFP tagged LC3 gene, promoter sequence and puromycin resistance gene. Clonal selection for positively transfected cells was obtained using a puromycin selection antibiotic, producing a stably transfected macrophage cell line with ubiquitous expression of the fluorescently tagged LC3 protein. The LC3-GFP macrophage cell line was cultured in RPMI-1640 + Glutamax Medium containing 10% (v/v) Foetal Bovine Serum (FBS) and 100µg/ml Penicillin and Streptomycin (Pen-Strep). Cells were also supplemented with a 1 in 1,000 dilution of puromycin selection antibiotic. Cells were maintained in 75cm² tissue culture flasks, containing 18ml supplemented media, at a temperature of 37°C in a 5% CO₂ / 95% O₂ humidified atmosphere. Cells were split at a ratio of 1 in 3, twice a week.

2.2.6 Hek-BlueTM-hTLR4 cells

Hek-BlueTM-hTLR4 cells are a commercially available cell line (Invitrogen) used in the study of Toll-Like Receptor – 4 (TLR4) signalling and NFκB activation. These cells are a transformed form of Human Embryonic Kidney (Hek) cells, transfected with reporter genes for hTLR4 and co-receptor proteins MD-2/CD-14, and which express no other receptors of the TLR family. Hek-Blue cells were cultured in DMEM + Glutamax Medium containing 10% (v/v) Foetal Bovine Serum (FBS), 100µg/ml Normocin, 100µg/ml Penicillin and Streptomycin (Pen-Strep). Cells were also supplemented with 1x Hek-Blue selection antibiotic (Invitrogen). Cells were maintained in 75cm² tissue culture flasks, containing 18ml supplemented media, at a temperature of 37°C in a 5% CO₂ / 95% O₂ humidified atmosphere. Cells were split at a ratio of 1 in 5, three times a week.

2.3 Cell maintenance, experimental set up and treatments

Cell lines grown in culture were maintained, seeded and treated under stringent aseptic conditions with all manipulations performed in a tissue culture hood, using sterilised plastic ware, tissue culture media and experimental reagents. For adherent cell lines (Mesothelioma and Human cervical cell lines), tissue culture media, PBS and 1 x Trypsin/EDTA was heated to 37°C prior to experimentation. Sub-confluent flasks of cells were washed in PBS and trypsinised at 37°C for 2-3 min. Cells were centrifuged at 300 x g for 5 min using a Sorvall T 436 centrifuge. The cell pellet was resuspended in 12ml culture media and cells were counted using a Haemocytometer. The desired amount of cells in the appropriate amount of media was seeded in tissue culture plates for each relevant experiment. Up to 18h was allowed for cells to adhere to the treatment plates prior to commencement of the assay. Suspension cells (Burkitt's Lymphoma cell lines), were counted and seeded as outlined above, but did not require trypsinisation.

Prior to cell treatment, all test compounds, vehicle and other experimental reagents were heated to 37°C in a water bath (Grant). In all experiments (single and dual agent incubations), the final vehicle composition and concentration was matched for all drug treatments.

2.4 Cell cryo-storage

Low passage number stocks of each cell type were stored in liquid nitrogen (-180°C) to ensure a consistent stock of cell lines for continual experimentation. Cryo-Stocks were generated for each cell type used by taking 5×10^6 cells of low passage number and resuspending them in 1.5ml of Freezing Media, containing 60% Tissue culture media, (v/v) 30% FBS and (v/v) 10% Dimethyl Sulphoxide (DMSO). Cells were placed in cryovials and incubated in a polycarbonate container containing iso-propanol and left overnight in a -80°C freezer – the freezing apparatus ensures cell integrity while freezing with a cooling rate of only $1^{\circ}\text{C}/\text{min}$. Subsequently, cryovials are stored in liquid nitrogen for long term cryopreservation of cells.

2.5 Western Blots

2.5.1 Preparation of whole cell lysates

Between 0.5×10^6 – 4×10^6 cells were seeded in tissue culture flasks, left to adhere for 18h and incubated with compound and controls for indicated time points. Post-treatment, cells were harvested via trypsinisation and centrifuged at $300 \times g$ for 5 min in a Sorvall T 6000D Centrifuge. The pellet was washed twice in ice cold PBS and resuspended in 50 - $150 \mu\text{l}$ (depending on pellet size) of 1 x RIPA lysis Buffer (50mM Tris, 150mM NaCl, 2mM EDTA, 1% (v/v) Nonidet P-40, 0.1% (w/v) sodium dodecyl sulphate (SDS)) supplemented with fresh 5mM Dithiothreitol (DTT) and 1x Complete Protease Cocktail. Cells were left to lyse for 1h on ice before being spun at $20,000 \times g$ and 4°C for 15min in an Eppendorf 5417R refrigerated centrifuge. Cellular debris and membranes were pelleted and whole cell lysate supernatants were removed and stored at -20°C prior to further experimentation.

2.5.2 BCA protein assay

Pierce BCA Protein assay® reagents were used to determine the protein concentrations in prepared lysates. Protein concentrations of lysates were equalised before resolving on SDS-PAGE to insure appropriate comparative analysis of protein expression by Western Blot. The BCA protein assay is a two-step colorimetric assay: The first exploits the Biuret reaction for detecting peptides. A peptide of three or more amino acids chelates a

cupric ion (Cu^{2+}) and is reduced to a cuprous cation (Cu^{1+}) in an alkaline environment containing sodium potassium tartrate. The copper ion then chelates to bicinchoninic acid (BCA) ($2 \text{ BCA} : 1 \text{ Cu}^{1+}$) producing an intense purple colour which is detectable on a spectrophotometric plate reader at 562nm. A strong linear relationship between protein concentration and BCA/ Cu^{1+} complex formation exists over a large protein concentration range (20 – 2,000 $\mu\text{g/ml}$) (Smith et al., 1985).

From each sample lysate, 4 μl was taken and diluted 1 in 25 with 96 μl distilled water (dH_2O). To a 96 well plate, 25 μl of diluted sample is added per well in triplicate. To each well, 200 μl of BCA “Working Detector” containing a 50:1 ratio of Reagent A to Reagent B is added to each test well and left to incubate in the dark, at 37°C, for 30min before reading the absorbance at 562nm, using a Versamax absorbance plate reader. A Bovine Serum Albumin (BSA) protein standard curve, spanning nine data points from 0 – 2,000 $\mu\text{g/ml}$, was assayed with sample lysates and was used to determine sample lysate protein concentrations from the BCA absorbance change.

2.5.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method of resolving denatured, negatively charged (SDS) coated proteins and polypeptide chains on a polyacrylamide gel, relating to their electrophoretic mobility – separating proteins with regard to protein molecular weight, charge and polyacrylamide gel pore size. Protein resolving gels ranging from 8 – 15% polyacrylamide were used (depending on molecular weight of protein of interest), with a 5% protein-stacking gel. A 5ml 10% resolving gel (enough for one mini gel) consisted of: 1.9ml dH_2O , 1.7ml ProtoGel™ (30% (w/v) acrylamide / 0.8% (w/v) bisacrylamide), 1.3ml 1.5M Tris (pH 8.8), 50 μl 10% (w/v) SDS, 50 μl 10% (w/v) ammonium persulphate and 2 μl N,N,N',N'-tetramethylethylenediamine (TEMED). The 5% stacking gel consists of: 2.1ml dH_2O , 0.5ml ProtoGel™, 0.38ml 1.0M Tris (pH 6.8), 30 μl 10% (w/v) SDS, 50 μl 10% (w/v) ammonium persulphate and 3 μl TEMED. A 12 pronged comb is used to generate 12 wells for sample lysate addition. Resolving and stacking gels were poured and let polymerise sequentially in a mini-gel plate apparatus and then placed in an electrophoresis gel box (ATTO AE6540) submerged in 1 x Running Buffer (25mM Tris, 192mM glycine, 0.1% (w/v) SDS).

Sample lysates were denatured in 5x SDS Sample Buffer (10% (w/v) SDS, 20% (v/v) glycerol, 0.2M Tris (pH 6.8), 0.05% (w/v) Bromophenol blue and fresh 50mM DTT) and boiled at 100°C for 5 min. To each well, 20µl of lysate containing 10 – 30µg of protein (depending on assay) was carefully added. Proteins were resolved by SDS-PAGE running at 80V (increasing to 100V) and 30mA for 2 – 3h.

2.5.4 Polyvinylidene Difluoride (PVDF) wet transfer

Once completed, proteins resolved in the polyacrylamide gel were transferred onto an Immobilon™ Polyvinylidene Difluoride (PVDF) membrane (pore size 0.45µm), applicable for immuno-probing in Western Blot experiments. A PVDF membrane was activated in 100% Methanol (10 – 30 sec) and washed in dH₂O for 1 min. PVDF membrane, gel, 6 filter papers and 2 sponges are soaked in ice-cold 1 x Transfer Buffer (25mM Tris, 192mM glycine, 10% (v/v) Methanol) for 10 min. In a BIORAD Mini-Trans Blot cassette, items were arranged as follows: Sponge, 3 filter papers, gel, PVDF membrane, 3 filter papers, Sponge. The cassette was placed in the transfer apparatus, submerged in ice cold 1 x Transfer buffer and placed with the PVDF membrane in between the gel and cathode, insuring protein transfer from gel to PVDF. The transfer was set at 70V and 170mA and run for 1.5 – 2h.

2.5.5 Blocking, Immuno-Blotting and protein detection

Once proteins were transferred to PVDF, the membrane was blocked using 5% (w/v) Dried Milk (Marvel) in 1 x Tris buffered saline, containing 0.1% Tween (TBS-T) (TBS: 10mM Tris and 150mM NaCl (pH 7.5)). Blocking was performed for 2h at room temperature or overnight at 4°C.

The PVDF membrane was incubated with the primary antibody of interest at a dilution factor of 1:500 – 1:10,000 in 5% (w/v) dried milk TBS-T (or 5% BSA TBS-T) for 2h at room temperature or overnight at 4°C. Post incubation, the membrane was washed in 5ml TBS-T three times for 5 min. Incubation with the secondary antibody (Anti-Mouse or anti-Rabbit, depending on primary Ab source), with a conjugated Horseradish Peroxidase (HRP) enzyme, was performed at a dilution factor of 1:2,500 – 1:20,000 for 1h at room

temperature. Non-specific secondary-Ab binding was washed off using 3 x 10 min washes in TBS-T and a final 5 min wash in TBS.

Proteins were detected by immunoblotting using electrochemiluminescence (ECL). Luminol, an ECL substrate, exposed to the secondary antibody conjugated HRP in alkaline conditions (H_2O_2) results in oxidation of Luminol to 3-aminophthalate and the release of light (chemiluminescence). This light was detected by exposing immuno-probed, ECL treated membranes to Kodak X-Omat LS film and developed using an X-RAY processor (Fugi). The amount of chemiluminescence and, in turn, the intensity of bands on exposed film corresponds to the amount of protein of interest present in each lane on the PVDF membrane.

2.5.6 Stripping blots and loading control re-probing

In order to validate differential expression levels observed between treatments in Western blot analysis, membranes needed to be stripped of antibody and re-probed for a loading control (β -Actin), a secondary assay control to insure equal protein loading per lane. Chemicon 1 x Re-Blot-Plus Strong stripping buffer (5ml) heated to 50°C was incubated with a blot for 20 – 30 min, after which, the blot was washed in TBS and re-blocked with 5% (w/v) Dried Milk TBST. Anti- β -Actin Mouse monoclonal antibody (AbCAM), diluted in 5% (w/v) Dried Milk TBST (1:5,000), was incubated with the stripped blot overnight at 4°C. Secondary antibody Anti-Mouse IgG-HRP (Promega), diluted 1:10,000 in 5% (w/v) Dried Milk TBST, was incubated with the blot for 1h at room temperature prior to wash (3 x TBST, 10 min; 1 x TBS, 10 min) and ECL detection, as described in Section 2.5.5.

2.6 Alamar Blue cell viability assay

Alamar Blue TM (AB) is a non-toxic, water soluble dye that can be used to calculate cell viability of cultured cells in a single-step, fluorescence-based assay. The oxidised form of Alamar Blue enters the cytosol and in viable cells with active mitochondrial enzyme activity is reduced by cytochromes and oxidative phosphorylation reducing substrates FMNH₂, FADH₂, NADH and NADPH. The reduced form of Alamar Blue is fluorescent, with excitation at 544nm and emission at 590nm, and the redox event also produces a

colorimetric change in media from the dark oxidised form to the pink reduced form of Alamar Blue. Fluorescence intensity of reduced Alamar Blue at 590nm is directly proportional to the amount of viable cells, as illustrated with HeLa cell seeding density vs. AB fluorescence in Figure 2.1.

0.5 x 10⁴ – 1.5 x 10⁵ cells per well, depending on cell type, were seeded in 96 well plates and left to adhere for 18h. Cells were treated with compound and controls in triplicate for indicated time points. Approximately 6h prior to endpoint, 20µl of Alamar blue was added to each test well and left in the dark at 37°C in a 5% CO₂ / 95% O₂ humidified atmosphere. Fluorescence intensity (FI) was read on a Spectramax Gemini fluorometric plate reader using a SOFTmax Pro version 4.0 (Molecular Devices) software package (Ex: 544nm, Em: 590nm). For data analysis, raw FI values of samples (Sample FI) were blanked by subtracting the FI of oxidised Alamar Blue of wells containing only Alamar Blue + media (Blank FI). Alamar Blue data was normalised by using the FI values of untreated control as representative of 100% viability (Untreated FI). Percentage viability of treated samples was generated using the following equation (*Formula 2.1*):

$$\frac{[(\text{Sample FI}) - (\text{Blank FI})]}{[(\text{Untreated FI}) - (\text{Blank FI})]} \times \frac{100}{1} = \text{\% Cell Viability of Treated Sample}$$

Formula 2.1

2.7 Propidium Iodide cell cycle Flow Cytometry analysis

Propidium iodide (PI) is a fluorescent, DNA intercalating agent which binds stoichiometrically to nucleic acid. Bound to DNA, PI’s fluorescence intensity increases 20 – 30 fold with excitation at 488nm and emission centred on 600nm. Through flow cytometry, a method of multi-parametric analysis of cells as they pass singly through a beam of light, PI staining can aid cell cycle analysis of a population of ethanol-fixed cells. Fluorescence intensity of DNA bound PI directly correlates to the DNA content of a cell and because of this, cells from a population present in G1 phase (diploid – 2n), G2/M phases (4n) or S Phase (2n – 4n) can be easily differentiated by plotting a histogram of PI fluorescence (Linear scale) V’s cell counts. G1 and G2/M populations are observed as

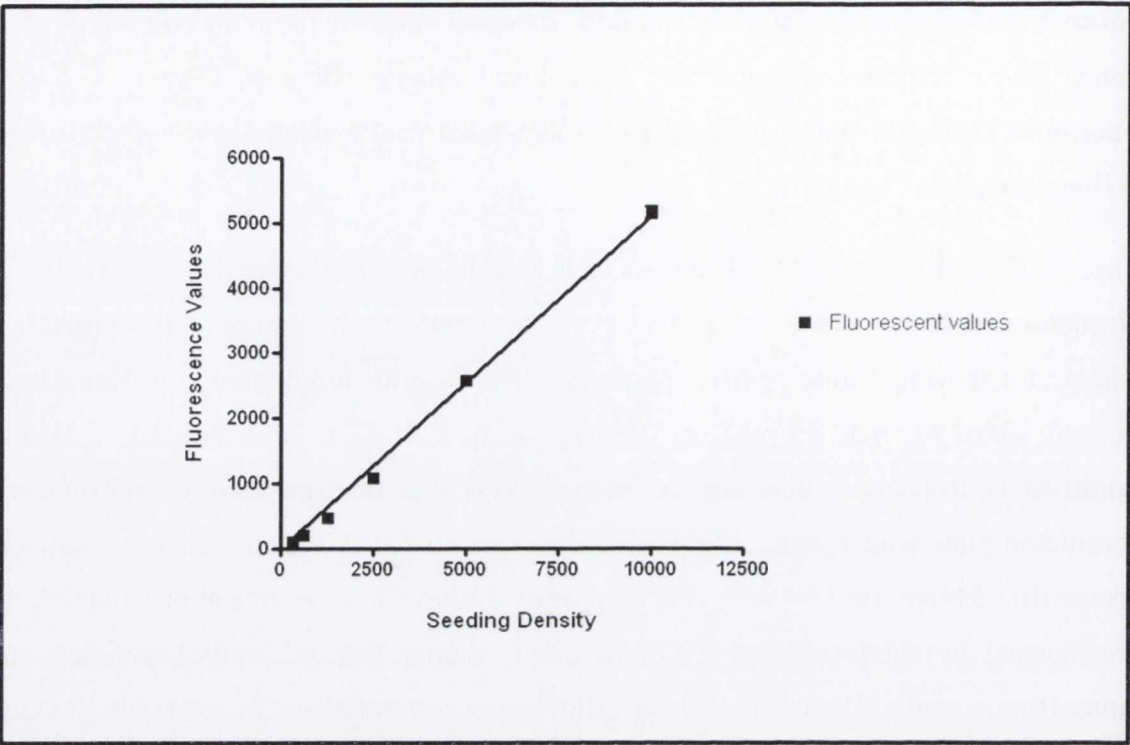


Figure 2.1: Linear relationship between Alamar blue fluorescence intensity and cell density.

HeLa cells seeded at indicated concentrations (0.03125×10^4 – 1.0×10^4 cells per well per 96-well plate - 200 μ l volume) were analysed by Alamar blue cell viability assay after 24h. Plotting raw fluorescence intensity values vs. seeding cell density, the fluorescence intensity of reduced Alamar blue was shown to be directly proportional to the amount of cells present in a test well, with a slope close to 1.

distinct peaks and S phase the population of events in between the two (Fig. 2.2.A). Cells having undergone apoptosis have a hypo-diploid or “pre G1” population indicative of cells and apoptotic bodies with low PI fluorescence due to the occurrence of DNA fragmentation, cell permeabilisation and low DNA content in apoptotic bodies (Fig 2.2B) (Riccardi and Nicoletti, 2006).

Cells were seeded at indicated densities of cells in 25cm² tissue culture flask (5ml Media) and left to adhere for 18h overnight. Cells were treated with compounds and controls for indicated time points. At experimental endpoints, cells were harvested by trypsinisation, centrifuged at 300xg for 5 min and washed once in ice cold PBS. The pellet was resuspended in 300µl of PBS and, while lightly vortexing, fixed with 2ml of 70% ethanol (EtOH) and left at 4°C overnight. Fixed cells were centrifuged at 500xg for 5 min and all EtOH decanted off. The pellet was resuspended in 300µl PBS and placed in 5ml polystyrene round bottom FACS tubes (BD Falcon). 30µl of RNase A (10mg/ml in PBS) and 75µl of propidium iodide (1mg/ml in PBS) were added to each sample and left to incubate in the dark at 37°C for 30 min. Flow cytometry analysis was performed on a FACScaliber (Bectin Dickson) Fluorescence Associated Cell Sorter (FACS) using Cell Quest and Quanti-Quest software. Appropriate gates were employed and data analysed for each experimental sample was representative of 10,000 gated events.

2.8 Caspase assays

2.8.1 Apoptotic inhibition using pan-caspase inhibitor, *z-VAD-FMK*

CRL 5915 cells were seeded at a density of 0.1x10⁶ cells per well (6 well plate) containing 2.5ml culture media. Cells were left to adhere in treatment wells for 18h prior to experimentation. Cells were pre-treated with 40µM pan-caspase inhibitor, *z-VAD-FMK*, for 1h prior to incubation with MDG 44. Experimental controls consisted of equivalent additions of *z-VAD-FMK* and MDG 44 solvent (Vehicle - 0.5% DMSO + 1% EtOH) and *z-VAD-FMK* treatment alone (*z-VAD-FMK* [40µM] + EtOH 1%). Cells were treated with MDG 44 for 6h and 24h time points. Cells were harvested by trypsinisation and apoptotic inhibition was analysed by PI staining and cell cycle Flow cytometry analysis, as outlined in Section 2.7.

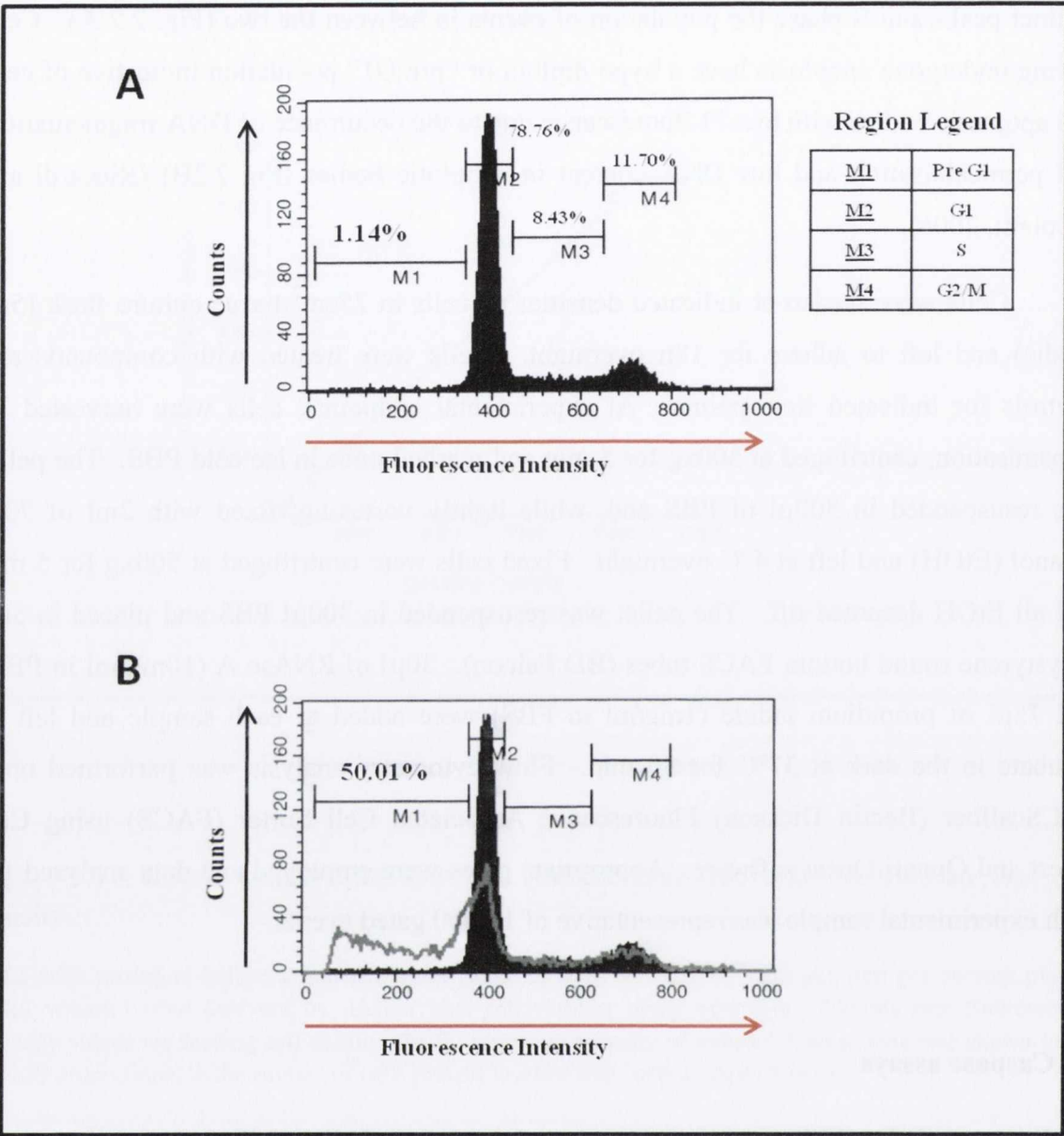


Figure 2.2: Propidium Iodide Cell Cycle Flow Cytometry analysis.

Graphical analysis for PI cell cycle flow cytometry assay. (A) Histogram analysis of a representative population (10,000 gated events) of healthy cells, differentiated by DNA content / cell cycle phase. Defined peaks represent the percentage of cells occupying the G1, S and G2/M phases of the cell cycle. (B) Induction of apoptosis in cells (Grey overlay) is represented by an increase in the “pre G1” population (50.01% of gated events) in histogram analysis of PI flow cytometry.

2.8.2 Apoptotic inhibition using Caspase-9 specific inhibitor, *z-LEHD-FMK*

CRL 5915 cells were seeded at a density of 0.1×10^6 cells per well (6 well plate) containing 2.5ml culture media. Cells were left to adhere in treatment wells for 18h prior to experimentation. Cells were pre-treated with 40 μ M of the Caspase-9 specific inhibitor, *z-LEHD-FMK*, for 1h prior to incubation with MDG 44. Experimental controls consisted of equivalent additions of *z-LEHD-FMK* and MDG 44 solvent (Vehicle - 0.5% DMSO + 1% EtOH) and *z-LEHD-FMK* treatment alone (*z-VAD-FMK* [40 μ M] + EtOH 1%). Cells were treated with MDG 44 for 8h and 48h time points. Cells were harvested by trypsinisation and apoptotic inhibition was analysed by PI staining and cell cycle Flow cytometry analysis, as outlined in section 2.7.

Cisplatin was used as a positive control for a Caspase-9 / Intrinsic apoptotic pathway dependant activation of apoptosis. CRL 5915 cells were seeded and treated with Cisplatin [25 μ M] with or without pre-treatment with *z-LEHD-FMK* [40 μ M], as described above. Cells were treated for 48h and apoptotic inhibition was analysed by PI staining and cell cycle Flow Cytometry analysis.

2.8.3 Fluorogenic caspase – 3, -8, & -9 activation assay

CRL 5915 cells were seeded at a density of 0.325×10^6 cells per 25cm² tissue culture flask in 5ml of culture media. Cells were left to adhere for 18h prior to incubation with vehicle or test compounds for indicated time points. At each experimental endpoint, cells were harvested by trypsinisation and centrifuged at 300xg for 5 min. Cells were washed twice in 2ml ice cold PBS and pellets resuspended in 120 μ l of 1 x Harvesting Buffer (20mM Hepes (pH 7.5), 10% (w/v) Sucrose, 0.1% (w/v) CHAPS, 1% (v/v) Nonidet P40 and 1mM Sodium EDTA) supplemented with fresh 2mM DTT, 1mM PhenylMethylsulphonyl Fluoride (PMSF), 1 μ g/ml Leupeptin and 1 μ g/ml Aprotinin. Cells were lysed on ice for 10 min, passed 12 times through a 21 gauge needle and incubated for a further 10 min on ice. Lysates were centrifuged at 20,000 x g, for 20 min, at 4°C. Supernatants were kept and stored at -20°C prior to investigation of caspase activation using a fluorescent based caspase activation assay.

The protein concentration of caspase lysates was determined by BCA assay, as described in Section 2.5.2. Lysates were diluted to a concentration of 100 μ g protein in 50 μ l

1 x Harvesting buffer. To the 100µg of lysate protein, 50µl of 2 x Caspase Reaction Buffer (1x: 100mM Hepes, 10% (w/v) Sucrose, 0.1% (w/v) CHAPS, 1mM EDTA and fresh 10mM DTT) was added. Fluorogenic substrates specific to caspase-3, caspase-8 or caspase-9 were added to the reaction mixture in independent experiments. These fluorescent substrates contained the fluorescent tag, 7-amino-4-methylcoumarin (AMC), and tetrapeptide cleavage motifs specific for either caspase -3 (DEVD), -8 (IETD) or -9 (LEHD). Active caspases bind their specific substrate, resulting in the cleavage and fluorescence of the AMC tag. The fluorescence from cleaved AMC is used to detect and quantify the amount of active caspase present in treated lysates.

To caspase lysates, 5µl of substrate (20x) was added and the reaction mixture left to incubate at 37°C, in the dark, for 2h. Final concentrations of 50µM of Caspase-3 substrate (Ac-DEVD-AMC), 50µM of Caspase-8 substrate (Ac-IETD-AMC), and 200µM of Caspase-9 substrate (Ac-LEHD-AMC) were used, with each caspase assay preformed separately. Fluorescence intensity (FI) was read on a Spectramax Gemini fluorometric plate reader using a SOFTmax Pro version 4.0 (Molecular Devices) software package (Ex: 380nm, Em: 460nm). Caspase activation data was analysed and presented as the fold induction of active caspase in drug and vehicle treated samples compared to the basal levels in untreated controls.

2.9 Annexin V / PI staining apoptosis assay

The Annexin V / Propidium iodide (PI) dual staining assay is a method of differential detection of early-stage and late-stage apoptosis by way of live cell flow cytometry. During the initial steps of the apoptotic program in cells, phosphatidylserine (PS), a phospholipid normally resident in the cytoplasmic leaflet of the plasma membrane, flips to the extracellular side of the cell membrane, a signal for phagocytosis by macrophages (Fadok et al., 1992). The protein Annexin V, in the presence of Ca^{2+} , has high affinity for PS and when conjugated to a fluorochrome can be used to detect cells at the early stage of apoptosis by Flow Cytometry. As the cell death program proceeds, late apoptotic / dead cells form permeabilised plasma membranes. This allows the cell entry and DNA binding of the fluorescent molecule PI, a marker for cell death. As such, dual staining of cells with Annexin V and PI allows for the differential detection of early apoptotic cells

(Annexin V +; PI -), late apoptotic (Annexin V +; PI +) and normal viable cells (Annexin V -; PI -) in cell populations (Fig 2.3).

CRL 5915 cells were seeded at density of 0.225×10^6 cell / 25cm^2 tissue culture flask (5ml) and left to adhere for 18h prior to experimentation. Cells were treated with vehicle or test compounds at indicated concentrations. At the experimental endpoint, cells were harvested by trypsinisation and centrifuged at 300xg for 5 min. Cells were washed twice in Ice cold 1 x PBS, followed by two additional wash steps in 1 x Ca^{2+} Annexin V binding buffer (1x: 0.545mM Hepes, 7mM NaCl and 125 μM CaCl_2 , pH to 7.4). The cell pellet was resuspended in $\sim 500\mu\text{l}$ of 1 x Ca^{2+} binding buffer, depending on pellet size, and 100 μl of cells were taken for staining. Cells were dual stained with 10 μl of Annexin V-Fitc (IQ products – IQP-120F) and 10 μl of Propidium iodide (50 $\mu\text{g}/\text{ml}$), covered and incubated on ice for 30 min. Control samples consisting of cells left unstained or single stained with Annexin V or PI alone, were prepared for determining fluorescence channel compensations during Flow Cytometry analysis. After incubation on ice, 400 μl of 1 x Ca^{2+} binding buffer was added to each sample and Flow Cytometry analysis completed within 1h of cell staining. Data acquisition and analysis was performed on a FACScaliber (Bectin Dickson) Fluorescence Associated Cell Sorter (FACS) using Cell Quest and Quanti-Quest software. Appropriate gates were employed and data analysed for each experimental sample was representative of 10,000 gated events.

2.10 Comet assay

The Comet assay is a method of single cell gel electrophoresis for the detection of DNA damage in individual cells (Singh et al., 1988). The assay involves the embedding of treated cells in a thin layer of agarose on a microscope slide. Cells are fixed and lysed, removing unwanted cellular material, leaving only nuclear DNA which is subsequently subjected to electrophoresis. The electrophoretic mobility of DNA through the agarose gel depends on its size and charge. As such, damaged or fragmented DNA will migrate much further through the gel during electrophoresis compared to intact nuclear DNA, forming a DNA smear or “Comet Tail” in the agarose gel. Propidium iodide DNA staining and fluorescent imaging is used to detect and quantify DNA damage in this way. This assay is used to detect DNA damage in cells in response to treatment with genotoxic agents or induction of programmed cell death.

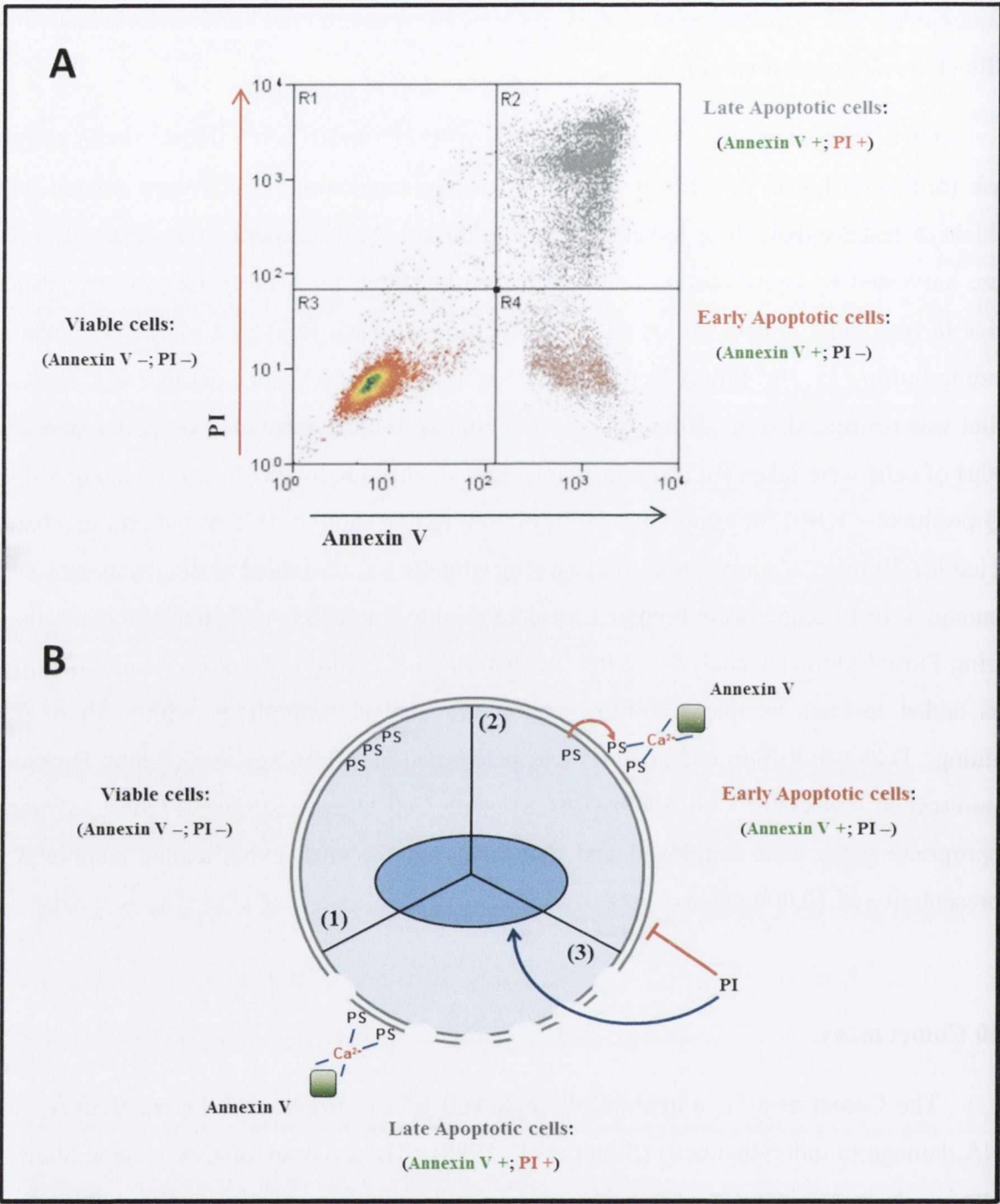


Figure 2.3: Annexin V / PI staining apoptosis assay

Flow Cytometry dot plot analysis of Annexin V / Propidium Iodide (PI) dual stained cells (A). Schematic analysis of Annexin V / PI dual staining of cells and differential detection of viable (Annexin V -; PI -), early apoptotic (Annexin V +; PI -) and late apoptotic (Annexin V +; PI +) cells (B).

2.10.1 Comet assay: Cell treatment

CRL 5915 cells were seeded at a density of 0.3125×10^6 cells / 25cm^2 tissue culture flask (5ml) and left to adhere for 18h prior to experimentation. Cells were treated with Vehicle (EtOH 1%) or MDG 44 [$50\mu\text{M}$] for 5h. At the experimental endpoint, cells were harvested by trypsinisation, using a 0.5% stock of trypsin-EDTA and centrifuged at $300\times g$ for 5 min. Cells were washed twice in ice cold PBS and the pellet was resuspended in $50\mu\text{l}$ $1 \times$ PBS and kept on ice.

2.10.2 Comet assay: Agarose slide preparation, cell lysis and Single Cell Gel Electrophoresis

Microscope slides were dipped in melted 1.5% agarose gel and left to dry at room temperature, forming a thin layer of Agarose on the slide. Low melting point agarose (LMPA – 0.5% in $1 \times$ PBS) was melted and maintained at a temperature of 37°C in a water bath. $75\mu\text{l}$ of LMPA was combined with $10\mu\text{l}$ of resuspended cells ($\sim 10,000$ cells), placed onto a 1.5% agarose coated microscope slide and covered with a coverslip. Once hardened, a third, “sandwich” layer of LMPA was added onto the slide and let set.

Cells embedded in the agarose gel were lysed using pre-chilled (4°C) $1 \times$ Comet Lysis buffer (1x: 2.5M NaCl, 100mM EDTA, 10mM Tris, 0.16g/ml NaOH, pH 10.0 and supplemented with fresh 1% Triton-X-100). Agarose slides were immersed in the $1 \times$ Lysis buffer and incubated at 4°C for 2h.

After lysis, slides were placed in a horizontal gel box, parallel to each other and perpendicular to the cathode end. Slides were covered in Electrophoresis alkaline buffer (1x: 300mM NaOH, 1mM EDTA, pH>13) and incubated for 20 min prior to starting electrophoresis. The gel was run at 24V and 330mAmps for 30 min.

2.10.3 Comet assay: Fixation of cells, Propidium iodide DNA staining and fluorescent imaging of “Comet Tails”

After electrophoresis, slides were washed three times in Neutralisation buffer (1x: 0.4M Tris, pH 7.5). Cells embedded on the slides were then fixed in 100% Methanol at -20°C , for 20 min and then stored at room temperature until ready for analysis. Prior to

imaging, slides were rehydrated in distilled H₂O for 30 min and then stained by coating the agarose gel with 10µg/ml Propidium Iodide for 20 min. Excess stain was removed by washing slides with distilled H₂O. Cellular DNA was imaged using an Olympus IX81-long focal length fluorescent microscope and pictures taken using an F-View II digital camera. The extent of DNA migration / Comet tail formation was compared between vehicle and MDG 44 treated samples.

2.11 Cytospin and RapiDiff staining of cells

Cells were seeded at a density of 0.25×10^6 cells in 25cm² tissue culture flasks (5ml) and left to adhere for 18h. Cells were treated with vehicle or drug for indicated time points and harvested by trypsinisation. Cell pellets were resuspended in 2ml of media, with 170µl taken and centrifuged onto 25mm x 75mm microscope slides (Thermo Scientific) at 500xg for 10 min using a Cytospin 3 specialised centrifuge (Shandon). Slides were left to air dry before staining.

Staining was performed using a RapiDiff Nuclear and cytoplasmic staining kit. Slides were dipped 10 times into RapiDiff Solution A (100% Methanol) to fix the cells. The cytoplasm is stained pink with 10 dips into Solution B (Eosin Y). The Nucleus is stained Blue with 8 dips in Solution C (Methylene Blue). Excess dye is washed off by careful rinse with distilled H₂O. Slides were visualised using a Zeiss Axiovert microscope and the effects of drug treatment on cellular morphology analysed and recorded using a 40x magnification lens.

2.12 XIAP-BIR3 fluorescence polarisation competitive binding assay

The Fluorescence polarisation (FP) principle describes the phenomenon in which fluorescent molecules in solution, excited with plane-polarised light will emit light directly back into a fixed plane, if the molecule remains stationary during the excitation and emission state (~ 4 nanoseconds). In FP binding assays this principle is employed to test ligand (agonist or antagonist) binding to a Protein / Ligand Binding Domain (LBD). A LBD of interest is incubated with a fluorescently labelled native ligand, excited vertically by plane-polarised light and fluorescence is monitored in parallel and perpendicular planes. In

solution, the small fluorescent probe has a very fast molecular rotation and, as such, does not remain stationary through the excitation state. This phenomenon gives low levels of fluorescent emission parallel to the excitation plane and emissions are said to be depolarised. When bound to the large LBD, the fluorescent probe / LBD complex has a much slower molecular rotation. The fluorescent probe remains stationary during the excitation state and fluorescent emission is polarised back into the plane of excitation. FP values are measured in milli-Polarisation units (mP) and calculated as follows (*Formula 2.2*):

$$\text{mP} = \frac{I_{//} - I_{\perp}}{I_{//} + I_{\perp}}$$

$I_{//}$ = Polarised Fluorescence Intensity

$I_{\perp}$ = Depolarised Fluorescence Intensity

Formula 2.2

Milli-Polarisation values measure the ratio of plane polarised light (Numerator) to total fluorescence in parallel and perpendicular directions (Denominator). FP competitive binding assays employ this technique by assaying the ability of test compounds to compete with a fluorescent probe for binding to the LBD. If a test compound has strong affinity for the LBD, it will out compete / displace the fluorescent probe from the binding pocket in a dose dependant manner. This results in a reduction in polarised fluorescent emission and a subsequent reduction in mP value (Illustrated in Fig 2.4). If a test compound has no affinity for the LBD, no change in fluorescent probe binding, polarised fluorescent emission or mP value will be observed.

A FP competitive binding assay was developed for the BIR3 domain of XIAP. The assay was designed and optimised in house, with reference to experiments performed by Nikolovska-Coleska et al (Nikolovska-Coleska et al., 2004). The assay was developed to test the affinity of computationally designed small-molecule SMAC mimetics for the Caspase-9 / SMAC-AVPI binding site of XIAP-BIR3.

In a 96 well black bottom plate, each assay well contained 0.15µM human recombinant XIAP-BIR3 (R&D Systems Cat. No. 895-XB), 0.005µM 5-carboxyfluorescein

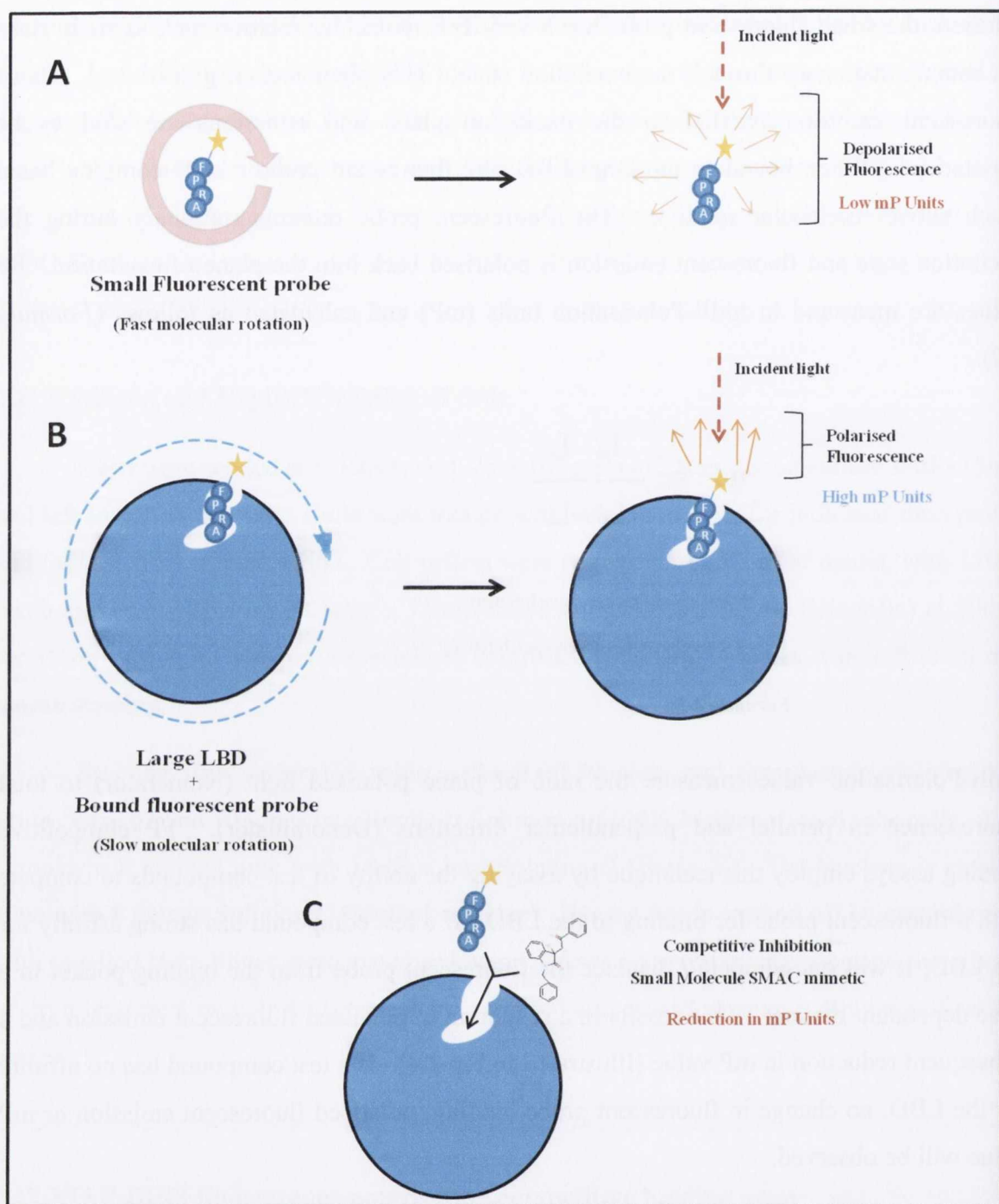


Figure 2.4 Fluorescence Polarisation competitive binding assay

(A) Small peptidic Fluorescent probe (Fluor) – ARPFAQK-5-FAM – has a fast molecular rotation and emits depolarised fluorescence (Low mP values). (B) Fluorescent probe bound to its Ligand Binding Domain (LBD). Larger LBD has a slow molecular rotation and once excited, bound fluor emits polarised fluorescence (High mP values). (C) Small molecule SMAC mimetic binds competitively to LBD, dissociating fluor and decreasing polarised fluorescence (Reduction in mP values).

tagged fluorescent probe, ARPFAQK-5-*FAM* (Genscript Corp), 1 x assay buffer (100mM potassium phosphate (pH 7.5), 100µg/ml bovine γ-globulins and 0.02% (w/v) sodium azide) and test compound (5µl of 25x running stock) in a final volume of 125µl. Experimental controls included assay incubations with test compound vehicle (Negative control – 0% Fluor displacement) and known XIAP-BIR3 agonist (Positive control – 100% Fluoromone displacement). Each assay was incubated in the dark for 2 – 3h prior to FP reading on a Pherastar fluorometer at excitation and emission wavelengths of 485nm and 530nm respectively.

2.13 TR-FRET PPAR-γ Competitive Binding assay

The Fluorescence Resonance Energy Transfer (FRET) principle describes the phenomenon in which two fluorophores in close proximity can transfer activation energy from one excited fluorophore (donor) to another (acceptor). This principle is employed in FRET competitive binding assays (Sharma et al., 2005). In these assays, the LBD is coupled to an antibody bound fluorophore and incubated with a fluorescently tagged ligand (fluoromone). Fluoromone binding to the LBD, followed by excitation of the antibody bound fluorophore, allows for the proximal transfer of activation energy from the fluorophore (donor) to the fluoromone (acceptor). This results in a FRET signal from the fluoromone and a decrease in detected emission from the donor fluorophore. Competitive binding of molecules to the LBD displaces the fluoromone, inhibiting energy transfer from donor to acceptor, due to reduced proximity, and abolishing the FRET signal. Competitive inhibition also results in an increase in the detected fluorescent emission from the donor, due to the inhibition of energy transfer from donor fluorophore to acceptor (Fig 2.5). Time Resolved FRET (TR-FRET) assays are more sensitive than normal FRET competitive binding assays, employing lanthanide fluorescent donors which have longer fluorescent emission half-lives (µsec – msec) compared to conventional fluorophores (nsec). Delaying FRET signal reading for 50 – 100 msec after excitation will prevent interference from autofluorescent reagents or test compounds in the assay.

A commercially available TR-FRET PPAR-γ competitive binding assay kit (LantaScreenTM) was used to investigate the affinity of test compounds for the PPAR-γ binding pocket. To a 384 well black bottomed plate, the following additions were made, in order, with final reagent concentrations indicated: 20µl of 2x test compound [0.1 - 50µM],

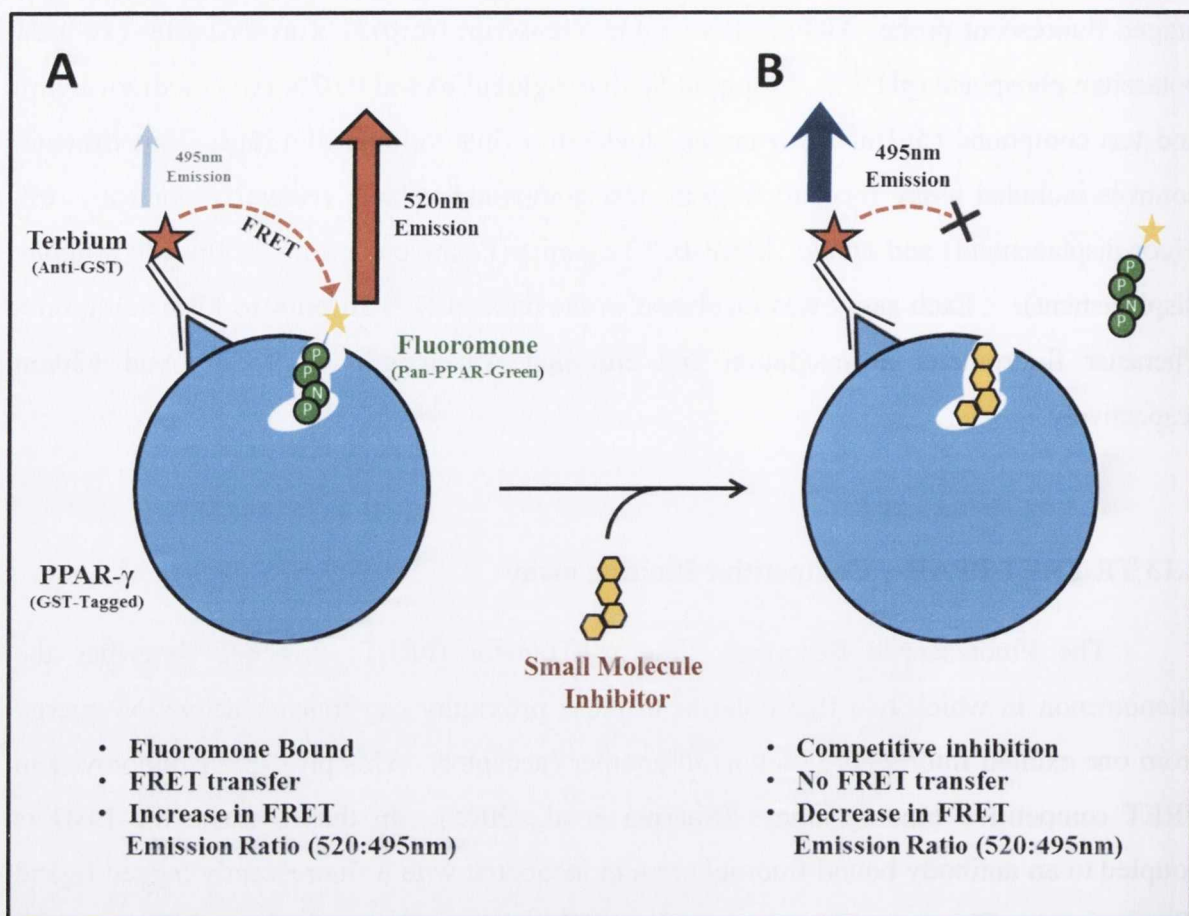


Figure 2.5: TR-FRET competitive binding assay

Schematic diagram of the Time Resolved, Fluorescence Resonance Energy Transfer (TR-FRET) principle; employed in a PPAR- γ competitive binding assay. **(A)** Fluoromone binding to the LBD allows the proximal transfer of energy from excited Terbium (donor) to the fluorescent probe (acceptor), resulting in fluorescent emission at 520nm and decreased detected fluorescent emission from terbium at 495nm. This causes a high FRET emission ratio (520nm emission / 495nm emission). **(B)** Displacement of the Fluoromone by a competitive inhibitor disrupts energy transfer to the Fluoromone. This is due to the reduced proximity of the Fluoromone to the LBD bound terbium, inhibiting FRET signal at 520nm and increasing detected fluorescent emission from terbium at 495nm. Competitive inhibition results in a decrease in the FRET emission ratio (520nm emission / 495nm emission).

10ul of 4x PanPPAR-Green Fluoromone [5nM] and 10μl of 4x PPAR-γ-LBD [0.5nM]/Terbium-anti-GST antibody [5nM] complex, giving a final assay volume of 40μl. All reagents were diluted to desired running stocks in a 1x TR-FRET PPAR-γ assay buffer, supplemented with 5mM DTT. Experimental controls included assay incubations with test compound vehicle (Negative control – 0% Fluoromone displacement) and the known PPAR-γ agonist, GW1929 [1μM] (Positive control – 100% Fluoromone displacement). Test plates were covered and incubated at room temperature for 1 – 6h prior to reading fluorescent emissions using a Pherastar fluorometer. The excitation wavelength of 340nm was used and emission was detected at 495nm and 520nm. The ratio of FRET emission (emission at 520nm / emission at 495nm) was calculated and graphed.

2.14 Catalase Spectrophotometric Enzyme assay

Catalase enzyme activity in CRL5915 drug treated and whole cell lysates was calculated using a spectrophotometric enzyme assay. Catalase catalyses; (1) – The decomposition of Hydrogen Peroxide to Water and Oxygen (Catalase Rxn); and (2) – The oxidation of alcohols to aldehydes (Peroxidic Rxn) (Chance, 1948):



Catalytic Rxn



Peroxidic Rxn

Formula 2.3

Enzyme activity in the Catalase Rxn was calculated spectrophotometrically by monitoring the decomposition of H₂O₂ (Abs @ 240nm) as the reaction proceeds, using a Unicam Helios γ Spectrophotometer and a LEM Servoger 102 chart recorder. Enzyme assays were performed at a constant temperature of 25°C, in a 3ml quartz cuvette containing 20mM H₂O₂ substrate in 50mM Potassium Phosphate Buffer, pH 7.0 (3ml assay volume). The chart recorder was set to a speed of 1cm min⁻¹ and an absorbance scale of 0 – 1 Abs units.

The Catalase activity of CRL 5915 whole cell lysate was assayed. Whole cell lysates were prepared using 1 x Ripa Buffer and lysate protein concentration was

determined, as described in Sections 2.5.1 and 2.5.2 respectively. The spectrophotometer was set to read absorbance at 240nm and blanked using 50mM Potassium phosphate buffer. Experiments were performed at 25°C. Upon the addition of H₂O₂, the blank rate was monitored for 2 – 3 min. Various concentrations of CRL 5915 protein (25µg - 400µg) was added to the cuvette and the decomposition of H₂O₂ (drop in absorbance @ 240nm) was observed for 2 – 5 min. Bovine liver Catalase [5 Units] (Sigma) was added to the reaction mixture after assaying CRL 5915 lysate as a positive control for Catalase activity. Initial reaction velocities for CRL 5915 lysate protein additions were calculated and enzymatic rate ($\Delta [\text{H}_2\text{O}_2] \text{ min}^{-1} - \text{mmol l}^{-1} \text{ min}^{-1}$) and specific activities (The total H₂O₂ decomposition, per min, per mg of protein added - $\mu\text{mol min}^{-1} \text{ mg}^{-1} / \text{Units mg}^{-1}$) were determined (Molar extinction coefficient 43.6 M⁻¹ cm⁻¹).

2.14.1 Catalase assay: Km Calculations

Attempts were made to calculate the Michaelis-Menten constant (K_m) of Catalase in CRL 5915 whole cell lysate and commercially available Bovine Liver Catalase. Catalase enzyme assays were performed as described using 400µg of CRL 5915 whole cell lysate and 4 Units Bovine liver Catalase. Separate experiments were performed with increasing concentrations of substrate (H₂O₂ – 2.5mM – 45mM). Initial velocities and enzymatic rates were calculated and K_m values predicted using a Hanes-Woolf Plot – $[S] \text{ v's } [S].V_0$; x-intercept = -K_m (Hanes, 1932).

2.14.2 Catalase assay: Sodium azide inhibition assay

Catalase enzyme assays were performed in the presence of sodium azide, a known inhibitor of Catalase enzyme activity (Gregory et al., 1977). Catalase activity was assayed using 300µg samples of CRL 5915 whole cell lysate, with or without pre-treatment with Sodium azide [1mM]. The assay and enzyme specific activity calculations were performed as outlined in Section 2.14. The Catalase enzymatic rate ($\Delta [\text{H}_2\text{O}_2] \text{ min}^{-1} - \text{mmol l}^{-1} \text{ min}^{-1}$) was compared between assays with or without pre-treatment with sodium azide.

2.15 Autophagic flux assay

The effect of drug treatment on “Autophagic Flux” – the increase or decrease in the level of degradation of cellular components by autophagy – was assayed by monitoring the protein expression levels of markers for autophagosome formation (LC3-II) and autolysosomal protein degradation (p62). Microtubule-associated protein Light chain 3 (LC3) exists in two forms; the cytosolic protein (LC3-I - ~16kDa) and lipidated protein (LC3-II - ~14kDa) – LC3-I conjugated to phosphatidylethanolamine (PE). Lipidation of LC3-I to form LC3-II is essential for autophagosomal formation (Kabeya et al., 2000) and the processing of LC3-I and accumulation of LC3-II in cells is commonly used as a marker for autophagy (Tanida et al., 2005). P62 is recruited to the autophagosome, binds to LC3-II, and upon autolysosomal formation, p62 is degraded (Bjorkoy et al., 2005). In this assay, the marker for true increase in autophagic flux in response to drug treatment was the accumulation of LC3-II and degradation of p62, monitored by Western blotting analysis.

2.15.1 Nutrient Starvation assay

CRL 5915 cells were seeded at a density of 0.13×10^6 cells / well (2.5ml) on a 6-well tissue culture plate, and left to adhere for 18h prior to experimentation. Cells were incubated in the presence of nutrient rich media (RPMI-1640 culture media supplemented with glucose, glutamine and FBS) or nutrient starvation media (HBSS media – Hank’s Balanced Salt Solution, independent of serum supplements) for 2h and 4h. Nutrient competent incubations involved replacing growth media with 2.5ml, fresh CRL 5915 cell culture media. Nutrient starved incubations involved removing growth media, washing cells three times in sterile 1 x PBS and incubating cells in 2.5ml HBSS. Nutrient rich and nutrient starvation incubations were also performed with or without the addition of cathepsin inhibitors, E64d [10µg/ml] and Pepstatin A [10µg/ml].

2.15.2 LC3-II and p62 Western Blot analysis

CRL 5915 cells were seeded at a density of 0.13×10^6 cells / well (2.5ml) on a 6-well tissue culture plate, and left to adhere for 18h prior to experimentation. Cells were treated in nutrient rich or nutrient starvation media (Section 2.15.1), with known autophagy inhibitors Chloroquine [25µM] and Bafilomycin A1 [10nM], MDG 44 [30µM - 40µM] and

Vehicle (EtOH 1%) for indicated time points. Treatments were performed with or without the addition of cathepsin inhibitors, E64d [10µg/ml] and Pepstatin A [10µg/ml]. At experimental end points, whole cell lysates were prepared and protein expression levels of LC3 and p62 was assayed by Western Blot analysis, as outlined in Section 2.5.

Specifically in LC3-II / p62 Western Blot experiments, 20µg of protein was resolved by SDS-PAGE on a 12.5% polyacrylamide gel (29:1 ratio Acrylamide:Methylene-bisacrylamide) and transferred to PVDF membrane (69V; 148mAmps for 1.5h). The blot was blocked with blocking buffer (5% Marvel TBS-Tween (0.1% Tween)) for 2h at room temperature. The blot was cut and probed with Rabbit Anti-LC3 (1 in 3,000 dilution, 5% w/v BSA in TBS-Tween, overnight, 4°C) and Rabbit Anti-p62 (1 in 2,500 dilution, 5% w/v BSA in TBS-Tween, overnight, 4°C) simultaneously. Blots were probed with Anti-Rabbit secondary Ab (1 in 5,000 dilution, 5% w/v Marvel in TBS-Tween) for 2h, at room temperature. Protein bands were imaged using Millipore ECL and Kodak X-Omat LS film.

2.16 Confocal Microscopy: LC3-GFP Macrophage imaging

The induction of autophagy and the accumulation of autophagosomes in cells was tested using confocal microscopy detection of LC3 positive, punctate structures within the cytoplasm of treated cells. A Macrophage cell line stably transfected with an LC3-GFP expression plasmid was used. Cells with GFP “Bright” punctuate structures within the cytoplasm, as opposed to a uniform distribution of GFP fluorescence, were indicative of LC3-II positive structures (autophagosomes).

LC3-GFP Macrophages were seeded at a density of $0.13 - 0.5 \times 10^6$ cell / well and left for 18h prior to experimentation. Cells were seeded on a glass bottom, confocal well in 2ml of culture media. Cells were incubated with Vehicle (EtOH 1%), known inducers of autophagosomal formation, Chloroquine [25µM] and Bafilomycin A1 [10nM], and MDG 44 [35 - 40µM]. Cells were also incubated in nutrient rich and nutrient starvation media (HBSS) – cell preparations for which were described in Section 2.15.1. After 4h incubation, cells were stained with the fluorescent nuclear dye DAPI (4,6-dimidino-2-phenylindole) [0.2µg/ml] for 15 – 20 min prior to imaging of cells. Cells were imaged using Olympus FV1000 Point-Scanning Confocal Microscope and in a separate experiment, an Andor Revolution XD Spinning Disk Confocal Microscope. The GFP fluorescent tag and DAPI

nuclear stain were excited using an Argon multi-line 457 / 488 / 515 nm laser and a 405 nm Near-Violet Laser diode respectively. Confocal microscopy pictures of cells were taken using a 60x oil immersion lens and FluoView Version 7.1 imaging software.

2.17 Hek-Blue NFκB Reporter assay

The Hek-Blue NFκB reporter assay was performed using Hek-BlueTM-hTLR4 cells. Hek-Blue-hTLR4 cells are a commercially available cell line transfected with expression plasmid containing hTLR4, its co-receptors MD2/CD14 and a Secreted Embryonic Alkaline Phosphatase (SEAP) reporter gene containing five NFκB response elements in its promoter region. Stimulation of TLR4 with its native ligand, Lipopolysaccharide (LPS), induces rapid activation of NFκB and consequential expression of SEAP, which is subsequently secreted out of Hek-Blue cells. Levels of SEAP and, in turn, the level of NFκB activation in response to LPS treatment can be quantified by incubating Hek-Blue cell supernatant with Quanti-BlueTM, a medium containing alkaline phosphatase substrate, which changes colour (Pink to Blue) in the presence of the enzyme.

Hek-Blue cells were seeded at a density of 0.25×10^5 cell / well (200μl) on a 96 well tissue culture plate and left incubate for 18h prior to experimentation. Cells were pre-treated for 1h with vehicle (EtOH 1%), or indicted concentrations of MDG 44 [0.1μM – 50μM]. Cells were subsequently incubated with LPS [100ng/ml] for 6h and 24h time points. Experimental controls included cells left untreated, treated with EtOH (1%) alone or pre-treated with the proteasome inhibitor, MG132, with or without incubation with LPS (100ng/ml). At experimental end points, Quanti-Blue analysis of NFκB activation and Alamar Blue cell viability analysis of Hek-Blue cells was performed simultaneously in each assay.

Quanti-Blue analysis was performed by taking 20μl of Hek-Blue supernatant and incubating it with 180μl of filter sterilised, Quanti-Blue media. Supernatants were incubated in the dark, at 37°C for 2h – 8h. In the presence of SEAP, the Quanti-Blue media changes from a pink/red to a purple/blue colour. This colour change was monitored by reading the absorbance at 650nm on a Versamax absorbance plate reader. The raw absorbance values for Quanti-Blue analysis were normalised in order to allow direct comparison between Quanti-Blue NFκB activation and Alamar Blue cell viability results –

percentage NFκB activation v's percentage Hek-Blue cell viability. All data points were normalised to be expressed between 1.0 – 0.0, by subtracting all values by the mean "EtOH only" control (Blank – 0% NFκB activation) and dividing all values by the mean "EtOH + LPS" control (Positive control – 100% NFκB activation).

Alamar Blue cell viability analysis was performed by adding 20μl of Alamar Blue dye to each test well, after 20μl of supernatant was removed from test wells for Quanti-Blue, NFκB activation analysis. Hek-Blue cells were incubated with Alamar blue at 37°C, in the dark, for a further 6h prior to fluorescence analysis using a Spectramax Gemini fluorometric plate reader and SOFTmax Pro version 4.0 (Molecular Devices) software package (Ex: 544nm, Em: 590nm). Raw Alamar Blue cell viability fluorescence results were normalised and graphed as outlined in Section 2.6.

2.18 IκB Degradation assay

CRL 5915 cells were seeded at a density of 0.12×10^6 cell / well (2.5ml culture media) and cells were left to adhere to the 6-well plate for 18h prior to experimentation. Cells were pre-treated with Vehicle (EtOH 1%), MDG 44 or MG132 for 1 – 2h and subsequently treated with Tumour Necrosis Factor-α (TNF-α [10ng/ml]) for 0 – 30min. The induced degradation of IκB in response to TNF-α treatment (NFκB activation) or inhibition of TNF-α induced IκB degradation by pre-treatment with test compounds (inhibition of NFκB activation) was observed by Western blotting analysis.

Preparation of whole cell lysates, BCA assay calculation of protein concentrations, SDS-PAGE and immunoblotting for IκB was performed, as outlined in Section 2.5. Specifically in IκB degradation experiments, 20μg of Protein was resolved by SDS-PAGE on a 10% polyacrylamide gel and transferred to a PVDF membrane (55V; 160mAmps for 1h). Blots were blocked in blocking buffer (5% Marvel TBS-Tween (0.1% Tween)) overnight and then probed with mouse Anti-IκB (1 in 2,500 in blocking buffer) for 2h, washed in TBS-Tween and probed with Anti-mouse secondary Ab (1 in 10,000 in blocking buffer) for 1h. Blots were stripped and re-probed with loading control anti-β-actin as outlined in Section 2.5.6.

2.19 Subcellular fractionation and mitochondrial hexokinase assay

2.19.1 Subcellular fractionation and mitochondrial isolation

Subcellular fractionation and mitochondrial isolation assays were performed in Burkitt's Lymphoma and MPM cell lines. Cells were grown in culture to 80 – 90% confluence in T175 tissue culture flasks (175cm²) and were harvested using a cell scraper. Cells were centrifuged at 300xg for 5 min and subsequently washed twice in ice cold PBS. Cells were resuspended in 1 x STE Buffer (250mM Sucrose, 5mM Tris, 2mM EGTA (pH 7.4)) supplemented with fresh 1 x complete protease cocktail. Cells were left on ice for 5 min before homogenisation with 60 strokes in a 1ml Dounce Homogeniser (Wheaton). Cells were again left incubate on ice for 5 min. 75µl of Fraction 1 (Homogenate) was stored for further analysis and the rest was centrifuged for a "slow spin" at 830xg and 4°C for 5min. The supernatant (Fraction 2 - Cytosolic) was retained and spun again to insure all cell debris was removed. 75µl of Fraction 2 was stored for further analysis and the rest was centrifuged at 14,000xg and 4°C for 30 min. The supernatant (Fraction 3 – Cytoplasmic) was stored for further analysis. The pellet (Fraction 4 – Mitochondria) was washed twice in ice cold 1 x STE buffer (14,000xg, 4°C, 5 min). The mitochondrial pellet was resuspended in 75µl of ice cold 1 x STE buffer and all fractions (1 – 4) were stored at -80°C before analysis by spectrophotometric enzyme assay (Schematic diagram – Fig 2.6.A).

2.19.2 Assessment of purity of fraction: Lactate Dehydrogenase and Citrate Synthase Assay

In order to assess the purity of fractions isolated by subcellular fractionation, spectrophotometric enzyme activity assays on Lactate Dehydrogenase (LDH) and Citrate Synthase (CSH) were performed. LDH, a cytoplasmically localised enzyme, is used to test cytoplasmic (Cyto) fraction contamination of the mitochondrial (Mito) fraction, while CSH, a mitochondrially localised enzyme, is used to test mitochondrial contamination of the cytoplasmic fraction. Enzyme activity assays were performed using a Unicam Helios γ Spectrophotometer and a LEM Servoger 102 chart recorder set to a speed of 1cm min⁻¹ and an absorbance scale of 0 – 1 Abs units.

Lactate Dehydrogenase (LDH) catalyses the reduction of pyruvate to lactate in the cytoplasm (Fig 2.6.B). This reaction requires the oxidation of cofactor NADH₂ to NAD⁺.

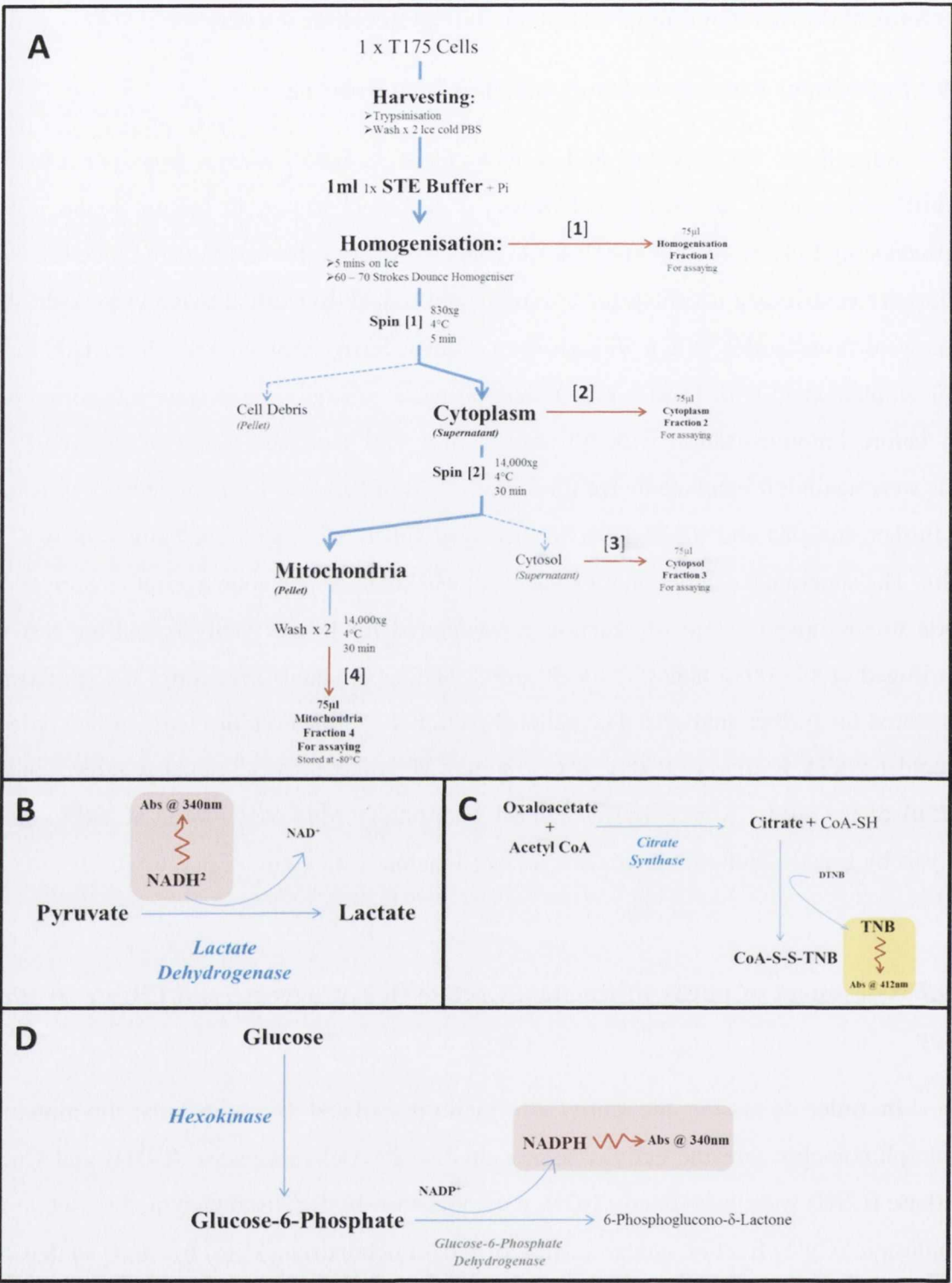


Figure 2.6: Mitochondrial isolation and Spectrophotometric Enzyme assays.

Mitochondria isolated from cells by subcellular fractionation (schematic diagram) (A). Reaction diagrams for spectrophotometric enzyme assays for Lactate Dehydrogenase (B), Citrate Synthase (C) and Hexokinase (D).

Spectrophotometrically, this reaction rate (measured as change in product concentration per unit time - $\mu\text{mol l}^{-1} \text{ min}^{-1}$) can be monitored by the loss of NADH_2 , which absorbs light at 340nm. Using the Beer-Lambert law (*Formula 2.4*) (Molar extinction coefficient of NADH_2 : $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 25°C), the reduction in absorbance as the reaction proceeds can be converted to concentration change.

$$A = \epsilon lc$$

Beer-Lambert Law

A = Absorbance

ϵ = Molar extinction coefficient

l = Path length

c = Concentration

Formula 2.4

Fraction purity and recovery was expressed as specific activity (μmol of product produced per min per mg of fraction protein added - $\mu\text{mol min}^{-1} \text{ mg}^{-1}$) of LDH in test fraction divided by specific activity of LDH in homogenate fraction.

To a 3ml plastic cuvette, 2.78ml of Tris/KCl Buffer (81.3mM Tris and 150mM KCl) was added and the spectrophotometer was zeroed at 340nm. 100 μl of 4mM NADH and test fraction was added and the blank rate of absorbance change monitored for 2 – 3 min. 100 μl of 48mM sodium pyruvate was added to the cuvette, stirred and initial rate was monitored for 5 – 6 min.

Citrate Synthase (CSH) catalyses the first step of the Citric acid cycle, the condensation of acetyl Coenzyme A and Oxaloacetate to Citrate and Co-A-SH (Fig 2.6.C). The subsequent spontaneous reaction of Co-A-SH with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) – DTNB) produces CoA-S-S-TNB and the yellow product TNB, which absorbs light at 412nm with an extinction coefficient of $13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 25°C . Reaction rate was measured spectrophotometrically and fraction purity and recovery were calculated as with LDH assays.

To a 3ml cuvette, 2.8ml of Tris-Sucrose buffer (0.1M Tris and 0.15M Sucrose (pH 8.1)) was added and spectrophotometer zeroed at 412nm. 50 μl of 4mg/ml DTNB, 50 μl of 2mM Acetyl CoA and test fraction were added and blank rate monitored for 2 – 3 min.

75µl of 60mM oxaloacetate was added to the cuvette, stirred and initial rate was monitored for 5 – 6 min.

2.19.3 Mitochondrial-Hexokinase dissociation assay: Spectrophotometric enzyme assay

A sample of isolated mitochondria was divided into 2 aliquots and treated with vehicle, test compounds (Clotrimazole, Econazole and Miconazole) or positive control (Glucose-6-phosphate) for 30min – 1h. Samples were centrifuged at 14,000 x g and 4°C for 15 min. Mitochondrial supernatants were retained and mitochondrial pellets were resuspended in 1 x STE buffer. Spectrophotometric hexokinase (HK) activity assays were performed on both samples (mito and supernatant) of drug and vehicle treatments. Various drugs ability to induce dissociation of HK from the mitochondria and cause free HK accumulation in the supernatant was assessed in this manner.

HK enzyme activity was measured by coupling to the reaction catalysed by glucose-6-phosphate Dehydrogenase (G6PD). HK catalyses the first step in glycolysis, the energy dependant phosphorylation of glucose to glucose-6-phosphate (G6P), resulting in no absorbance change of products. G6PD converts G6P to 6-phosphoglucano-δ-lactone, involving the reduction of NADP^+ to NADPH. Change in absorbance at 340nm due to accumulation of NADPH as the reaction proceeds is used to measure HK activity (Fig 2.6.D).

To a 3 ml cuvette, 1.2ml of 0.25mM Tris buffer (pH 6.9), 300µl of 75mM MgCl_2 , 300µl of 5U/ml G6PD, 300µl of 7.5mM ATP, 600µl of 2mM NADP^+ and 300µl of glucose was added. The blank rate was observed for 2 – 3 min. Test sample was added to the cuvette, stirred and the initial rate was observed for 5 – 6 min. Rates were calculated using the molar extinction coefficient of NADPH at 25°C of $6.22 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$. Specific activities of HK in test samples were calculated (μmol of product produced per min per mg of fraction protein added - $\mu\text{mol min}^{-1} \text{mg}^{-1}$) and used to compare vehicle and drug treated samples for a drugs ability to dissociate HK from the mitochondria.

2.20 Mitochondrial-Hexokinase dissociation assay: Western Blot analysis

Forced dissociation of HK from the mitochondria was also assayed by Western Blot analysis. Cells were seeded at a density of 2.5×10^6 – 15×10^6 cells in 75cm² tissue culture

flasks containing 15ml culture media and were left to incubate for 18h prior to experimentation. Cells were incubated with vehicle or test compounds – Clotrimazole, Econazole and Miconazole – for 1h – 24h time points. At experimental endpoints, STE-buffer cell lysates were prepared and subcellular fractionation and mitochondrial isolation was performed as described in Section 2.19.1. Protein concentration of mitochondrial and cytoplasmic fractions was determined by BCA assay (Section 2.5.2). Protein (30µg) from vehicle and test compound treated mitochondrial and cytoplasmic fractions was resolved by SDS-PAGE on an 8% gel and transferred to PVDF membrane (60V; 160mAmps for 1.5h). Blots were blocked in blocking buffer (5% Marvel TBS-Tween (0.1% Tween)) overnight and then probed with rabbit Anti-HK-II (1 in 1,000 in blocking buffer) for 2h, washed in TBS-Tween and probed with Anti-rabbit secondary Ab (1 in 4,000 in blocking buffer) for 1h. Blots were stripped (Section 2.5.6) and re-probed for mitochondrial and cytoplasmic fraction markers – Heat shock protein-60 (rabbit Anti-HSP60; 1 in 5,000 in blocking buffer) and β -actin (mouse anti- β -actin; 1 in 5,000 in blocking buffer).

Chapter 3

Investigating the potential of SMAC mimetic therapy to overcome IAP-mediated chemotherapeutic resistance in Malignant Pleural Mesothelioma

Introduction

The IAP protein family regulate caspase activation in normal somatic cells, a failsafe against inappropriate activation of cell death. Once PCD is induced in cells, SMAC is released from the mitochondrial inter-membrane space and can directly inhibit or induce proteosomal degradation of IAPs, circumventing IAP-mediated resistance and allowing apoptosis to proceed. In cancer, this network of caspase checkpoints can be co-opted through overexpression of IAP family proteins to confer a resistant and defective apoptotic phenotype in tumours. Specific overexpression of Survivin, c-IAP1/2 and XIAP has been associated with resistance and poor prognosis in many cancer sub-types including neuroblastoma (Islam et al., 2000), breast carcinoma (Bockbrader et al., 2005), acute myeloid leukaemia (AML) (Sung et al., 2009) and multiple myeloma (Nakagawa et al., 2006). Targeting this altered IAP expression profile specific to cancer is potentially a very effective strategy in developing rational therapies against susceptible cancers. Anti-sense knockdown of IAPs (McManus et al., 2004), peptide (Fulda et al., 2002) and small molecule mimics of SMAC (Nikolovska-Coleska et al., 2004) have been shown to inhibit overexpressed IAPs in cancer and directly kill or sensitise cells to chemotherapy.

IAP targeting was originally proposed as a method of overcoming IAP-mediated apoptotic resistance by relieving the direct sequestration of active caspases by IAP family proteins and sensitising cells to chemotherapeutic challenge. XIAP inhibition is of particular interest due to its ability to confer apoptotic resistance to cancer in this manner (Deveraux et al., 1998, Deveraux et al., 1997). SMAC mimetics have been shown to target BIR2 and BIR3 domains of XIAP, preventing binding of active caspase-3/7 and active caspase-9, respectively (Wu et al., 2003, Schimmer et al., 2006). This prevents the apoptotic blockade of overexpressed XIAP and thus chemosensitises cells to induction of apoptosis through intrinsic apoptotic pathway drug challenge. This effect of IAPs mediating apoptotic resistance and SMAC mimetic / IAP targeting being able to chemosensitise resistant cancer cells is succinctly illustrated in a study by Nikolovska-Coleska *et al* (Nikolovska-Coleska et al., 2004). In Jurkat T-lymphocytes, artificial overexpression of XIAP conferred increased apoptotic resistance to the topoisomerase II poison, Etoposide. However, co-treatment with the small-molecule SMAC mimetic embelin, was able to overcome XIAP mediated resistance and re-sensitise cells to apoptosis.

SMAC mimetic therapy has also been shown to elicit single-agent apoptotic effects, as well as chemosensitisation in cancer. This response is incongruous to conventional theories on XIAP targeting, which require SMAC mimetic therapy in conjunction with stimulus of the intrinsic apoptotic pathway to induce cell death. Explanations for this include a study by Yang *et al*, which propose that high IAP protein expression, in particular XIAP, is in response to high levels of active initiator and effector caspases present in cancer cells (Yang et al., 2003a). The study proposes that SMAC mimetic treatment releases the sequestration of these active caspases, stimulating apoptosis without the requirement of co-treatment with chemotherapeutics. Other explanations include particular SMAC mimetics engaging alternate cellular targets with the ability to induce PCD. These effects were documented in numerous embelin studies (Ahn et al., 2007, Siegelin et al., 2009) and suggested to explain data reported in Oost *et al* in which novel SMAC mimetics were shown to have affinity for XIAP-BIR3, but single agent drug potency did not correlate with XIAP protein expression in treated cells (Oost et al., 2004). The third and recently most well documented explanation for SMAC mimetic, single-agent apoptosis is the targeting of alternate IAP family members, specifically cIAP-1/2.

Numerous recent studies have investigated the apoptotic potential of targeting cIAP-1 and cIAP-2 using SMAC mimetic compounds (Vince et al., 2007, Yang and Du, 2004, Varfolomeev et al., 2007, Petersen et al., 2007). The cIAP-1/2 proteins have a number of diverse cellular functions, which were fully detailed in Chapter 1. Specifically for SMAC mimetic therapy in cancer, their role in regulation of TNFR / Caspase-8/10 signalling and upstream ubiquitination control of the canonical and non-canonical NF κ B pathway is of particular importance. Native SMAC, as well as peptide (Vince et al., 2007) and small molecule SMAC mimetics (Varfolomeev et al., 2007, Petersen et al., 2007) have been shown to bind to the BIR-2/3 domains of cIAP-1/2, inducing a conformational change in its tertiary structure. This structural change allows for the autoubiquitination of the protein, catalysed by its RING-E3 ubiquitin ligase domain, tagging cIAP-1/2 for subsequent proteosomal degradation. The loss of cIAP-1/2, induced by SMAC, modulates standard control of TNF- α apoptotic signalling. In light of this, studies have shown that SMAC and SMAC mimetics can facilitate the formation of the RIP1/FADD/Caspase-8 death receptor complex, caspase-8 activation and apoptotic induction when treated in conjunction with TNF- α (Wang et al., 2008). SMAC mimetic targeting of cIAP-1/2 has also been shown to induce single-agent, caspase-8 dependant apoptosis, completely independent of exogenous

co-treatment with TNF- α . SMAC mimetic degradation of cIAP-1/2 blocks its negative regulation of NIK, an upstream activator of non-canonical NF κ B signalling. In susceptible cell lines, NF κ B activation in this way results in expression of TNF- α and engagement of an autocrine signalling loop. Autocrine ligation of the TNFR, coupled with the loss of cIAP-1/2 control of death receptor signalling, results in caspase-8 dependant apoptosis induced by SMAC mimetic targeting of cIAP-1/2 alone (Petersen et al., 2007).

The ability of SMAC mimetic therapeutics to overcome IAP resistance through either chemosensitisation or inducing direct apoptotic effects is of great interest for the development of treatments for chemoresistant malignancies, in particular Malignant Pleural Mesothelioma (MPM). MPM is a highly aggressive neoplasm of the cell lining of the pleural cavity and is an incurable cancer with poor response rates and median survival times with conventional chemotherapy treatment strategies (Belli et al., 2009). MPM resistance and poor prognosis in patients has recently been associated with overexpression of IAP family proteins, in particular cIAP-1 (Gordon et al., 2007a, Kleinberg et al., 2007). Also, TNF- α signalling is believed to be a causative factor in the aetiology of MPM and has been associated with conferring a protective effect against apoptotic challenge in MPM (Yang et al., 2006). The protective effect of TNF- α is NF κ B dependent (Yang et al., 2006) and a recent study by Gordon *et al* has shown that TNF- α can elicit this protective effect through inducing increased protein expression of IAP family proteins, specifically cIAP-1, cIAP-2 and XIAP (Gordon et al., 2007b). Using SMAC mimetic therapy to target the reliance of MPM on IAP expression and TNF- α signalling for apoptotic resistance and survival may be an effective treatment strategy for this fatal, chemoresistant disease.

The aims of this Chapter was to first, confirm the importance of IAP family member expression in conferring chemotherapeutic resistance to a panel of human Malignant Pleural Mesothelioma cell lines and investigate a link between high IAP expression in MPM and apoptotic resistance to a range of known chemotherapeutic agents. Of particular interest were XIAP and c-IAPs due to their defined role in resistance in cancer and as targets for SMAC mimetic drugs. Subsequently, SMAC mimetic therapy was investigated for its ability to overcome resistance and challenge the proposed tumour survival reliance of MPM on the cellular alteration of IAP overexpression. This was investigated using small molecule and peptide mimetics of the SMAC N-terminal tetrapeptide AVPI sequence in combination with cisplatin and TNF- α , intrinsic and extrinsic apoptotic pathway activators,

respectively. These experiments aimed to investigate sensitisation of cells to apoptosis through SMAC mimetic challenge of both XIAP and cIAP-1/2.

Results

3.1 IAP expression levels in Mesothelioma cell lines

In order to validate the hypothesis that chemoresistance in Mesothelioma is conferred through IAP overexpression, it was first necessary to determine the basal cellular expression levels of the IAP family members of interest. The differential protein expression levels of c-IAP-1, c-IAP-2, XIAP and Survivin, all proteins implicated in apoptotic resistance in cancer, were determined in a panel of four Mesothelioma cell lines (CRL 5915, CRL 5820, One 58 and Ju77) and, for comparative analysis, in the well-established HeLa cervical cancer cell model.

3.1.1 Mesothelioma cell lines have high basal expression levels of c-IAP-1

Protein expression levels of c-IAP-1 (72kDa) in the Mesothelioma cell line panel and HeLa cells were determined by way of Western blot analysis (Fig 3.1.A), as outlined in Section 2.5. Results revealed high expression levels of c-IAP-1 in all four Mesothelioma cell lines analysed, compared to HeLa. Mesothelioma expression levels of c-IAP-1 were also elevated compared to Burkitt lymphoma cell lines Mutu-1 and DG-75 (Data not shown). These results are in line with previous reports that c-IAP-1 levels are highly elevated in Mesothelioma tumour specimens (Gordon et al., 2002). This study by *Gordon et al* also reports that high c-IAP-1 expression in Mesothelioma plays a role in promoting tumour cell survival against cisplatin challenge. A follow up report by the same lab describes a correlation between elevated c-IAP-1 levels and shorter survival times in Mesothelioma patients (Gordon et al., 2007a).

3.1.2 CRL 5820 cells have high basal expression levels of c-IAP-2, XIAP and Survivin

Protein expression levels of c-IAP-2 (72kDa), XIAP (53kDa) and Survivin (16.5kDa) in the Mesothelioma cell line panel and HeLa cells were determined by way of

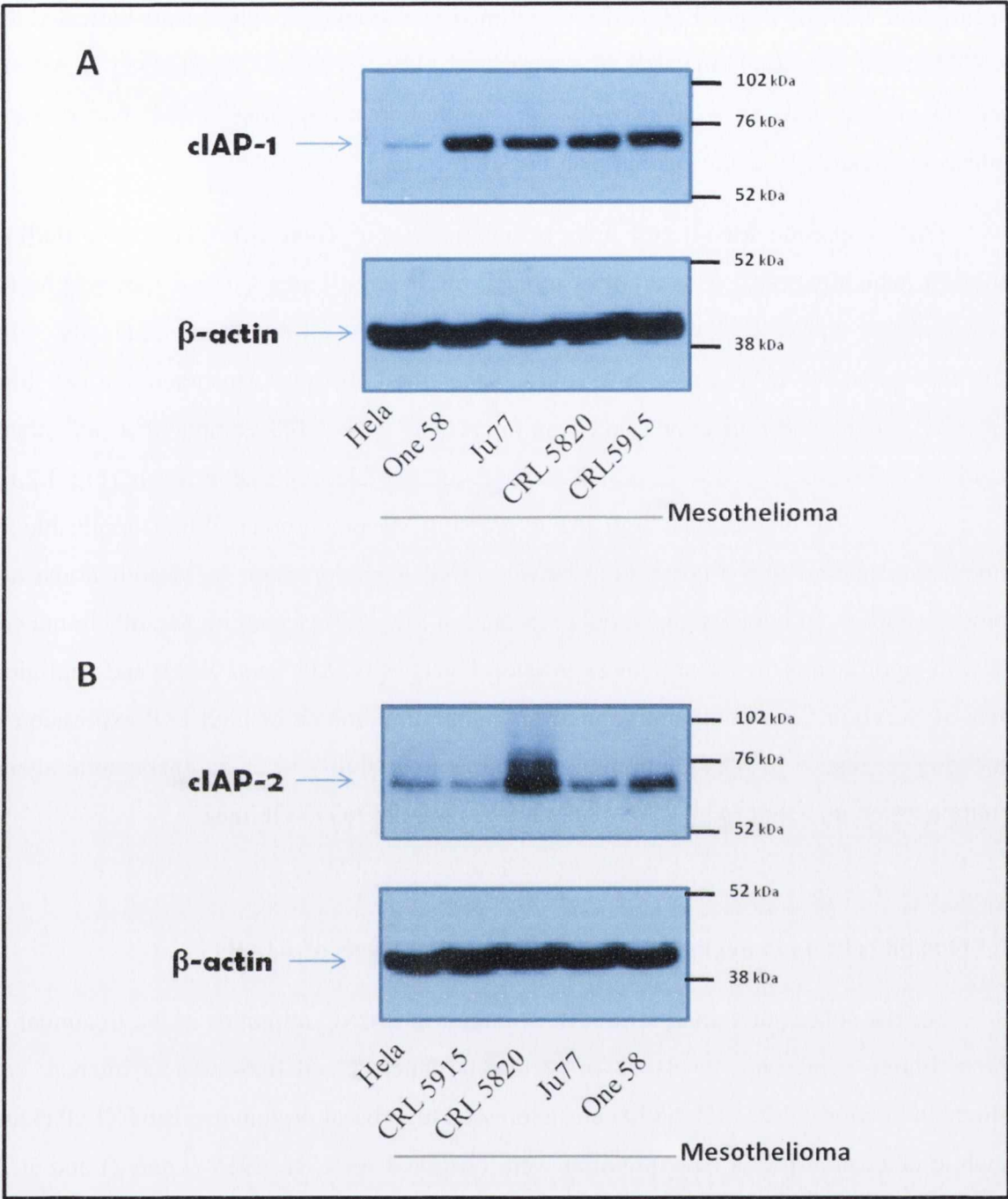


Figure 3.1: All MPM cell lines showed high basal expression levels of cIAP-1; CRL 5820 and One58 show high basal expression levels of cIAP-2.

Whole cell lysates were prepared from sub confluent cultures of One 58, CRL 5820, CRL 5915 and Ju77 (Mesothelioma) and HeLa (epithelial cervical cancer) cell lines. Proteins (30μg) were resolved on 10% SDS-PAGE gel and transferred to PVDF membrane, as outlined in Section 2.5. Blots were blocked in 5% Marvel TBS-Tween, probed with anti-cIAP-1 (A) or anti-cIAP-2 (B) and re-probed with anti-β-actin as a loading control, as outlined in Section 2.5.6. Results are representative of three independent experiments

Western blot analysis (Fig 3.1.B – 3.2), as outlined in Section 2.5. The results indicated that the CRL 5820 Mesothelioma cell line expressed elevated levels of c-IAP-2, XIAP and Survivin protein and, as well as with all Mesothelioma cell lines tested, had elevated expression of c-IAP-1, as outlined in Section 3.1.1.

IAP expression for all cell lines is summarised in Table 3.1. This table outlines results in which expression of c-IAP-2 in CRL 5820 cells (Lane 3, Fig 3.1.B) was highly elevated above expression levels of all other Mesothelioma cell lines and HeLa cells. CRL 5820 expression of XIAP was significantly higher than all other Mesothelioma cell lines (Fig 3.2.A) and Survivin expression was highest in CRL 5820 compared to all others, except CRL 5915 cells (Lane 2) which showed comparable expression levels (Fig 3.2.B). As such, CRL 5820 represents a high IAP expressing Mesothelioma cell line, applicable for further investigation into a correlation between IAP overexpression in Mesothelioma and chemoresistance. In contrast, Ju77 cells represent a low IAP expressing Mesothelioma cell line with significantly lower protein expression levels of c-IAP-2 and XIAP and negligible levels of Survivin. For comparative analysis of the importance of high IAP expression in conferring resistance in Mesothelioma, apoptotic susceptibility to chemotherapeutic agents would be tested in “High IAP” CRL 5820 and “Low IAP” Ju77 cell lines.

3.1.3 One 58 cells have negligible protein expression levels of XIAP

For the subsequent analysis of XIAP targeting SMAC mimetics in the treatment of Mesothelioma, expression levels of XIAP in this panel of cell lines was of interest. As outlined in Section 3.1.2, CRL 5820 cells expressed high basal protein levels of XIAP (Lane 3), while detectable levels of expression were observed for CRL 5915 (Lane 2) and Ju77 (Lane 4) cell lines (Fig 3.2.A). However, in One 58 cell lines, negligible levels of XIAP expression were detected (Lane 5, Fig 3.2.A).

3.2 High IAP expression mediates chemoresistance in Mesothelioma cell lines

As outlined in Section 3.1.2, in order to test the theory that high IAP expression in Mesothelioma confers apoptotic resistance, the sensitivity and apoptotic response of “High IAP” CRL 5820 cells and “Low IAP” Ju77 cells to a screen of chemotherapeutic agents was tested. CRL 5915 cells expressed consistently the highest levels of IAPs in Mesothelioma

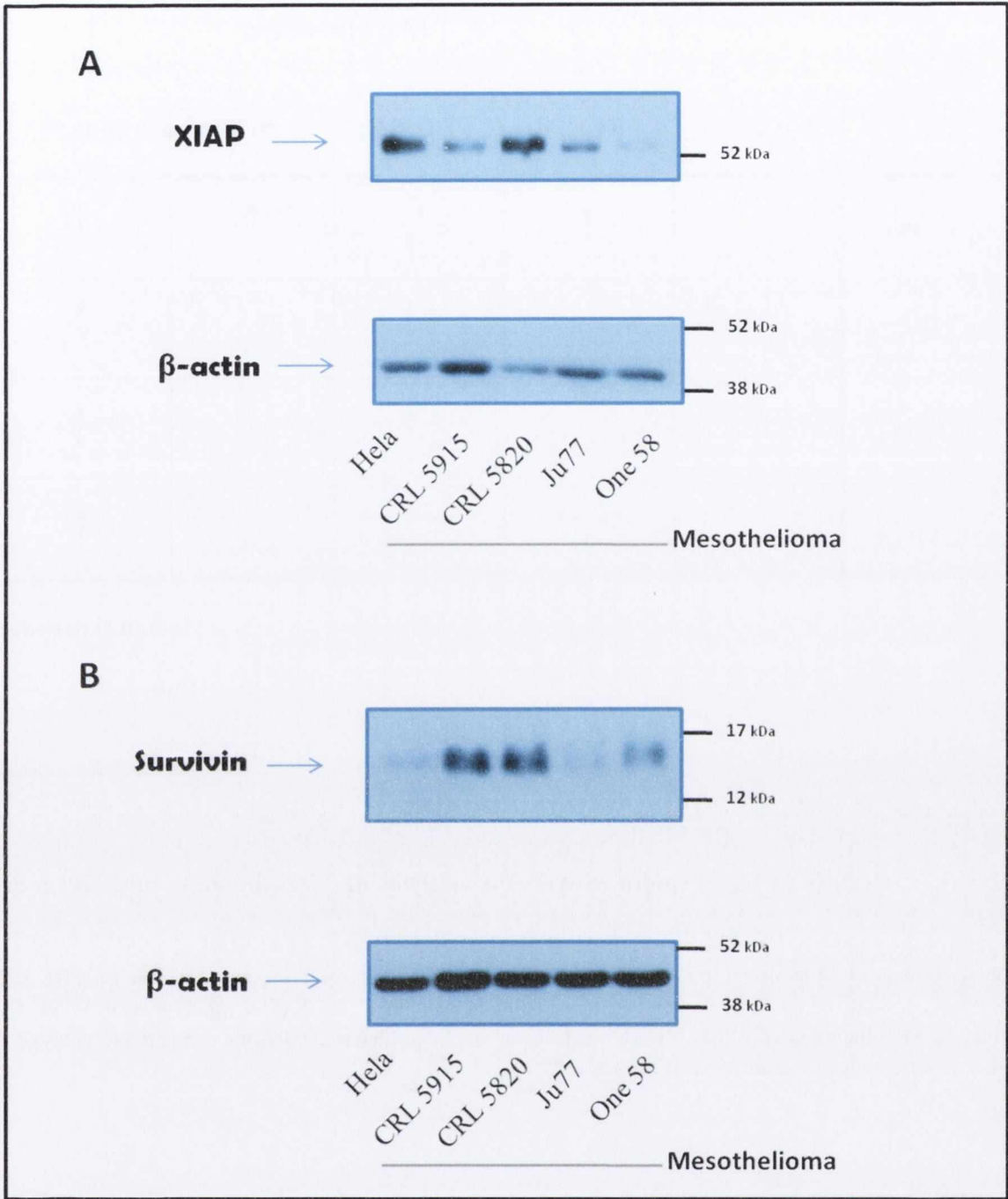


Figure 3.2: MPM cell line CRL 5820 showed high basal expression levels of XIAP; CRL 5820 and CRL 5915 show high basal expression levels of Survivin.

Whole cell lysates were prepared from sub confluent cultures of One 58, CRL 5820, CRL 5915, Ju77 and HeLa cell lines. Proteins (30μg) were resolved on 10% SDS-PAGE gel and transferred to PVDF membrane, as outlined in Section 2.5. Blots were blocked in 5% Marvel TBS-Tween, probed with anti-XIAP (A) or anti-Survivin (B) and re-probed with anti-β-actin as a loading control, as outlined in Section 2.5.6. Results are representative of three independent experiments.

Mesothelioma					
	HeLa	CRL5915	CRL5820	Ju77	One 58
c-IAP-1	—	↑	↑	↑	↑
c-IAP-2	—	—	↑↑	—	↑
XIAP	↑	—	↑↑	—	↓
Survivin	—	↑↑	↑↑	—	↑

— Median expression

↑ High

↓ Low

Table 3.1: Results of IAP protein expression analysis of Mesothelioma and HeLa cell lines.

Table of results of IAP family protein expression levels in Mesothelioma cell lines and HeLa cells. CRL 5820 cells (blue) represent a high IAP expressing Mesothelioma cell line. Ju77 cells (red) represent a low IAP expressing Mesothelioma cell line. “High” and “Low” rankings determined from comparative analysis of protein expression levels in all five cell lines tested.

cell lines (Fig 3.1 – 3.2). Ju77 cells were chosen because, they expressed only comparable levels of c-IAP-1 and either median (c-IAP-2 & XIAP) or lower levels (Survivin) of IAPs compared to all Mesothelioma cell lines. Chemotherapeutic agents chosen for testing were available compounds with distinct cellular targets and modes of action and include therapies approved for treatment of Mesothelioma in patients (Belli et al., 2009, Bertino et al., 2009, Ceresoli et al., 2009). These drugs included;

(A) DNA damaging agents – Etoposide, doxorubicin and cisplatin – the latter two common treatments or part of combination therapies and trials for MPM (Belli et al., 2009, Bertino et al., 2009, Tomek et al., 2003).

(B) Microtubule targeting agents (MTAs) – Vinblastine and taxol. Vinblastine, a vinca alkaloid, induces microtubule depolymerisation, disruption of mitotic spindle assembly and M phase arrest leading to apoptosis. Taxol, in contrast, stabilises microtubules and prevents disassembly prior to cell division leading to arrest and apoptosis.

(C) Anti-folate therapy – raltitrexed and methotrexate. Anti-folate therapy works by inhibiting thymidylate synthase and dihydrofolate reductase, enzymes involved in purine and pyrimidine synthesis. Disrupting nucleotide synthesis affects DNA replication and leads to apoptosis. Anti-folate therapy is of strong interest in MPM due to presence of high affinity folate carriers in MPM (Bueno et al., 2001) and the use of anti-folate drugs, in combination with cisplatin, is the preferred treatment regime in MPM (Belli et al., 2009).

3.2.1 High IAP expressing CRL 5820 cells are more resistant than Ju77 cells to cell death from Etoposide challenge

Firstly, etoposide was used to challenge the cell viability of high IAP expressing CRL 5820 cells and low IAP expressing Ju77 cells. Cells were treated with etoposide in a dose dependent manner for 24h or 48h and cell viability analysed by way of Alamar Blue assay, as outlined in Section 2.6. Figure 3.3 illustrates a significant difference in cell viability in response to etoposide challenge. CRL 5820 cells were significantly more resistant to induction of cell death from etoposide than Ju77 cells. After 48h incubation with etoposide, EC₅₀ values (concentration of test compound constituting 50% cell viability) for CRL 5820 and Ju77 cell lines were 20.7µM and 4.7µM, respectively.

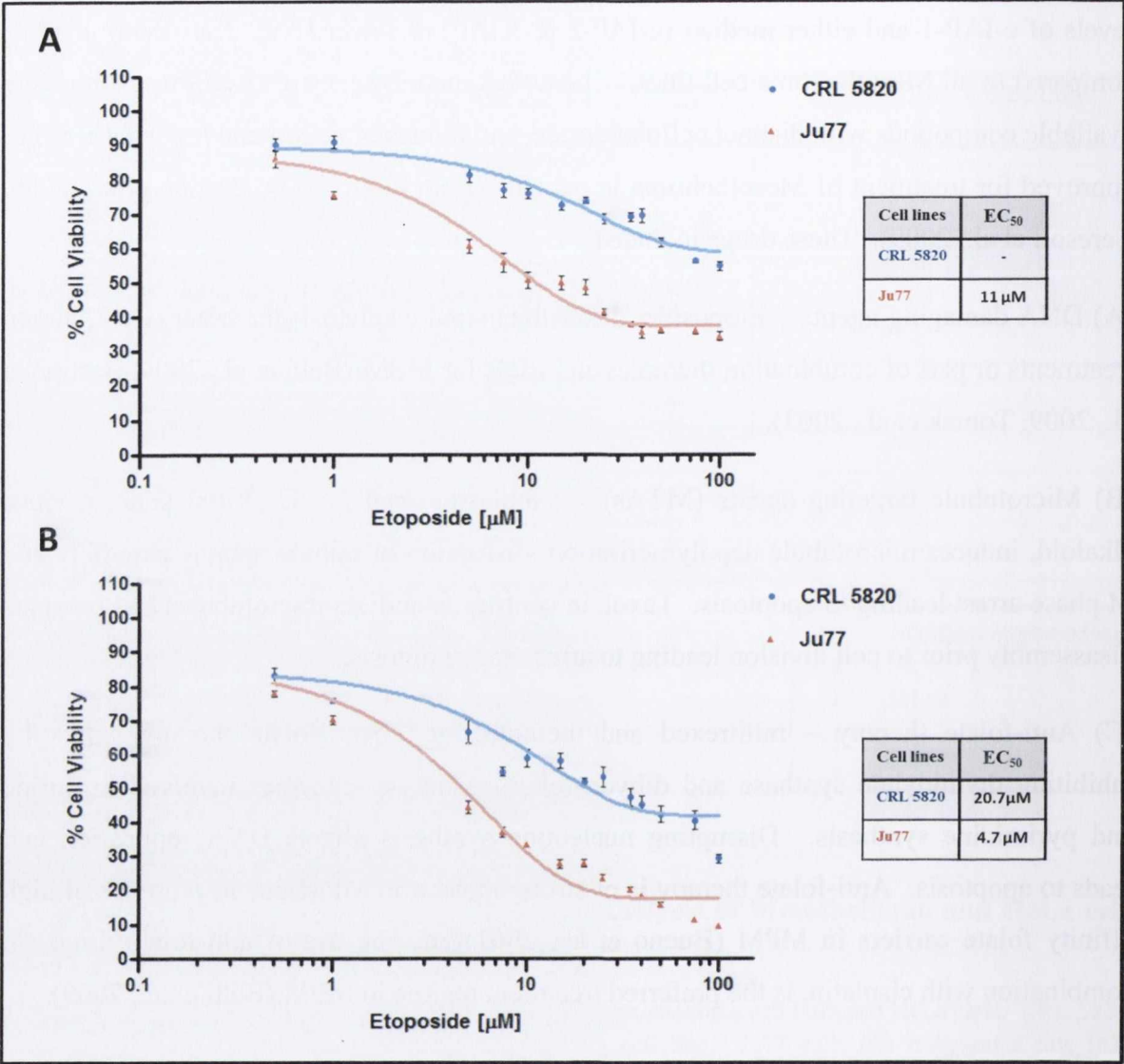


Figure 3.3: High IAP expressing CRL 5820 cells are more resistant to etoposide challenge.

CRL 5820 (●), and Ju77 (▲) cells treated with Etoposide for 24h (A) and 48h (B). Cells were seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200μl medium and left to adhere for 18h. Cells were treated at indicated concentrations of Etoposide and left to incubate for 24h or 48h before analysis of cell viability by way of Alamar Blue assay, outlined in Section 2.6. Results are representative of at least two independent experiments performed in triplicate. Regression curves were generated using a sigmoidal dose-response equation with variable slope. Comparative analysis was performed using a One-way ANOVA analysis (Graphpad Prism 4 statistical software).

Table of EC₅₀ values for 24h and 48h treatments in Mesothelioma cells lines are included on the graph.

3.2.2 CRL 5820 cells are more resistant to apoptotic induction from DNA damaging agents

After this initial test, CRL 5820 cells and Ju77 cells were analysed for apoptotic induction in response to a panel of chemotherapeutic drug agents, as indicated above. Cells were treated and induction of apoptosis was analysed by propidium iodide (PI) cell cycle flow cytometry analysis, as outlined in Section 2.7. Briefly, PI intercalates between DNA nucleic acid bases in fixed cells and analysis by flow cytometry can differentiate between cells of different cell cycle stages (G1, S & G2/M) due to DNA content and associated intercalated PI fluorescence. Apoptosis can also be quantified by the detection of events with apparent hypodiploid (pre-G1) DNA content, indicative of cells and apoptotic bodies with fragmented DNA, a hallmark of apoptosis.

Cells were treated with the DNA-damaging agents, etoposide, doxorubicin and cisplatin for 24h and 48h (Fig 3.4). For each drug treatment, Ju77 cells were shown to be significantly more susceptible to induction of apoptosis compared to CRL 5820 cells, analysed by two-way ANOVA statistical analysis. Etoposide treatment of 10 μ M induced 25.1% ($\pm$ 0.7%) apoptosis after only 24h and a maximal response of 44.4% ($\pm$ 0.6%) apoptosis with 25 μ M etoposide treatment after 48h in Ju77 cells. This compared with no significant increase in apoptosis in CRL 5820 cells with 24h or 48h incubations (Fig 3.4.A – 3.4.B). A similar highly significant difference in apoptotic response between Ju77 and CRL 5820 cell lines is seen in treatment with doxorubicin (Fig 3.4.C – 3.4.D) and cisplatin (Fig. 3.4.E – 3.4.F). In Ju77 cells, significant apoptosis is induced at lower drug concentrations and shorter time points with doxorubicin and cisplatin treatment compared to CRL 5820, with an approximate 3 fold difference in maximal apoptotic response in each case. These results strongly indicate that “High IAP” CRL 5820 MPM cells were more resistant to apoptotic induction than “Low IAP” Ju77 cells.

3.2.3 CRL 5820 cells are more resistant to apoptotic induction from Microtubule Targeting Agents (MTA)

Microtubule targeting agents were also used to investigate chemoresistance in the Mesothelioma IAP test model. Cells were treated with microtubule depolymerising agent vinblastine and microtubule polymerising agent taxol for 24h in a dose dependent manner

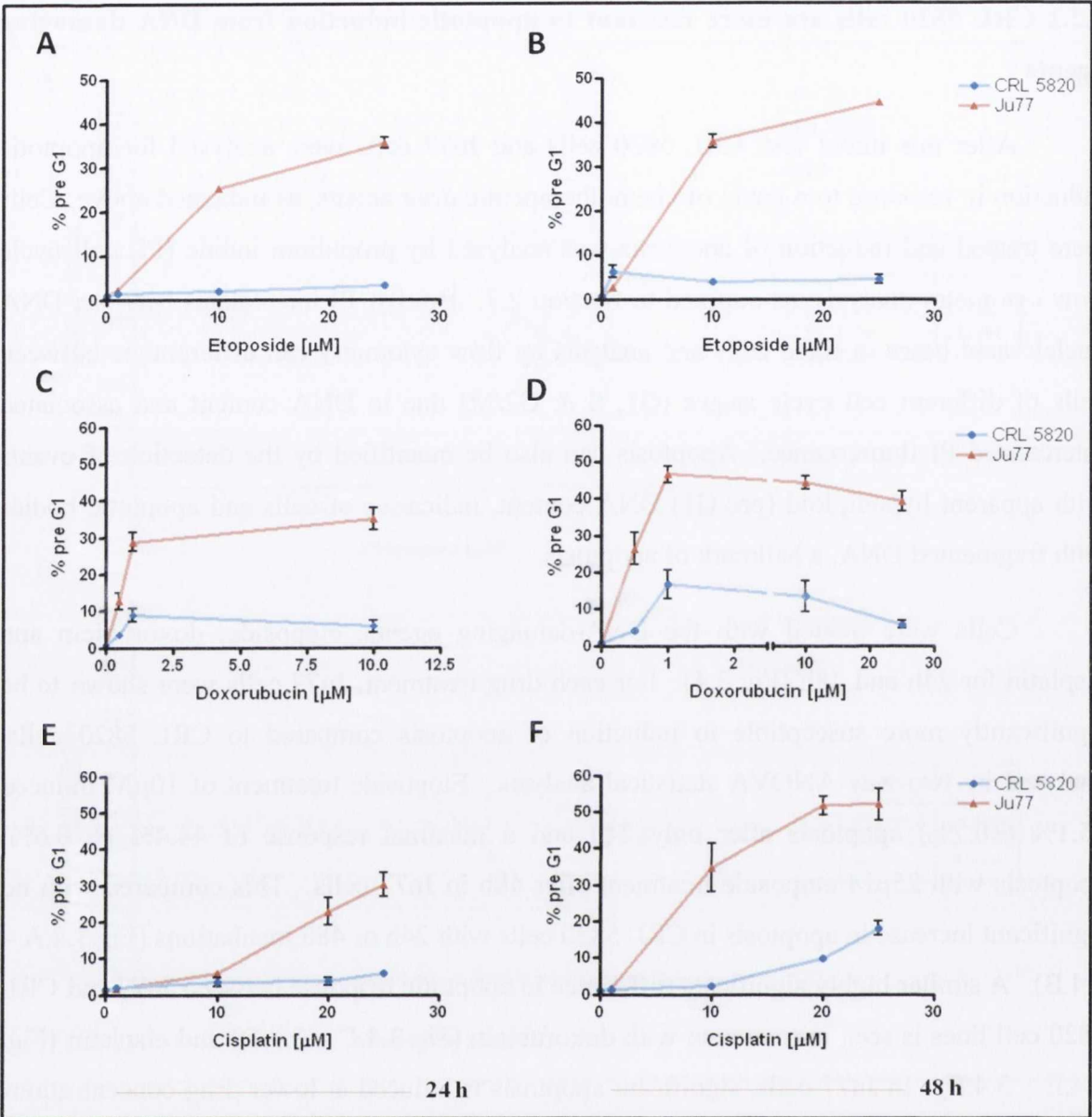


Figure 3.4: Mesothelioma chemotherapeutic screen: CRL 5820 cells are more resistant to apoptotic induction from DNA damaging agents.

CRL 5820 (●), and Ju77 (▲) cells treated with Etoposide (A & B), Doxorubicin (C & D) or Cisplatin (E & F) for 24h (A, C & E) and 48h (B, D & F). Cells seeded at a density of 0.25×10^6 cells / 5ml and left to adhere for 18h. Cells were treated with DNA damaging agents at indicated concentrations for 24h or 48h, upon which time cells were harvested and fixed for propidium iodide cell cycle flow cytometry analysis, as outlined in Section 2.7. Percentage apoptosis is determined from the proportion of cells present in the sub-G1 population of a total of 10,000 gated events.

and induction of apoptosis was analysed by PI cell cycle analysis (Fig 3.5). In Figure 3.5.A, results clearly showed that CRL 5820 cells were significantly more resistant to challenge from Vinblastine than Ju77 cells. Apoptotic induction was observed in Ju77 cells at 1nM concentrations, while no increase in pre-G1 populations is observed in CRL 5820 cells up to 250nM.

Similarly to vinblastine, CRL 5820 cells were significantly more resistant to taxol treatment than Ju77 cells (Fig 3.5.B). Ju77 cells induced approximately 40% apoptosis at low nanomolar concentrations of taxol. In contrast, CRL 5820 cells displayed up to a 21.6% increase in apoptosis with 5nM taxol, but apoptotic induction reduced with increasing concentrations. This can be explained as a greater proportion of cells arrested in G2/M with increasing taxol concentrations (Data not shown). 5nM taxol effect in CRL 5820 represented apoptotic induction, pre arrest of the cell cycle.

3.2.4 CRL 5820 and Ju77 cells show similar susceptibility to apoptosis from anti-folate therapy

Anti-folate chemotherapeutics, a component of combinational therapy employed in treatment of Mesothelioma, were also used to investigate IAP mediated chemoresistance. CRL 5820 and Ju77 cells were treated with raltitrexed and methotrexate in a dose dependent manner for 24h and 48h and percentage apoptosis was measured using PI cell cycle analysis (Fig 3.6). Treatment for 24h, with up to 50 μ M drug concentration resulted in negligible levels of apoptosis in CRL 5820 and Ju77 cells (Fig 3.6.A & 3.6.C). An increase of cells arrested in S phase was observed however in both cell lines (Data not shown), consistent with drug targeting DNA replication. Treatment for 48h resulted in a significant increase in apoptosis with raltitrexed and methotrexate in both cell lines (Fig 3.6.B & 3.6.D). The magnitude of apoptotic induction was comparable in both Ju77 and CRL 5820 cell lines and both were deemed to have similar susceptibility to anti-folate therapy.

3.3 SMAC mimetic therapy relieves IAP mediated resistance in Mesothelioma

Having established a correlation between high IAP expression in MPM and resistance to apoptosis induced by genotoxic and microtubule targeting agents, we next investigated the potential of SMAC mimetic therapy to overcome IAP mediated resistance

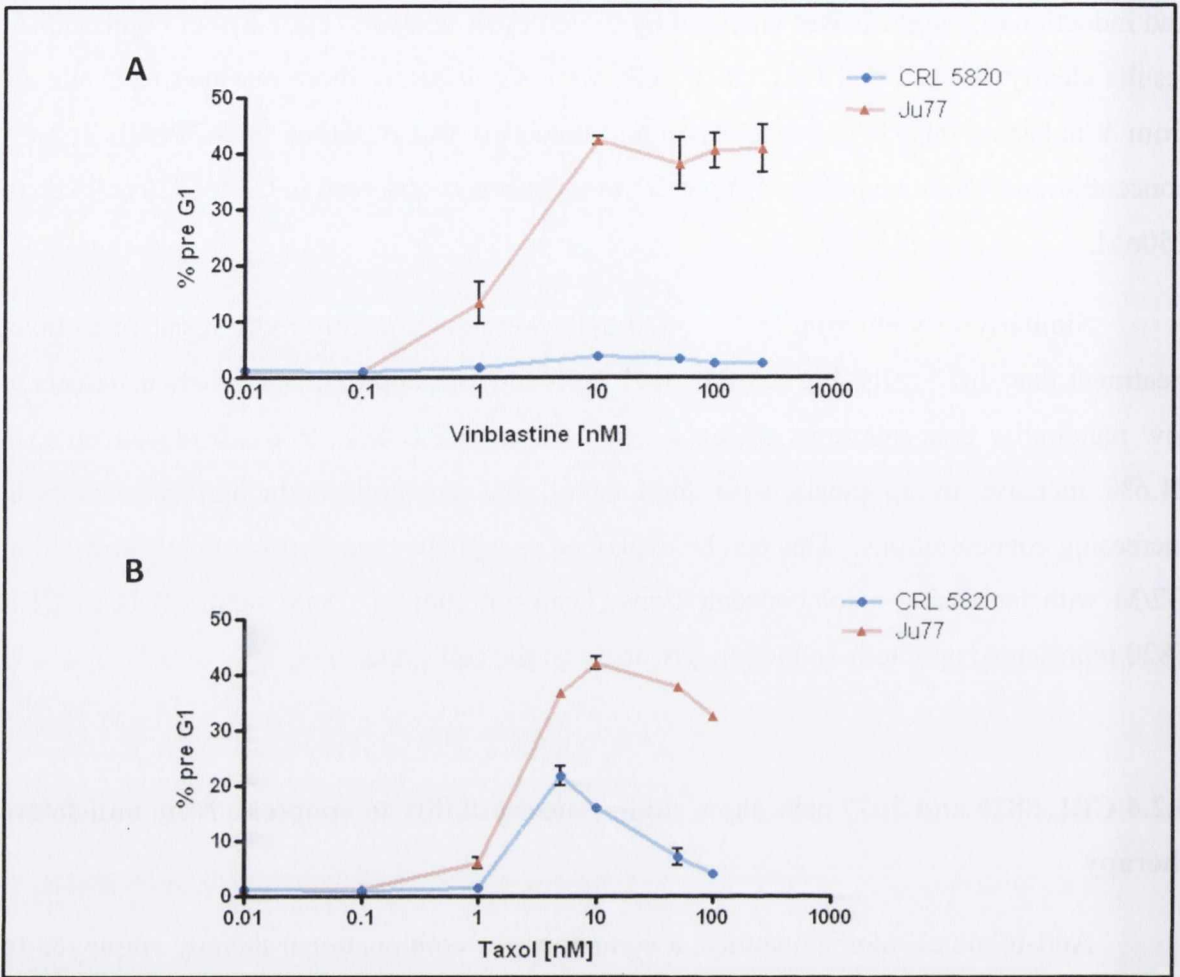


Figure 3.5: Mesothelioma chemotherapeutic screen: CRL 5820 cells were more resistant to apoptotic induction from Microtubule targeting agents.

CRL 5820 (●), and Ju77 (▲) cells treated with Vinblastine (A) or Taxol (B) for 24h. Cells seeded at a density of 0.25×10^6 cells / 5ml and left to adhere for 18h. Cells were treated with Microtubule targeting agents at indicated concentrations for 24h, upon which time cells were harvested and fixed for propidium iodide cell cycle flow cytometry analysis, as outlined in Section 2.7. Percentage apoptosis is determined from the proportion of cells present in the sub-G1 population of a total of 10,000 gated events.

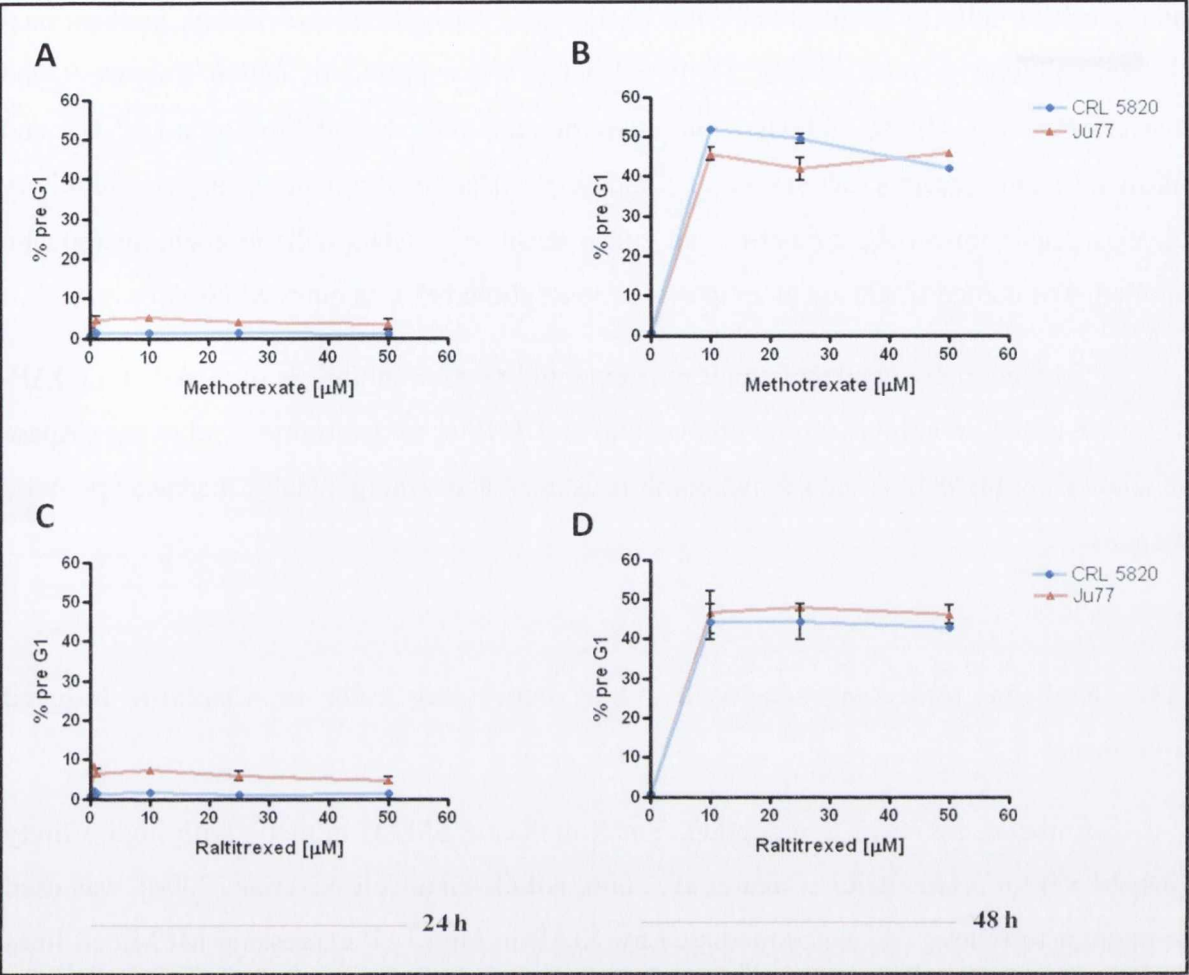


Figure 3.6: Mesothelioma chemotherapeutic screen: Ju77 and CRL 5820 cell lines show similar susceptibility to anti-folate therapy.

CRL 5820 (●), and Ju77 (▲) cells treated with Methotrexate (A) or Raltitrexed (B) for 24h (A & C) and 48h (B & D). Cells seeded at a density of 0.25×10^6 cells / 5ml and left to adhere for 18h. Cells were treated with Anti-folates at indicated concentrations for 24h or 48h, upon which time cells were harvested and fixed for propidium iodide cell cycle flow cytometry analysis, as outlined in Section 2.7. Percentage apoptosis is determined from the proportion of cells present in the sub-G1 population of a total of 10,000 gated events.

and sensitise cells to chemotherapeutic challenge. As outlined previously, conventional SMAC mimetic therapy targets XIAP, releasing sequestration of active Caspase-9 and Caspase-3/7, or c-IAPs, inducing autoubiquitination and degradation of c-IAP-1/2 and allowing death receptor activation of Caspase-8. Deregulating these caspase inhibitory check points with SMAC mimetics can either sensitise resistant cells to chemotherapeutic challenge or induce single agent apoptosis in susceptible cells, as outlined previously.

Commercially available small molecule and peptide inhibitors of XIAP and c-IAP-1/2 were tested, in combination with cisplatin and TNF- α , to determine if relieving caspase regulation by these IAPs could overcome resistance and synergistically increase apoptotic response.

3.3.1 Embelin treatment sensitised XIAP expressing cells to Cisplatin induced apoptosis

Embelin, a known non-peptidic, small molecule SMAC mimetic with high affinity (0.4 μ M *K_i*) for XIAP-BIR3 (Chen et al., 2006, Nikolovska-Coleska et al., 2004), was used to attempt to relieve resistance mediated by XIAP in high IAP expressing MPM cell lines and sensitise cells to apoptosis induced by chemotherapeutic challenge. Embelin's ability to sensitise resistant cells to apoptosis has been reported previously in a number of cancer models (Nikolovska-Coleska et al., 2004). Cisplatin was the chemotherapeutic co-treatment partner of choice as induction of the intrinsic apoptotic pathway was required – XIAP primarily abrogates intrinsic pathway caspase signalling (Caspase-9 & Caspase-3/7) – and cisplatin is part of the prescribed combinational treatment regimen for Mesothelioma.

All four MPM cell lines were treated with embelin (1, 10 & 25 μ M) and cisplatin (25 μ M) alone or in combination and percentage apoptosis (% pre G1) was analysed by PI cell cycle analysis. In XIAP expressing CRL 5915, CRL 5820 and Ju77 MPM cell lines (Fig 3.7.A – 3.7.C), 25 μ M embelin co-treatment induced a synergistic increase in cisplatin induced apoptosis compared to cisplatin and embelin treatment alone.

In the high XIAP expressing CRL 5820 cell line, cisplatin alone produced no increased induction of apoptosis after 48h treatment – 3.8% ($\pm$ 0.4%) – (Fig 3.7.B). However, co-treatment of CRL 5820 cells with embelin was able to completely overcome XIAP mediated apoptotic resistance to cisplatin, inducing a highly significant level of

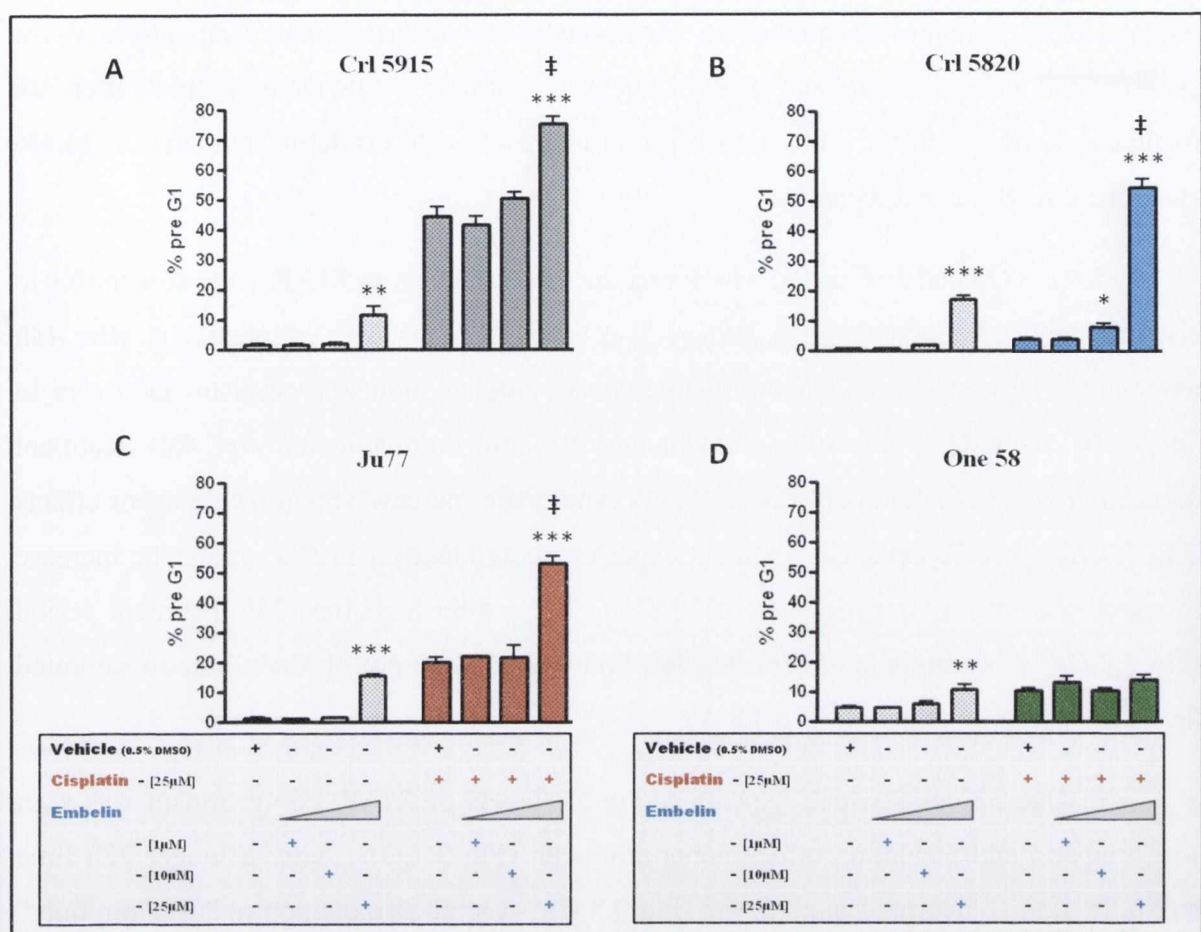


Figure 3.7 Embelin sensitises XIAP expressing CRL 5820, CRL 5915 and Ju77 cell lines to cisplatin induced apoptosis, but not low XIAP expressing One 58 cells

CRL 5915 (A), CRL 5820 (B), Ju77 (C) and One 58 (D) cells were treated with cisplatin alone or in combination with embelin. Cells were seeded at a density of 0.25×10^6 cells / 5ml and left to adhere for 18h. Cells were treated with indicated concentrations of drug combinations for 48h (A, B & D) or 24h (C), upon which time cells were harvested and fixed for propidium iodide cell cycle flow cytometry analysis, as outlined in Section 2.7. Percentage apoptosis is determined from the proportion of cells present in the sub-G1 population of a total of 10,000 gated events. Values are representative of mean $\pm$ SEM of at least three independent experiments. * $P < 0.5$; ** $P < 0.01$; *** $P < 0.001$ (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software). Synergism determined by two-way analysis of variance (ANOVA) of interaction effect of single agent and co-treatment response ($\ddagger$ $P < 0.01$).

54.1% ($\pm 2.8\%$) apoptosis, equating to a synergistic increase of 33.4% in apoptosis above cisplatin and embelin treatments alone (Table 3.2). Similar results were observed after 72h incubations (Fig 3.8.B), with embelin co-treatment with cisplatin inducing a 26.4% synergistic increase in apoptosis.

CRL 5925 and Ju77 cells, which express median levels of XIAP, were susceptible to cisplatin induced apoptosis – 44.2% ($\pm 3.5\%$) and 43.1% ($\pm 2.6\%$), respectively, after 48h treatment. However, embelin co-treatment was able to induce synergistic increases in apoptosis. In CRL 5915 cells, embelin and cisplatin co-treatments after 48h treatment induced 74.9% ($\pm 2.5\%$) apoptosis, a 19.3% synergistic increase above single agent effects (Fig 3.7.A). In Ju77 cells, co-treatment experiments produced a 17.6% synergistic increase in apoptosis, with a total response of 52.8% ($\pm 2.1\%$) after a shorter, 24h treatment period (Fig 3.7.C). A synergistic effect after 48h time points were not observed due to saturated levels of apoptotic induction (Fig 3.8.A).

In One 58 cells, which express negligible levels of XIAP, no synergism was seen with embelin and cisplatin co-treatments after 48h (Fig 3.7.D). After a longer 72h time point, the same outcome was observed (Fig 3.8.C). Apoptotic induction in “cisplatin only” treatments was also noted as being negligible after both 48h and 72h time points, possibly indicating a cellular mechanism for cisplatin resistance other than one mediated by XIAP.

The sensitivity of cells to embelin directly correlated with XIAP expression levels and indicated specific targeting of XIAP by embelin. High XIAP expressing CRL 5820 cells were fully resistant to cisplatin. However, embelin co-treatment allowed inhibition of XIAP sequestration of active caspases, relieving XIAP mediated apoptotic resistance and fully potentiating cisplatin induced apoptosis. CRL 5915 and Ju77 cell lines, expressing median XIAP protein levels were susceptible to a degree of cisplatin induced apoptosis, but embelin was able to overcome some degree of XIAP mediated cisplatin resistance in these cells, inducing synergistic increases in apoptosis. One 58 cells, deficient in XIAP expression, lack this apoptotic blockade and as such embelin did not sensitise this cell line to cisplatin induced apoptosis and no synergistic response to embelin and cisplatin co-treatment was observed.

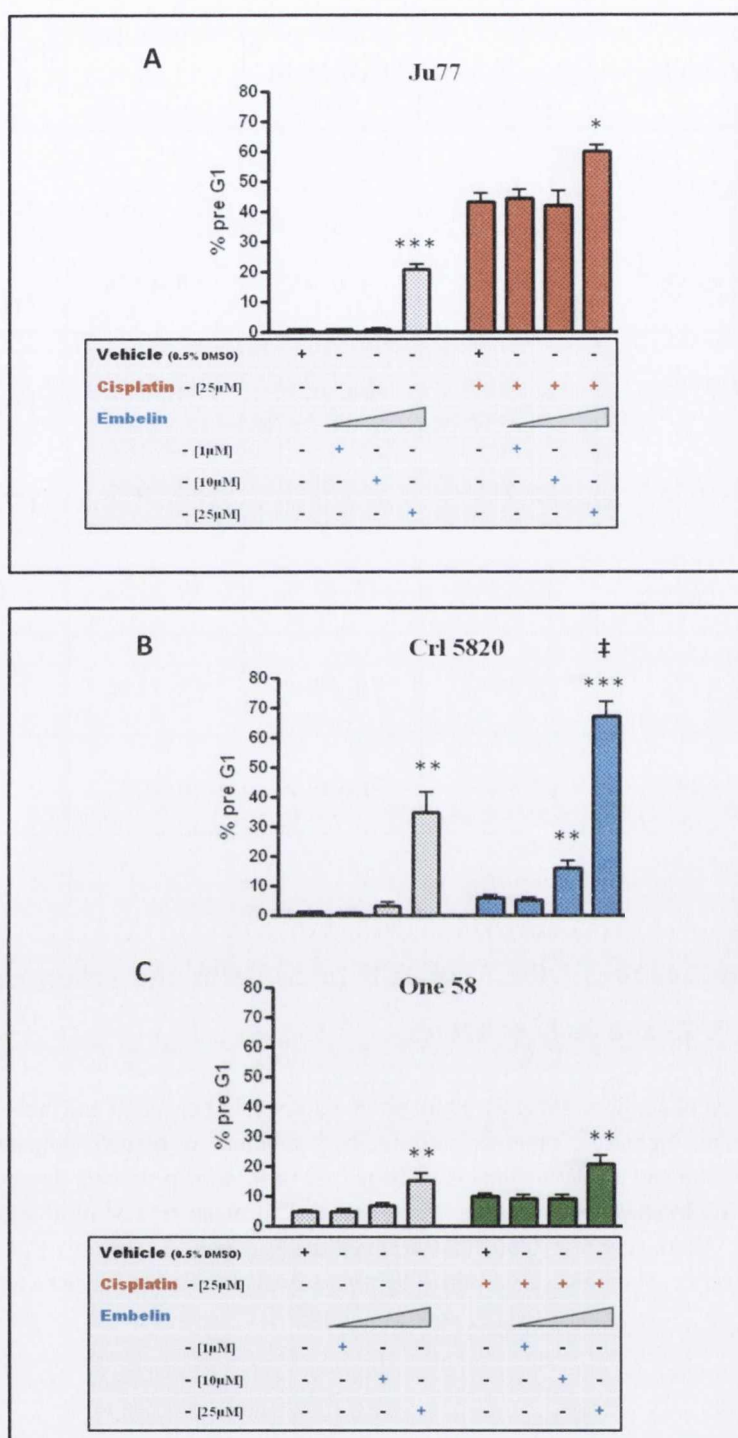


Figure 3.8 Embelin sensitised XIAP expressing CRL 5820, CRL 5915 and Ju77 cell lines to cisplatin induced apoptosis, but not low XIAP expressing One 58 cells

Ju77 (A), CRL 5820 (B), One 58 (C) cells were treated with cisplatin alone or in combination with embelin. Cells were seeded at a density of 0.25×10^6 cells / 5ml and left to adhere for 18h. Cells were treated with indicated concentrations of drug combinations for 48h (A) or 72h (B - C), upon which time cells were harvested and fixed for propidium iodide cell cycle flow cytometry analysis, as outlined in Section 2.7. Percentage apoptosis is determined from the proportion of cells present in the sub-G1 population of a total of 10,000 gated events.

Cell line	Vehicle	Embelin [25µM]	Cisplatin [25µM]	Embelin + Cisplatin	Percentage Increase	Synergism (P statistic)
CRL 5820 (48h)	0.67% (±0.17%)	16.96% (±1.49%)	3.77% (±0.41%)	54.08% (±2.84%)	↑ 33.35%	<0.0001 [‡]
(72h)	0.97% (±0.13%)	34.77% (±6.77%)	5.92% (±0.87%)	67.07% (±4.84%)	↑ 26.38%	<0.01 [‡]
CRL 5925 (48h)	1.57% (±0.30%)	11.50% (±2.72%)	44.16% (±3.49%)	74.94% (±2.49%)	↑ 19.28%	0.0002 [‡]
Ju77 (24h)	1.18% (±0.13%)	15.44% (±0.35%)	19.70% (±1.58%)	52.76% (±2.11%)	↑ 17.62%	0.0001 [‡]
(48h)	0.72% (±0.10%)	20.85% (±1.29%)	43.07% (±2.62%)	59.71% (±2.26%)	(n/s)	(n/s) 0.3363
One 58 (48h)	4.54% (±0.54%)	10.49% (±1.80%)	10.27% (±0.94%)	13.73% (±1.88%)	(n/s)	(n/s) 0.3106
(72h)	4.85% (±0.41%)	15.16% (±2.26%)	9.99% (±0.82%)	20.58% (±2.89%)	(n/s)	(n/s) 0.9244

Table 3.2 Embelin sensitises CRL 5820 cells to cisplatin and synergistically increases apoptosis in CRL 5915 and Ju77 cell lines.

Table of apoptotic effects of single agent and combination treatments of cisplatin and embelin in all four MPM cells lines. The percentage increase in apoptosis induced by “embelin + cisplatin” compared to the cumulative apoptotic effects of embelin and cisplatin alone is indicated, as well as the p statistic for synergism for embelin and cisplatin combination treatments. Values are representative of mean ± SEM of at least three independent experiments. *P<0.5; **P<0.01; ***P<0.001 (Determined using unpaired Student’s t-test, part of Graphpad Prism 4 statistical software). Synergism determined by two-way analysis of variance (ANOVA) of interaction effect of single agent and co-treatment response (‡ P<0.01).

3.3.2 Embelin induces single agent cell cycle arrest, cytotoxicity and apoptosis in all Mesothelioma cell lines.

As well as synergistically increasing the apoptotic potential of cisplatin in XIAP-expressing Mesothelioma cells, embelin also had potent single agent effects on cellular growth arrest, cytotoxicity and apoptosis in all cell lines tested, irrespective of XIAP status. Embelin treatment (25µM) induced apoptosis as a single agent in all four Mesothelioma cell lines; highest apoptotic response was seen in Ju77 and CRL 5820 cells but also observed in XIAP deficient One 58 cells (Fig 3.7 – 3.8; Table 3.1). A number of possible explanations exist for the single agent effect of embelin in cancer cell lines. While XIAP inhibition of embelin has been attributed as being the main factor in its anti-cancer activity, embelin has been shown to regulate alternate targets, which include c-FLIP (Siegelin et al., 2009), p21 (Joy et al., 2010) and NFκB signalling (Ahn et al., 2007). This “off XIAP” targeting has been proposed to contribute to single agent cell arrest, cytotoxic and apoptotic effects of embelin.

Mesothelioma cell line treated for 48h with 1 – 25µM embelin were analysed by PI cell cycle analysis (Fig 3.9.B), as outlined in Section 2.7. In all four Mesothelioma cell lines, embelin treatment (25µM) induced accumulation of cells in the S phase of the cell cycle. This was significant in high XIAP expressing CRL 5820 (29.1%) and low expressing One 58 (25.9%), and highest in CRL 5915 cells (46.2%). Cellular cytotoxicity was also observed in HeLa and all Mesothelioma cell lines. Cells were treated with embelin in a dose dependent manner and cell viability analysed by Alamar Blue assay (Fig 3.9.A). All cells showed comparable responses to embelin, indicating low micro-molar EC₅₀ values.

3.4 Cell permeable AVPI did not sensitise Mesothelioma cells to cisplatin or TNF-α induced cell death

In this study, a cell permeable form of the SMAC N-terminal tetrapeptide AVPI was used in an attempt to target and potentially overcome IAP mediated resistance from XIAP and / or c-IAP-1/2. AVPI was conjugated to a cell-permeable polyarginine sequence, containing eight arginine residues - AVPI-RRRRRRRR (AVPI-R(8)). A polyarginine-conjugated cell permeable form of AVPI has been shown previously to overcome XIAP mediated resistance in non-small cell lung carcinoma (NSCLC) H460 cells and sensitise

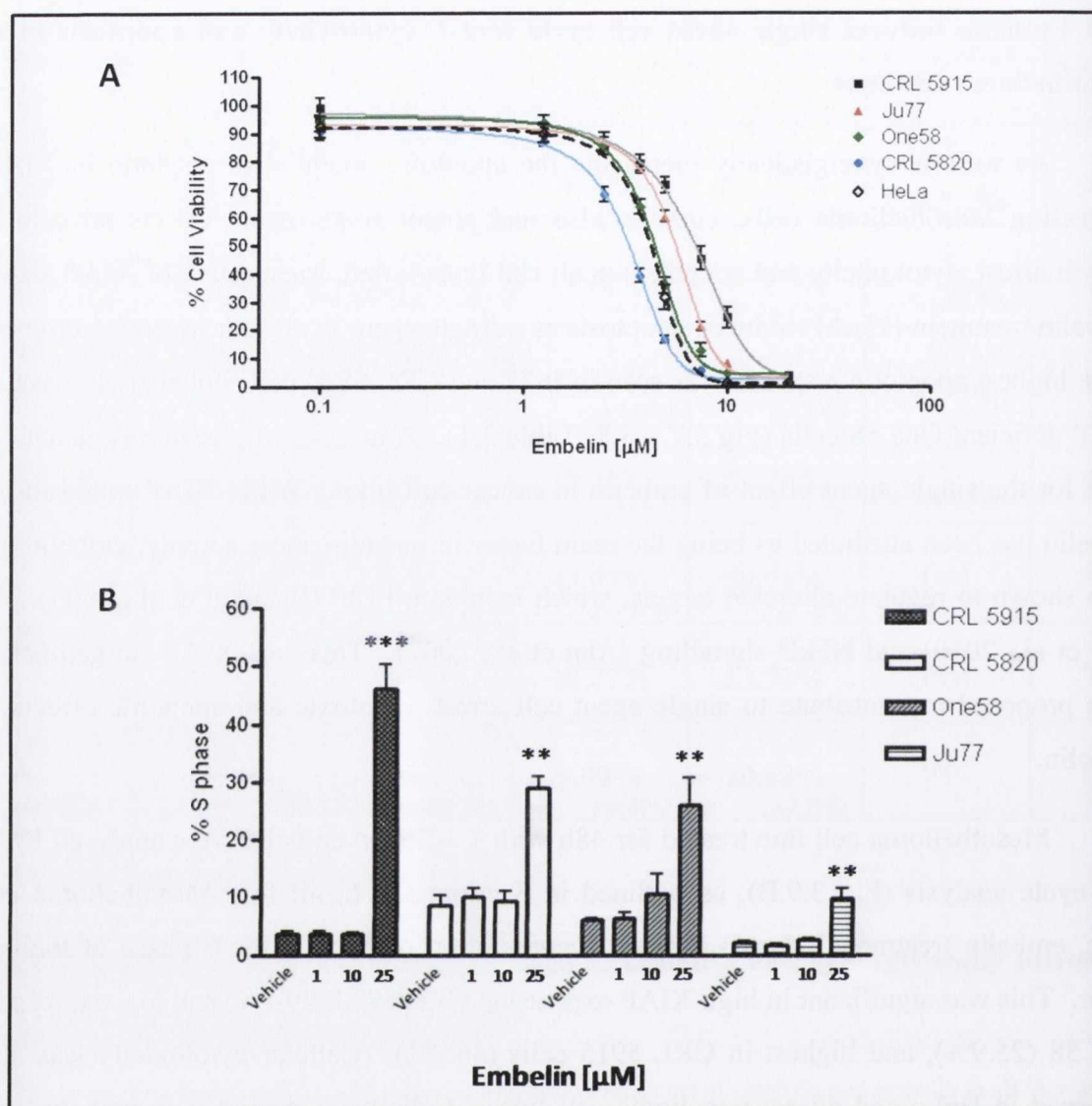


Figure 3.9 Embelin induced single agent cytotoxic and cell arrest effects in Mesothelioma cell lines, irrespective of XIAP expression status

(A) Mesothelioma and HeLa cells were seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200 μ l medium and left to adhere for 18h. Cells were treated with indicated concentrations of Embelin and left to incubate for 24h before analysis of cell viability by way of Alamar Blue assay, as outlined in section 2.6. Experiments were performed at least three times in triplicate.

(B) Cells seeded at 0.25×10^6 cells/5ml and treated with Embelin (at indicated concentrations). After 48h incubation, cells were harvested and fixed in 70% Ethanol. Cell cycle flow cytometry analysis was performed as outlined in section 2.7. Values are representative of mean $\pm$ SEM of three independent experiments. **P<0.01; ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

cells to cisplatin mediated cell death (Yang et al., 2003b). Treating cells with Native-SMAC protein (Vince et al., 2007) or SMAC mimetics computationally designed using the AVPI (& AVPF) tetrapeptide motif (Li et al., 2004, Wang et al., 2008) have also shown ability to bind c-IAP-1/2 and potentiate TNF- α mediated cell death.

All four Mesothelioma cells were treated with AVPI-R(8) alone or in combination with Cisplatin and TNF- α for 48h and analysed for cell viability by Alamar Blue assay (Fig 3.10). Single agent treatment with AVPI-R(8) (0.1 μ M – 10 μ M) had no effect on cell death in any of the MPM cell lines. Combination treatments of AVPI-R(8) with either cisplatin (20 μ M) or TNF- α (10ng/ml) did not sensitise or induce any synergistic increase in cell death.

Discussion

In this Chapter it was proposed to examine the IAP expression profile of a panel of Malignant Pleural Mesothelioma cell lines to investigate the link between high IAP expression in MPM cells and apoptotic resistance. Upon identifying an MPM cell line model that displayed both high levels of IAP and chemoresistance, investigation of the ability of conventional SMAC mimetic therapy to inhibit IAP mediated resistance and sensitise cells to apoptotic challenge was performed. Of particular interest was the significance of XIAP and c-IAP-1/2 IAP family proteins in resistance and the potential of targeting these IAPs using SMAC mimetics. Both are well established negative regulators of caspase and known to confer apoptotic resistance in cancer (Checinska et al., 2007, Gordon et al., 2002). XIAP acts as a central regulator of caspase signalling, inhibiting apoptosome activation of caspase-9, and intrinsic and extrinsic pathway activated effector caspases-3/7 and cIAP-1/2 regulates the apoptotic potential of death receptor signalling. This study aimed to inform us on the potential of SMAC mimetic therapy as a treatment strategy in resistant MPM and also, whether MPM could be a good model for the further development of novel SMAC mimetic compounds.

IAP expression analysis was performed on c-IAP-1/2, XIAP and also Survivin. Survivin expression in cancer has been linked with apoptotic resistance (Zaffaroni et al., 2002), poor prognosis in neuroblastoma (Islam et al., 2000) and Mesothelioma (Gordon et al., 2007a) and can directly inhibit caspase-9 when in complex with other proteins. Its

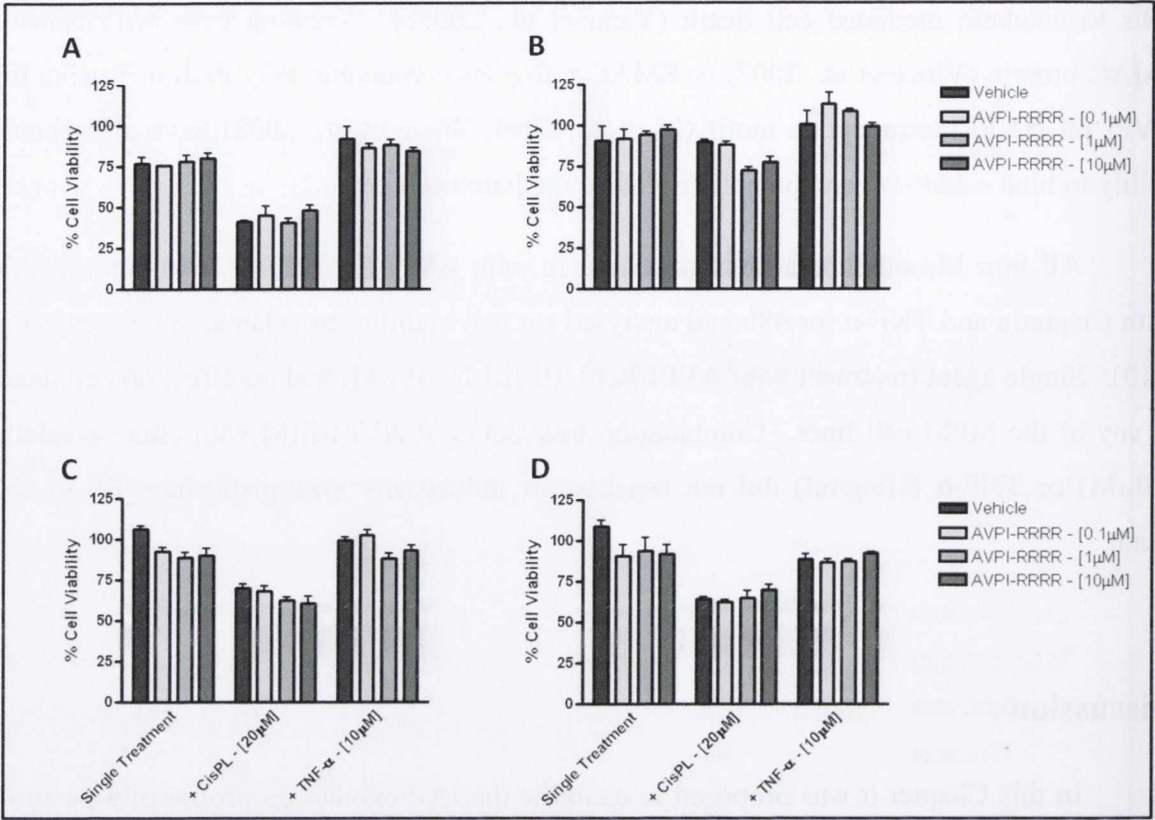


Figure 3.10: Cell permeable AVPI peptide did not induce cell death as a single agent or in combination with Cisplatin or TNF- α in Mesothelioma cell lines.

Mesothelioma cell lines CRL 5915 (A), CRL 5820 (B), Ju77 (C) and One 58 (D) treated with cell permeable AVPI-RRRR alone or in combination with Cisplatin or TNF- α for 48h. Cells were plated on 96 well plates at a density of 0.5×10^4 cells per well, containing 200 μ l medium and left to adhere for 18h. Cells were treated with indicated concentrations and left to incubate for 48h before analysis of cytotoxicity by way of Alamar Blue cell viability assay, outlined in Section 2.6. Values are representative of mean $\pm$ SEM of a triplicate experiment. Statistical analysis of data using unpaired Student's t-test, part of Graphpad Prism 4 statistical software.

apoptotic mechanism of action is not fully elucidated and has not previously been found as a target for small molecule SMAC mimetic therapy. Results revealed c-IAP-1 was highly expressed in all Mesothelioma cell lines, in comparison to cIAP-1 expression levels detected in Hela cells. This result correlated with previous reports of high c-IAP-1 expression in MPM tumours, which was also linked to tumour resistance (Gordon et al., 2002). One Mesothelioma cell line, CRL 5820, was revealed to also express high levels of c-IAP-2, XIAP and Survivin. This line was paired with Ju77, an MPM cell line with low levels of IAP protein expression, to form a comparative model to test association between IAP expression and chemoresistance.

Treatment with known chemotherapeutics of different classes revealed a direct correlation between high IAP expression in CRL 5820 cells and resistance to cell death and apoptosis. CRL 5820 cells were significantly more resistant than Ju77 cells to challenge from a collection of DNA damaging drugs (etoposide, doxorubicin and cisplatin) and MTAs (vinblastine and taxol). These drugs induce an intrinsic apoptotic response and as such, elevated IAPs in CRL 5820, in particular XIAP, are proposed to be the causative factor in mediating resistance to this type of challenge. However, this paradigm was not true for all drug treatments. Anti-folate therapy induced comparable rates of apoptosis in both CRL 5820 and Ju77 cells.

Having identified the correlation that high IAP expression in MPM conferred apoptotic resistance, conventional SMAC mimetic therapy was used to attempt to inhibit this mechanism of resistance. Co-treatment experiments with the XIAP inhibitor embelin were able to overcome resistance and synergistically increased cisplatin induced apoptosis in susceptible MPM cell lines. Susceptibility of cells to sensitisation by embelin correlated with the XIAP expression profile of the cell line. In the Cisplatin resistant, high XIAP expressing CRL 5820 cell line, embelin co-treatment fully sensitised cells to cisplatin challenge, increasing cellular response from 3.8% to 54.1% apoptosis after 48h treatment. Synergistic increases in apoptosis was observed with co-treatment in XIAP expressing CRL 5915 and Ju77 cell lines, but embelin had no effect on sensitising XIAP deficient One 58 cells to cisplatin induced apoptosis. These results further indicate that XIAP can successfully inhibit apoptotic induction in expressing MPM cells and SMAC mimetic therapy was an effective method of subverting this resistance mechanism and re-sensitising cells to chemotherapy.

Embelin was also observed to induce single agent cell arrest, cytotoxic and apoptotic effects in cells irrespective of XIAP expression levels. Alternate target binding, as opposed to single agent SMAC mimetic mechanisms, was believed to induce some of these effects. Previous reports have shown embelin can induce cell arrest in S Phase through p21 targeting (Joy et al., 2010), and cytotoxicity through c-FLIP (Siegelin et al., 2009) and NF κ B signalling regulation (Ahn et al., 2007). While data and previous reports suggest off target effects of embelin in cancer cells, other possible mechanisms of induction of single agent cell death exist. SMAC mimetic therapy in cells with high steady state expression levels of active-caspase has been shown to induce apoptosis as a single agent (Yang et al., 2003a). Under these conditions, targeting XIAP alone (at BIR2 and BIR3) can release sequestered active caspases and induce an increased rate of apoptosis, without exogenous apoptotic stimulus. It is proposed that a high IAP expression phenotype in some cells can occur to counterbalance and inhibit the expression of high levels of endogenous active caspases (Sensintaffar et al., 2010, Yang et al., 2003a). A final possible mechanism of action for SMAC mimetic single agent effect is cIAP-1/2 targeting and autocrine TNF- α signalling, as discussed in the introduction. While these alternate targets exist, XIAP binding is the primary target in its cell death activity and was employed successfully as an effective XIAP-BIR3 SMAC mimetic in this study.

A second SMAC mimetic, a proposed cell permeable form of the SMAC N-terminal tetrapeptide, AVPI-R(8), was tested for its potential to target XIAP and c-IAP1/2 and sensitise cells to cell death. AVPI-R(8) was treated in cells in combination with cisplatin and TNF- α , but no increase in cisplatin mediated cell death or induction of TNF- α induced cell death was observed. This result was determined to be a failure in assay as opposed to a failure in concept. A previous study reported efficacy of a modified, cell permeable form of the SMAC N-terminal tetrapeptide AVPI being able to inhibit XIAP and cIAP-1/2 and synergistically increase cisplatin and TNF- α induced cell death in an NSCLC cell line (Yang et al., 2003b). This peptide was structurally different to the simple tetra-peptide used in these experiments, containing the last seven SMAC N-terminal residues as well as a polyarginine tail. Differences in peptide were believed to cause the lack of response. Increase in concentration of AVPI-R(8) and experiments confirming cellular internalisation of the peptide need to be performed with this assay.

In conclusion, we report in this Chapter a link between high IAP expression and chemotherapeutic resistance in the CRL 5820 Mesothelioma cell line. Through the use of

SMAC mimetic treatments, we have shown XIAP to be of specific importance in mediating this resistance and that SMAC mimetic targeting of XIAP is an effective strategy in relieving this apoptotic resistance in Mesothelioma. This confirmed the theory that IAP inhibition was a rational target for the design of treatments for chemoresistant MPM and a good test model for the screening and development of SMAC mimetic therapies. Following on from these conclusions, in the next Chapter, a series of novel computationally designed SMAC mimetic compounds were developed in an effort to find more potent agonists of IAP inhibition. Biochemical analysis of the novel series of MDG SMAC mimetic compounds was performed, including “on target” and cellular functional assays to determine if the computationally designed SMAC mimetics sensitises cells to apoptosis and mediate effects through IAP inhibition.

Chapter 4

***Biochemical analysis of a series of novel computationally-designed SMAC
mimetics in Malignant Pleural Mesothelioma***

Introduction

Investigations in Chapter 3 examined the role of IAP family proteins in mediating apoptotic resistance in MPM cell lines and confirmed, using an *in vitro* model, the therapeutic potential of SMAC mimetic inhibitors of IAPs to re-sensitise resistant cells to chemotherapy. High IAP expression in MPM cell lines was shown to confer apoptotic resistance to a screen of conventional chemotherapeutic drugs. Embelin, a small molecule SMAC mimetic inhibitor of XIAP-BIR3, was able to fully sensitise resistant, high XIAP expressing CRL 5820 cells to cisplatin induced apoptosis and synergistically increase apoptotic induction of cisplatin in other XIAP expressing MPM cell lines. Sensitisation of cells to apoptosis was mediated specifically through XIAP inhibition as no synergistic combinational effects were observed in an XIAP deficient MPM cell line. These results confirmed the basic hypothesis that high IAP expression in MPM can confer apoptotic / chemotherapeutic resistance. MPM is proposed as an appropriate model for the study of IAP inhibition in anti-cancer drug discovery and aid the development of novel SMAC mimetic compounds of possible therapeutic benefit in this highly resistant form of cancer.

Following on from this, a novel series of computationally designed SMAC mimetic compounds were developed by collaborators in the Molecular Design Group, Trinity College Dublin. Small molecule design incorporating ligand-based pharmacophore design techniques was preferred due to fewer efficacy, delivery and pharmacokinetic limitations over interfering RNA and peptidic inhibitors. In light of the positive results for embelin inhibition of XIAP-BIR3, reported in Chapter 3, this site was chosen as a primary putative target for SMAC mimetic development. The x-ray crystal structure of the biological pose of native ligand SMAC-AVPI resident in the binding pocket of XIAP-BIR3 was the basis for the SMAC mimetic pharmacophore (Wu et al., 2000). The primary objectives of experimentation described in this Chapter was to evaluate the potential of these computationally predicted SMAC mimetic compounds as direct inhibitors of IAP and their ability to sensitise IAP resistant MPM cell lines to chemotherapy. Both “on target” and biochemical functional assays were employed to test these compounds SMAC mimetic potential.

A non-cell based fluorescence polarisation (FP) competitive binding assay was developed and used in the laboratory to test binding of compounds to the BIR3 domain of XIAP. A cellular functional assay was used to test for indications of XIAP targeting by

MDG SMAC compounds in MPM cell lines. Cells were treated with SMAC compounds in combination with cisplatin to investigate if these compounds can relieve resistance by directly inhibiting XIAP sequestration of active caspases and synergistically increase cisplatin induced apoptosis. The cytotoxic and apoptotic activity of these compounds as single agent inducers of PCD was also examined in MPM cell lines.

Secondary IAP targets, including cIAP-1 and cIAP-2, were also proposed as potential binding partners of these compounds. As outlined in Chapter 1, Native SMAC-AVPI and SMAC mimetics have been shown to bind to BIR domains of cIAP-1/2 and induce autoubiquitination and proteosomal degradation of cIAPs. Degradation of cIAP-1/2 causes a loss of regulation of TNFR signalling and NF κ B activation, allowing formation of the Caspase-8 activating death receptor complex in response to extrinsic or autocrine exposure to TNF- α . MDG compound targeting of cIAP-1/2 was investigated by Western blot, analysing degradation of cIAP-1/2 protein in response to compound treatment in MPM cell lines. A cellular functional assay was also employed to test targeting of cIAP-1/2, investigating the ability of MDG compounds to sensitise or synergistically increase cell death in response to death receptor ligation.

As well as inducing the extrinsic apoptotic pathway, death receptor signalling has long been linked with a role in promoting tumour growth and cell transformation in a variety of cancers (Scott et al., 2004, Suganuma et al., 1999). TNF- α is of particular interest in Mesothelioma, as it is believed to participate in asbestos-mediated tumorigenesis (Lemaire and Ouellet, 1996, Xie et al., 2000). Alveolar macrophages phagocytose the long-form asbestos fibres, causing chronic release of cytokines, including TNF- α . This "Frustrated Phagocytosis" of the long asbestos fibres causes persistent inflammation which sustains mesothelial cells containing mutations and genetic insults and aids tumorigenesis (Tanaka et al., 2000, Yang et al., 2006). High amounts of TNF- α are also released by human mesothelial cells exposed to crocidolite asbestos (Yang et al., 2006) and patients with MPM tumour burden present with high serum levels of TNF- α (Gordon et al., 2007, Partanen et al., 1995), strongly indicating that large amounts of TNF- α are present and persist in the tumour microenvironment. TNF- α is believed to be a contributing factor in resistance in MPM cells as treatment with TNF- α has been shown to induce a protective effect against induction of cell death (Yang et al., 2006). This protective effect is dependent on NF κ B signalling, indicating the potential increased expression of survival proteins transcriptionally controlled by NF κ B, including IAPs (Gordon et al., 2007).

The role of TNF- α in MPM resistance and evidence that this is mediated through increased IAP expression further supports the potential of SMAC mimetic therapy in treating this resistant form of cancer. SMAC mimetic mediated inhibition of XIAP and induced degradation of cIAP-1/2 could nullify TNF- α induced resistance and switch TNF- α signalling from NF κ B activation and a protective response to formation of the caspase-8 death complex and apoptosis.

In summary, in this Chapter the “on target” binding of computationally designed small molecule SMAC mimetic compounds was analysed for affinity to the primary target of XIAP-BIR3 and the secondary putative target cIAP-1/2. Lead compounds were tested for their ability to act as SMAC mimetics in MPM cell lines, sensitising cells to cisplatin or death receptor induced apoptosis. In subsequent experiments, the role of TNF- α inducing a protective effect against drug induced cell death was examined and the role of IAP expression in mediating this protective effect was investigated.

Results

4.1 Computational Ligand Based Design of a series of compounds based on the SMAC N-terminal tetra-peptide, AVPI

Having investigated the potential and efficacy of the known SMAC mimetic, embelin, in relieving IAP mediated apoptotic resistance in MPM cell lines, attempts were made to develop novel SMAC mimetic compounds. Collaborators in the Molecular Design Group (MDG), School of Biochemistry and Immunology, Trinity College Dublin designed a series of compounds based on a pharmacophore of the AVPI N-terminal tetra-peptide sequence of human SMAC (Fig. 4.1). The competitive binding of SMAC N-terminal AVPI for the Baculovirus IAP repeat number 3 (BIR3) domain of XIAP, the site responsible for sequestration of Caspase-9, was the target interaction of interest in this study for the identification of novel SMAC mimetic compounds. This AVPI / XIAP-BIR3 interaction site had been the basis of computational SMAC mimetic based design in a number of previous reports (Sun et al., 2004, Crisostomo et al., 2009).

A Computational ligand based design approach was taken, constructing a pharmacophore feature map of the AVPI sequence of SMAC, through which commercially available compound databases could be virtually screened (VS) for similarity to AVPI. A

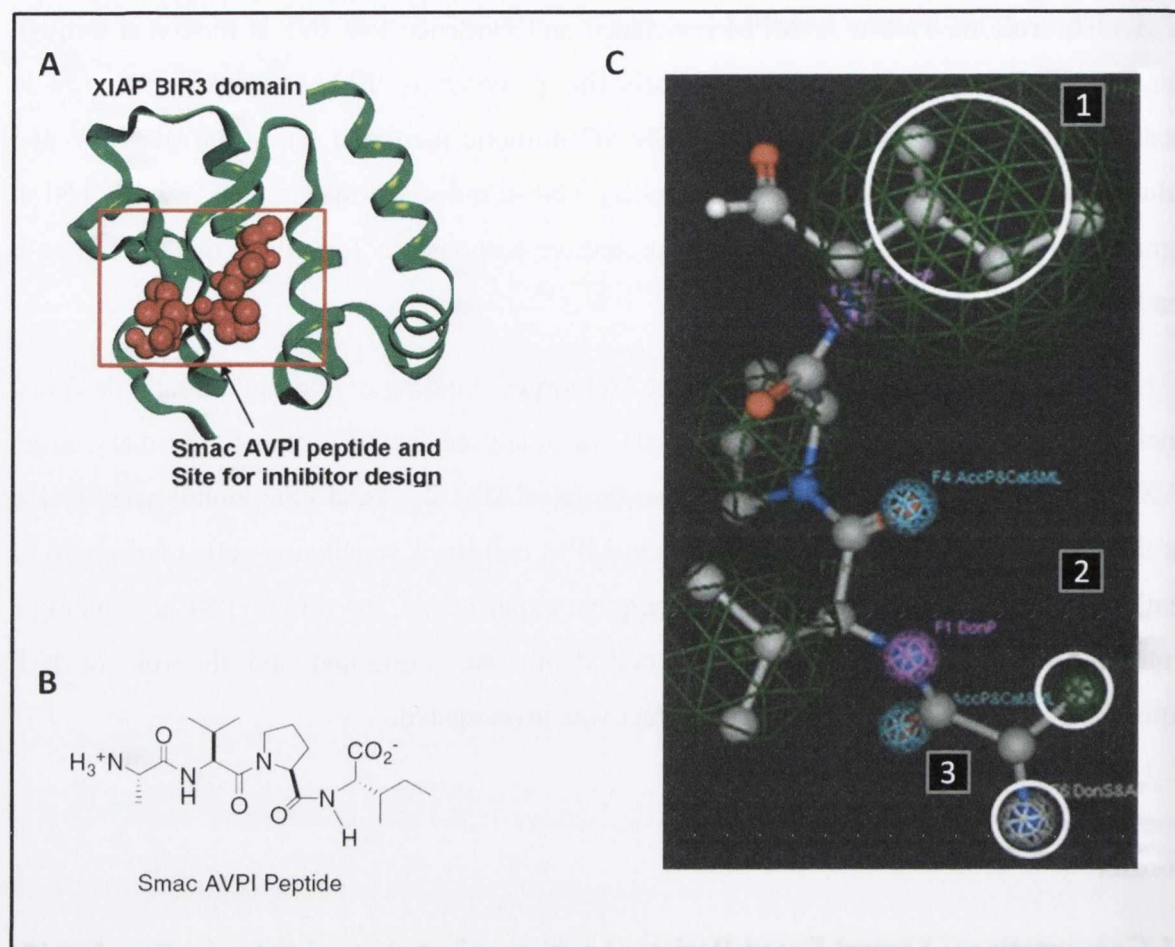


Figure 4.1: Pharmacophore feature map of SMAC N-terminal tetrapeptide AVPI.

(A) Structural representation of SMAC N-terminal AVPI peptide associated with binding pocket of XIAP-BIR3 domain (Nikolovska-Coleska et al., 2004), the crystal structure of which gave the bioactive conformation of AVPI, the basis for the ligand based pharmacophore (Wu et al., 2000). (B) Chemical diagram of AVPI tetra peptide (Chen et al., 2006).

(C) Structural diagram of SMAC N-Terminal tetra-peptide AVPI in its bioactive conformation when bound to BIR3 domain of XIAP is shown. The 9 chemical features that contributed to the constructed pharmacophore used in the Virtual screen for novel SMAC mimetics are displayed (Green: Hydrophobic region; Cyan: H-bond acceptor; Grey: H-bond donor / acceptor; Purple: H-Bond donor) – [Design by Darren Fayne, MDG Trinity College Dublin]. The molecular features circled and marked 1 (hydrophobic carbon chain), 2 (hydrophobic methyl group) and 3 (H-Bond donor ammonium cation)

previously published X-ray structure of SMAC complexed with XIAP (Wu et al., 2000) provided a picture of the bioactive conformation of AVPI bound to XIAP-BIR3, upon which the constructed pharmacophore was based. This pharmacophore consisted of 9 molecular features describing distinct physiochemical properties of AVPI, 3 of which were believed to be of particular importance for binding (Fig. 4.1.C). The pharmacophore also included positional, volume and chemical constraints to better select “Hits” and improve likelihood of “Drug-like Hits”. Virtual screening of commercial compound databases (Specs, Peakdale, ZINC), each containing ~150,000 distinct structures, through the constructed AVPI pharmacophore generated *in silico* compound “Hits” mapping to AVPI. Numerous “hit” compounds matched 6 of 9 pharmacophore features and eight selected compounds went forward for *in vitro* and cell based biochemical validation (Table 4.1 & Table 4.2). MDG 42, 43, 44, 330 & 331 consisted of alterations to a primary core scaffold (Table 4.1), while MDG 39 and 40 and MDG 45 were “hits” of distinct alternate scaffold structures (Table 4.2).

4.2 Optimisation and validation of an *in vitro* fluorescence polarisation assay for competitive binding analysis of computational “Hit” compounds to XIAP-BIR3 domain

To validate an on-target effect of computational “Hit” MDG SMAC mimetic compounds, an *in vitro* FP competitive binding assay was developed and optimised. Optimisation of this assay protocol involved the testing of various concentrations of the recombinant human XIAP-BIR3 (hXIAP-BIR3) Ligand Binding Domain (LBD) and its fluorescently labelled peptide ligand (fluor), ARPFAQ-[K-5-FAM], in order to obtain a maximal dynamic millipolarisation (mP) range for greatest assay sensitivity. The highest hXIAP-BIR3 concentration tested (0.15 μ M) gave the largest assay range (Fig. 4.2.A). Conversely, increasing fluor concentration, from 0.005 μ M to 0.01 μ M fluor, did not significantly alter assay range (Fig. 4.2). The optimised protocol taken forward was to test with 0.15 μ M LBD and 0.005 μ M fluor in competitive binding assays. This protocol gave a maximum dynamic assay range of between 50 – 90 mP units with vehicle treatment (Fig. 4.2.B), which is consistent with previous reports (Glover et al., 2003).

Validation of the assay itself was determined by testing competitive inhibition by known peptide inhibitors of hXIAP-BIR3. These included a synthetic cell permeabilised

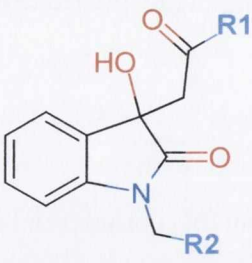
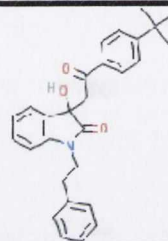
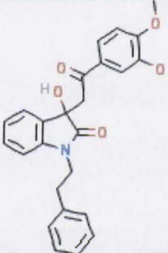
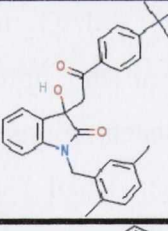
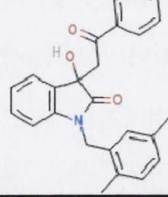
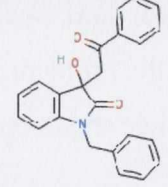
MDG SMAC Mimetics		
		MDG SMAC Mimetic Primary Scaffold
<u>Chemical structure</u>	<u>Chemical Name</u>	<u>MDG designation</u>
	3-[2-(4-tert-butylphenyl)-2-oxoethyl]-3-hydroxy-1-(2-phenylethyl)-1,3-dihydro-2H-indol-2-one	42
	3-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(2-phenylethyl)-1,3-dihydro-2H-indol-2-one	43
	3-[2-(4-tert-butylphenyl)-2-oxoethyl]-1-(2,5-dimethylbenzyl)-3-hydroxy-1,3-dihydro-2H-indol-2-one	44
	1-(2,5-dimethylbenzyl)-3-hydroxy-3-(2-oxo-2-phenylethyl)indolin-2-one	330
	1-benzyl-3-hydroxy-3-(2-oxo-2-phenylethyl)indolin-2-one	331

Table 4.1: Chemical structure and formulae of MDG SMAC mimetics based on primary SMAC N-terminal tetra-peptide (AVPI) pharmacophore scaffold.

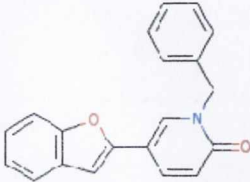
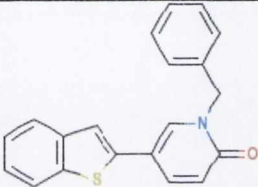
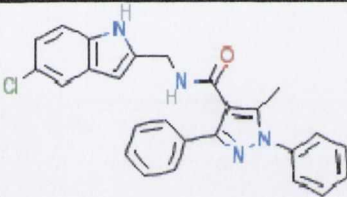
<u>MDG SMAC Mimetics</u>		
<u>Chemical structure</u>	<u>Chemical Name</u>	<u>MDG designation</u>
	5-(benzofuran-2-yl)-1-benzylpyridin-2(1H)-one	39
	5-(benzo[b]thiophen-2-yl)-1-benzylpyridin-2(1H)-one	40
	N-[(5-chloro-1H-indol-2-yl)methyl]-5-methyl-1,3-diphenyl-1H-pyrazole-4-carboxamide	45

Table 4.2: Chemical structure and formulae of MDG SMAC mimetics based on alternate AVPI pharmacophore scaffolds.

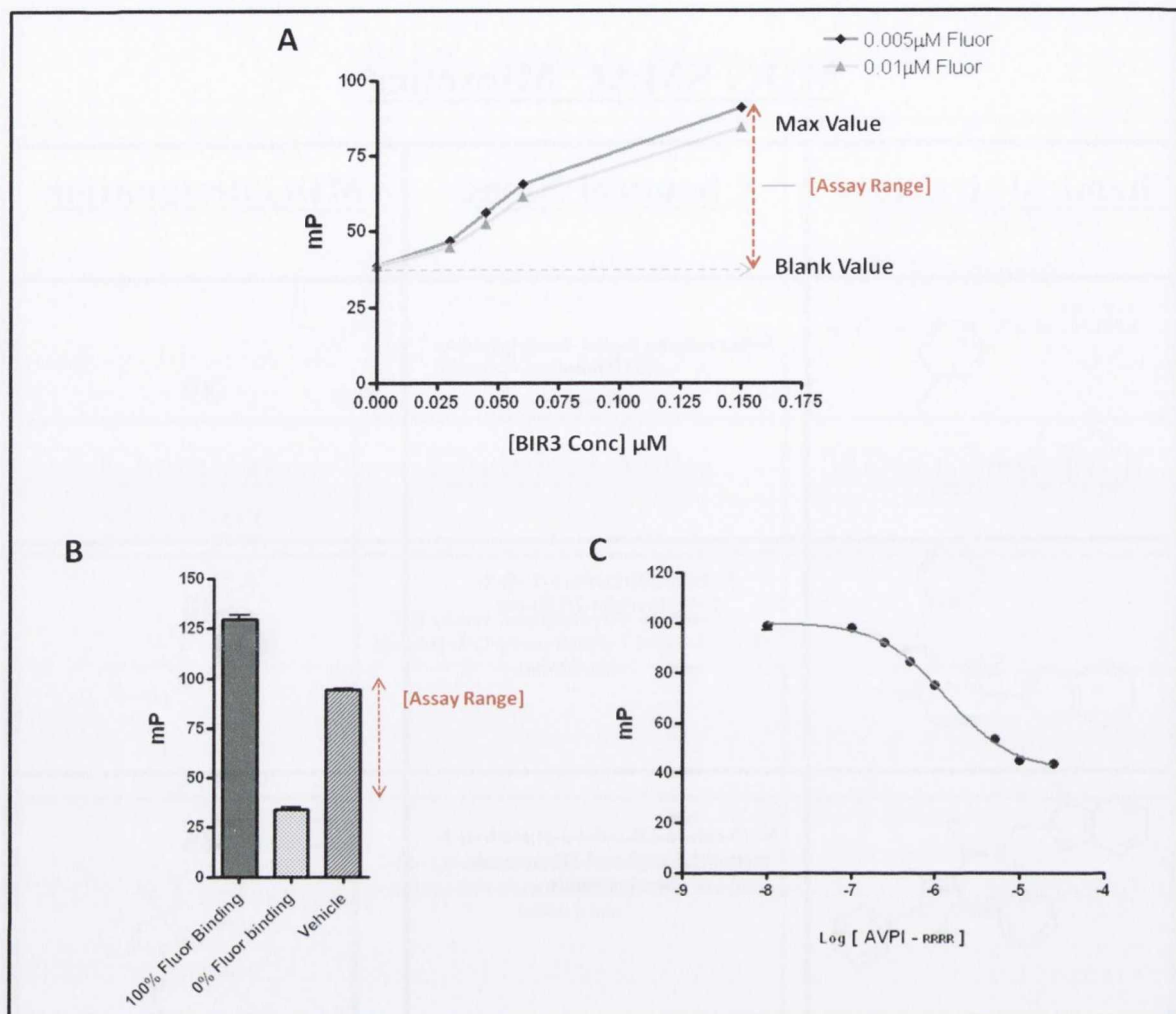


Figure 4.2: Optimisation and testing of fluorescence polarisation competitive binding assay for XIAP-BIR3 Caspase 9 / SMAC binding pocket.

Various concentrations of recombinant human XIAP-BIR3 (hXIAP-BIR3) ligand binding domain were assayed at two different concentrations of fluorescent ligand (fluor) (A). Fluorescence polarisation was as outlined in Section 2.8. Max polarisation represents control incubations with hXIAP-BIR3 and fluor only, indicative of 100% bound fluor, giving highest mP (millipolarisation units) value at indicated assay conditions. Blank polarisation level represents a control treatment with fluor only, giving a blank mP value indicative of total competitive dissociation of fluor. Results are representative of 2h incubation in dark, at room temperature and in triplicate.

In vitro, non-cell based fluorescence polarisation assay of competitive inhibition of test agents to the Caspase 9 / Smac binding pocket of purified, recombinant human XIAP-BIR3. (B) 100% fluor binding represents a control incubation with hXIAP-BIR3 and fluorescent probe (fluor) only, giving highest fluorescence polarisation (mP – millipolarisation units) with no competition. 0% fluor binding represents a control treatment with fluor only, giving a blank mP value akin to total competitive dissociation of fluor. (C) Dose dependent competitive inhibition of synthetic cell permeable form of AVPI (AVPI-RRRR) to hXIAP-BIR3.

form of native SMAC-AVPI (AVPI-RRRRRRRR or AVPI-R(8)), used in experimentation in Chapter 3, and a non-fluorescently labelled form of the fluor peptide (ARPFAQK). AVPI-R(8) induced dose dependent reduction in fluorescence polarization, with an IC_{50} value of $1.13\mu M (\pm 0.04\mu M)$ (Fig. 4.2.C). This value is consistent with previous reports on IC_{50} values for fluorescence polarisation competitive binding studies of AVPI peptide binding to XIAP-BIR3 (Glover et al., 2003, Nikolovska-Coleska et al., 2004). ARPFAQK was a more potent inhibitor of BIR3 domain (Fig. 4.3), with $1\mu M$ treatment inducing an approximate 90% inhibition of fluor binding to hXIAP-BIR3. These results are in line with reported IC_{50} values of $0.13 - 0.68 \mu M$ of ARPFAQK peptide for hXIAP-BIR3 (Nikolovska-Coleska et al., 2004).

4.3 Computationally designed MDG compounds did not bind to the XIAP-BIR3 domain

MDG “hit” compounds MDG 39, 40, 42 43, 44, 45, 330 and 331 were tested at $10\mu M$, $50\mu M$ and $100\mu M$ for potential binding to hXIAP-BIR3 in the optimised fluorescence polarisation competitive binding assay. None of the compounds tested showed binding to XIAP-BIR3, with no competitive inhibition of the binding of fluor to the hXIAP-BIR3, (Fig. 4.3). No dose dependent effects on MDG compound binding were observed, however MDG 39 induced an apparent increase in fluorescence polarisation in a dose dependent manner. XIAP targeting positive controls were used, including AVPI-R(8) and ARPFAQK peptides and small-molecule SMAC mimetic embelin, were used to insure experimental validity. Experimental positive controls induced significant, dose dependent reductions in fluorescent probe binding, confirming affinity for XIAP-BIR3 and validating test results.

4.4 Evaluation of the cytotoxic and apoptotic potential of MDG compounds in Mesothelioma cell lines

Whilst this series of compounds did not show significant direct binding to XIAP-BIR3, its predicted molecular target, in an “on target” FP competitive binding assay, cellular functional assays analysing the capacity of MDG compounds to sensitise MPM cells to chemotherapeutic induction of apoptosis were performed. The cytotoxic and apoptotic

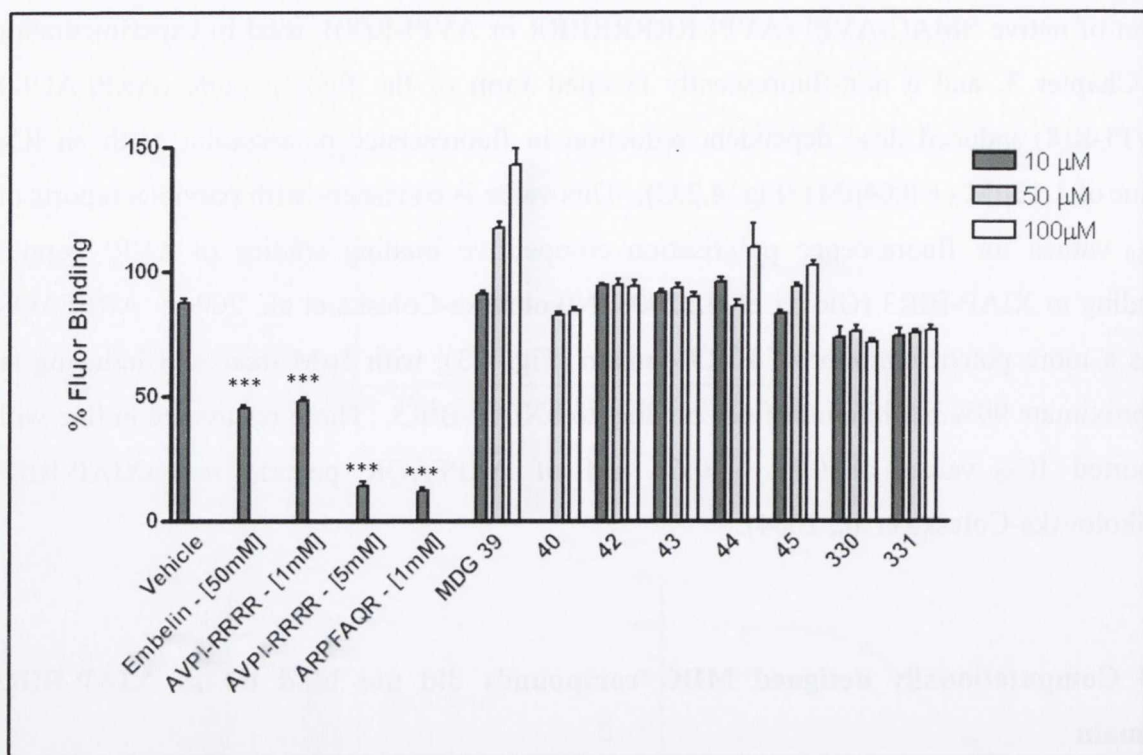


Figure 4.3: MDG SMAC mimetics did not show binding to XIAP-BIR3 Caspase 9 / SMAC binding pocket.

In vitro, non-cell based fluorescence polarisation assay of competitive binding of MDG 39, 40, 42, 43, 44, 45, 330 & 331 to the Caspase 9 / SMAC binding pocket of purified, recombinant human XIAP-BIR3. Test compounds (at indicated concentrations) and fluor (5nM) were incubated with 0.15 μ M hXIAP-BIR3 at room temp in the dark for 4h. Millipolarisation (mP) values were recorded using Pherastar fluorescence plate reader and software. mP values were transformed and affinity of test compounds to hXIAP-BIR3 were illustrated by their percentage inhibition of fluor binding to the LBD. Values are representative of mean $\pm$ SEM of three independent experiments. ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

potential of these novel compounds in MPM cell lines were still of particular interest in light of positive results observed in previous cell death experimentation (data not shown). The compounds MDG 39, MDG 42 and MDG 44 were selected for further analysis due to significant cytotoxic effects observed when screened previously in alternate *in vitro* cancer cell models, including breast and prostate cancer cell lines. The capacity for both single agent and combinatorial treatment regimens to induce or synergistically increase apoptosis in cells was tested. Similarly with embelin co-treatment experiments, performed in Chapter 3, which analysed sensitisation of MPM cells to apoptosis, cisplatin was chosen as the co-treatment chemotherapeutic of choice, due to its clinical use in Mesothelioma treatment.

4.4.1 MDG 39, 42 and 44 induced cell death in selected Mesothelioma cell lines

The ability of MDG 39, MDG 42 and MDG 44 to induce cell death in CRL 5915, CRL 5820, Ju77 and One 58 cell lines was tested after 24h and 48h incubation times, by way of Alamar Blue cell viability assay. Cells were incubated with vehicle or MDG SMAC mimetic at a screening concentration of 25 μ M (Fig 4.4).

MDG 44 was the most potent compound of the three tested in all Mesothelioma cell lines (Fig 4.4). MDG 44 induced significant levels of cell death in the CRL 5820 cell line at 24h and 48h (28.3% $\pm$ 2.5 & 33.0 $\pm$ 3.7, respectively) and a $\sim$ 10% increase in cell death in all other MPM cell lines after 48h incubation. Interestingly, CRL 5820 cells, the high IAP expressing MPM model, were again the most sensitive cell line to MDG 42 and 39 incubation, inducing significant levels of cell death after 24h ($\sim$ 10%) and 48h ($\sim$ 15%). Besides CRL 5820 susceptibility, MDG 42 was only able to induce a marginal increase in cell death in One 58 cells after 48h (8.1% $\pm$ 1.5), with MDG 42 and 39 inducing negligible effects at 25 μ M in all other incubations.

4.4.2 MDG 42 and MDG 44 synergistically increased cisplatin induced apoptosis in CRL 5915 and One 58 cell lines, but not Ju77 or CRL 5820 cell lines

After observing the cytotoxic effects of MDG compounds as single agents in MPM cells, the apoptotic induction of single agents and, importantly, the capacity to synergise with cisplatin to increase induction of apoptosis was next tested. While XIAP-BIR3 target binding was not confirmed for these compounds, the ability to combine with cisplatin, the

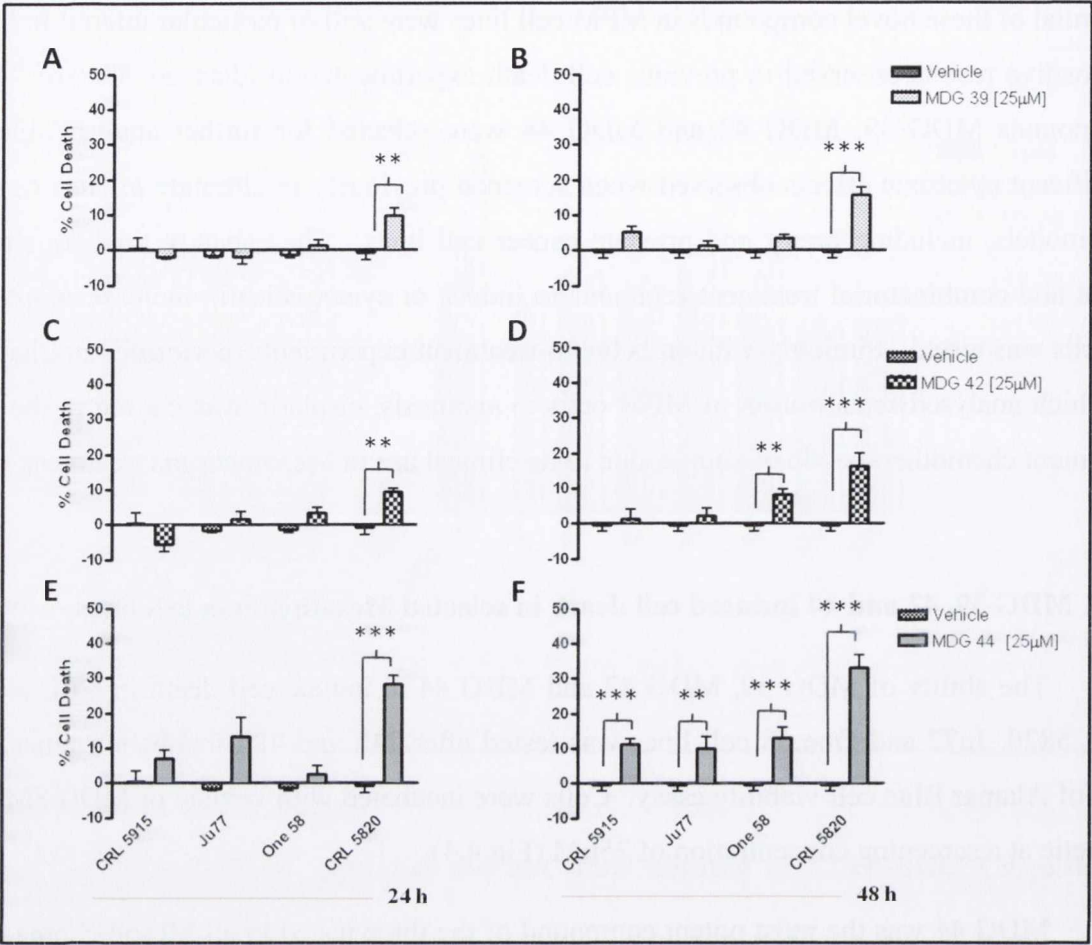


Figure 4.4: MDG SMAC mimetics induced single agent cytotoxic effect in Mesothelioma cell lines.

Mesothelioma cell lines treated with 25µM MDG 39 (A & B), MDG 42 (C & D) or MDG 44 (E & F) for 24h (A, C, E) or 48h (B, D, F). Cells were plated on 96 well plates at a density of 0.5×10^4 cells per well, containing 200µl medium and left to adhere for 18h. Cells were treated with indicated concentrations of MDG SMAC Mimetics and left to incubate for 24h or 48h before analysis of cytotoxicity by way of Alamar Blue cell viability assay, outlined in Section 2.6. Values are representative of mean $\pm$ SEM of at least three independent experiments. **P<0.01; ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

primary combinational treatment partner in MPM therapy (Belli et al., 2009), was of particular interest. Cells were treated with MDG compounds in a dose dependent manner of drug (1 μ M, 10 μ M & 25 μ M) alone or in combination with cisplatin (25 μ M) for 48h. Supplementary to this, Ju77 cells were treated as indicated previously for 24h and One 58 cells were treated with MDG 44 $\pm$ cisplatin for 72h, in order to optimise detection of a synergistic effect. After treatment, cells were fixed and stained with propidium iodide for flow cytometry cell cycle analysis, as outlined in Section 2.7. Apoptosis was quantified by the percentage hypodiploid or “pre G1” apoptotic population of cells compared with the total number of total gated events.

Single agent apoptotic induction mirrored the cytotoxicity results for MDG compounds (Fig 4.4). MDG 44 was the most potent compound and CRL 5820 the most susceptible cell line tested. Significant induction of single agent apoptosis was only observed with MDG 44 treatment in high IAP expressing CRL 5820 cells (Fig 4.5.D). Treatment with MDG 44, MDG 42 or MDG 39 in all other cell lines did not induce single agent apoptosis (Fig 4.5 – 4.7).

In combinational treatments of MDG compounds and cisplatin, only two of the four MPM cell lines induced a significant increase in apoptosis compared to cisplatin treatment alone – the CRL 5915 cell line (A) and the ONE 58 (B) cell line (Fig 4.5 – 4.7). In these two cell lines MDG 42 and MDG 44 (25 μ M), but not MDG 39, were effective at synergistically increasing apoptosis with cisplatin treatment – a synergistic response was deemed significant by two-way ANOVA statistical analysis of the interaction effect of MDG compound and cisplatin. Co-treatment responses were most effective in CRL 5915 cells. MDG 42 and 44 had no apoptotic effect on their own at 25 μ M, but in co-treatment with cisplatin, MDG 42 induced a 22.0% increase in apoptosis, while MDG 44 elicited almost a fold increase (doubling) in apoptosis (28.6%) compared to cisplatin treatment alone. While the magnitude of response in One 58 cells was lower (cisplatin induced 31.2% $\pm$ 1.8 and 9.9% $\pm$ 0.9 apoptosis in CRL 5915 and One 58 respectively) comparable synergistic increases were observed with MDG 42 and MDG 44 co-treatments at 48h and 72h.

In synergism experimentation in Ju77 and CRL 5810 cell lines, co-treatment with any of the compounds MDG 39, MDG 42 or MDG 44 in combination with cisplatin was not effective at synergistically increasing apoptosis. In the CRL 5820 cell line, high IAP

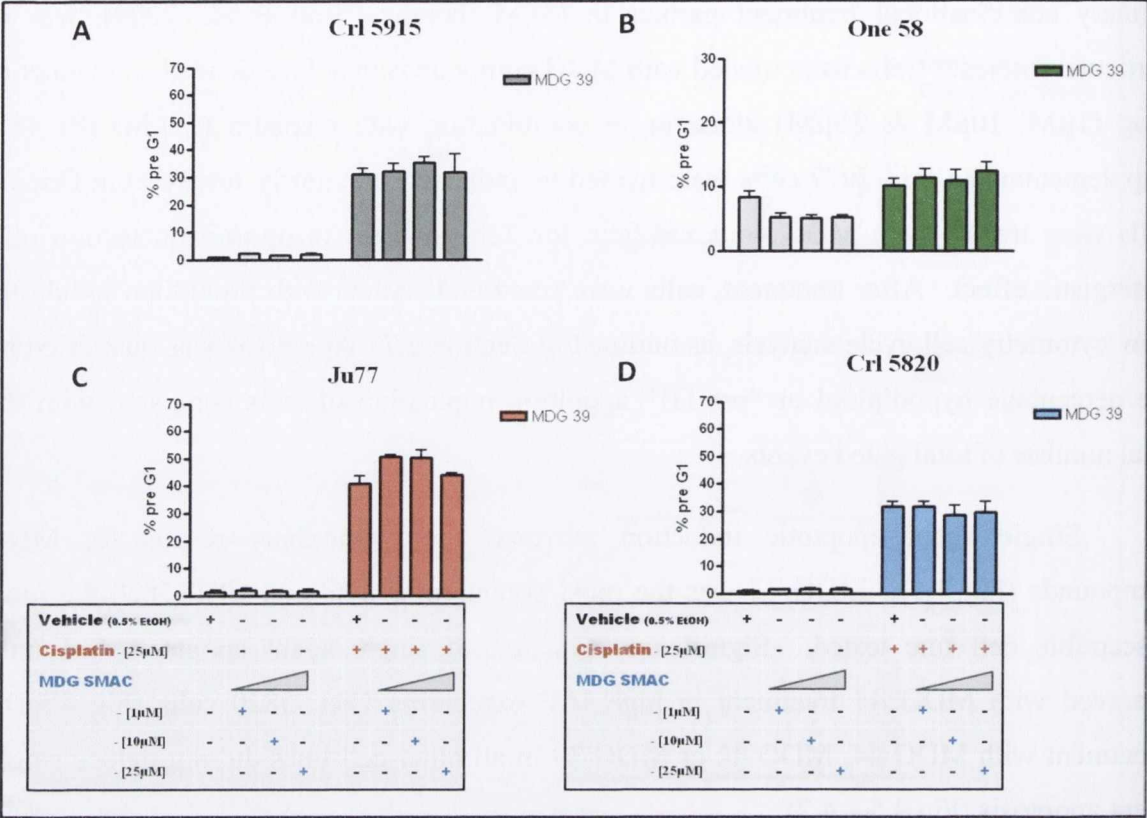


Figure 4.5: MDG 39 did not synergistically increase cisplatin induced apoptosis in Mesothelioma cell lines

CRL 5915 (A), One 58 (B), Ju77 (C) and CRL 5820 (D) cells were treated with MDG 39 for 48h alone or in combination with Cisplatin. Cells were seeded at 0.25 x 10⁶ cells / 5ml in 25cm² tissue culture flasks. After 48h incubation, cells were harvested and fixed in 70% Ethanol and PI cell cycle flow cytometry analysis was performed, as outlined in Section 2.7. Cells in pre G1 population represented apoptotic cells. Values are representative of mean ± SEM of at least three independent experiments.*P<0.05; **P<0.01 ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software). Synergism determined by two-way analysis of variance (ANOVA) of interaction effect of single agent and co-treatment response (‡ P<0.01).

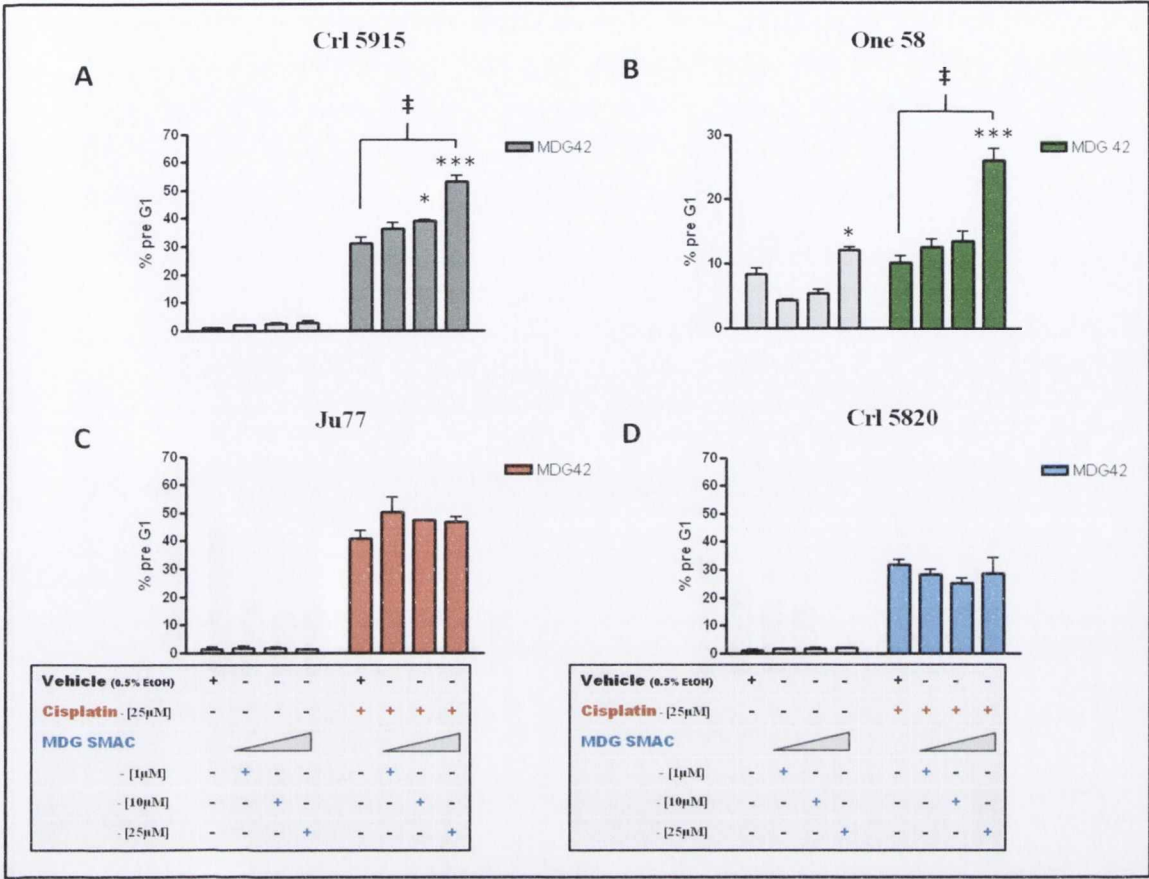


Figure 4.6: MDG 42 synergistically increased cisplatin induced apoptosis in CRL 5915 and One 58 Mesothelioma cell lines

CRL 5915 (A), One 58 (B), Ju77 (C) and CRL 5820 (D) cells were treated with MDG 42 for 48h alone or in combination with Cisplatin. Cells were seeded at 0.25×10^6 cells / 5ml in 25cm² tissue culture flasks. After 48h incubation, cells were harvested and fixed in 70% Ethanol and PI cell cycle flow cytometry analysis was performed, as outlined in Section 2.7. Cells in pre G1 population represented apoptotic cells. Values are representative of mean $\pm$ SEM of at least three independent experiments.*P<0.05; **P<0.01 ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software). Synergism determined by two-way analysis of variance (ANOVA) of interaction effect of single agent and co-treatment response ($\ddagger$ P<0.01).

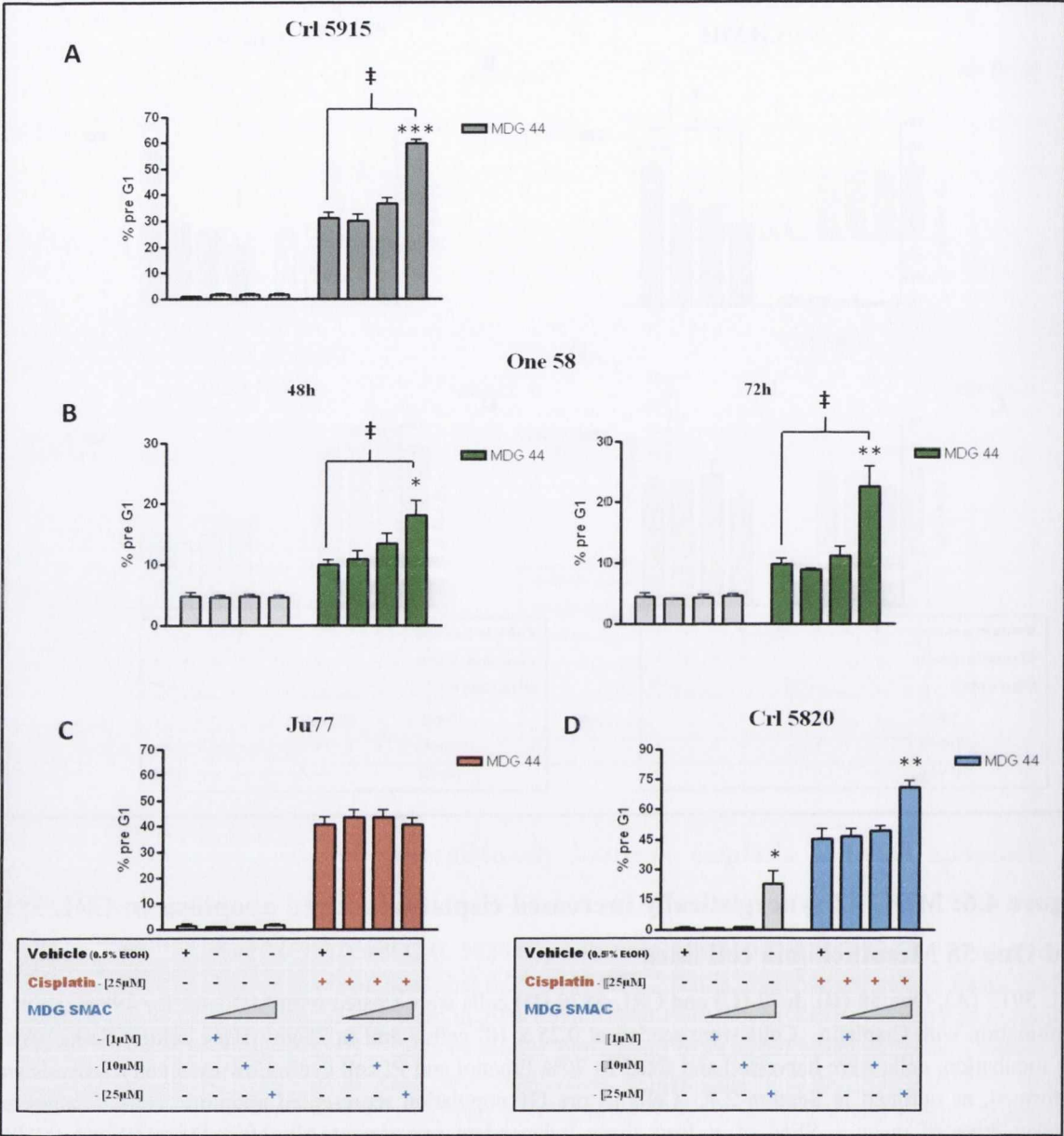


Figure 4.7: MDG 44 synergistically increased cisplatin induced apoptosis in CRL 5915 and One 58 Mesothelioma cell lines

CRL 5915 (A), One 58 (B), Ju77 (C) and CRL 5820 (D) cells were treated with MDG 44 for 48h or 72h alone or in combination with Cisplatin. Cells were seeded at 0.25×10^6 cells / 5ml in 25cm² tissue culture flasks. After 48h incubation, cells were harvested and fixed in 70% Ethanol and PI cell cycle flow cytometry analysis was performed, as outlined in Section 2.7. Cells in pre G1 population represented apoptotic cells. Values are representative of mean $\pm$ SEM of at least three independent experiments. *P<0.05; **P<0.01 ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software). Synergism determined by two-way analysis of variance (ANOVA) of interaction effect of single agent and co-treatment response (‡ P<0.01).

expressing cells sensitised to cisplatin induced apoptosis with embelin co-treatment, no synergistic effects with MDG compound treatment were observed. In the Ju77 cell line, cells described in Chapter 3 as being very susceptible to apoptotic challenge, reached saturating apoptotic response with 25 μ M cisplatin after 48h (Fig 4.5 – 4.7). A shorter 24h incubation time was employed but synergism was still not seen in this cell line (Fig 4.8).

Synergism results for each compound and cell line were summarised in Table 4.3. While these results report the discovery of two novel compounds which can synergistically increase the apoptotic potential of cisplatin in two specific MPM cell lines, the profile of susceptible and non-susceptible cell lines does not correlate with results for XIAP cellular expression or embelin / cisplatin synergism experiments performed in Chapter 3.

4.5 MDG compounds do not bind to cIAP-1 or cIAP-2, a secondary putative IAP binding partner

Having established that the MDG compounds do not elicit their effect through inhibition of XIAP, possible alternate targets were investigated for these computationally designed SMAC mimetics. While the primary target of SMAC mimetic therapy involves direct disruption of IAP / caspase interactions, relieving caspase sequestration and amplification of an intrinsic apoptotic stimulus (Chai et al., 2000, Srinivasula et al., 2001), cIAP-1/2 targeting by MDG compounds was investigated as a possible mechanism of action. As outlined in Chapter 1, SMAC mimetics are also able to bind cIAP-1/2, inducing proteosomal degradation which leads to loss of regulation of TNFR signalling and apoptosis induced by autocrine or extrinsic TNF- α / TNFR activation of caspase-8 (Varfolomeev et al., 2007, Wang et al., 2008, Vince et al., 2007). As well as this, having already observed MDG compounds ability to synergise with cisplatin, but not elicit this effect through XIAP, a 2010 report in *Cell Death and Differentiation* further suggested cIAP-1/2 as a putative target for these compounds (Probst et al., 2010). The paper reported the ability of genotoxic chemotherapeutic drugs, including cisplatin, to synergise with SMAC mimetics in a TNF- α dependent manner. SMAC mimetics target cIAP-1/2 for degradation while genotoxic chemotherapeutics inhibit cell proliferation and cause canonical NF κ B activation of TNF- α , increasing the ability of SMAC mimetics to drive autocrine activation of an extrinsic pathway of apoptosis.

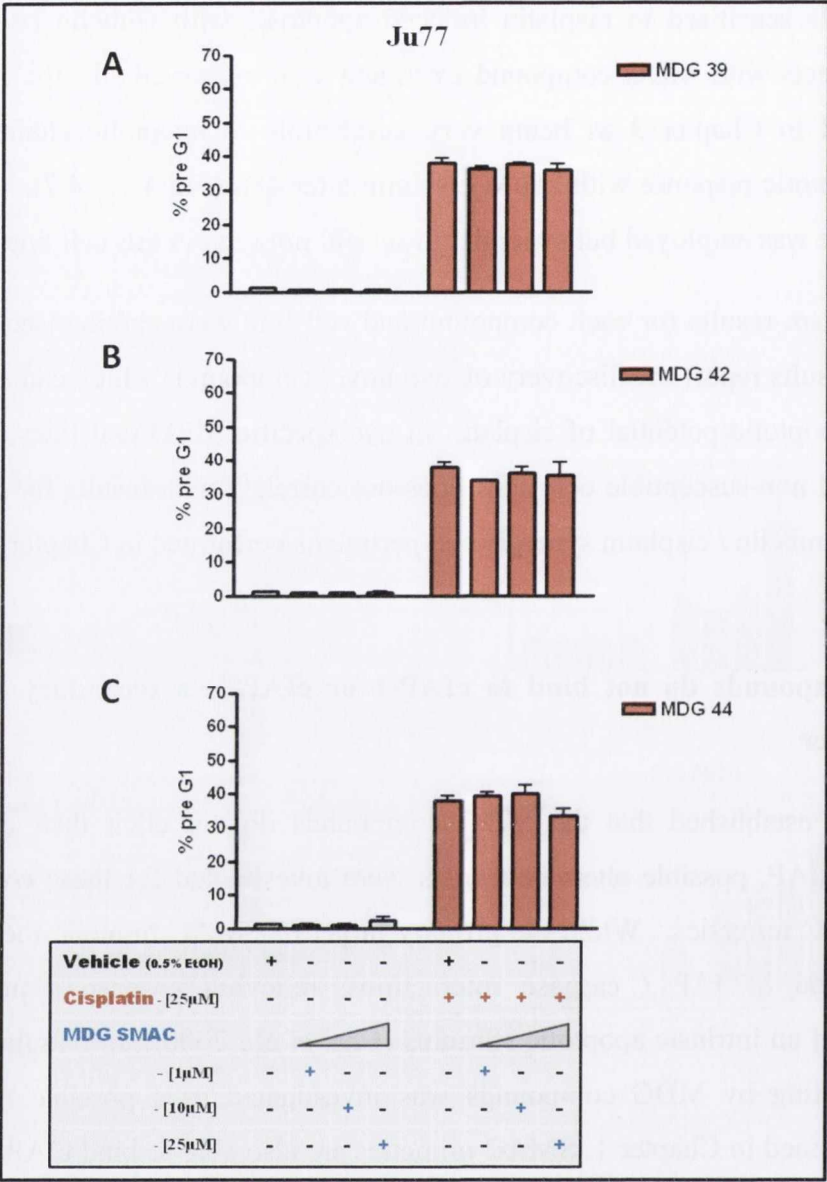


Figure 4.8: MDG SMAC mimetic treatment for 24h did not synergistically increase cisplatin induced apoptosis in Mesothelioma cell lines

Ju77 cells were treated with MDG 39 (A), MDG 42 (B) and MDG 44 (C) for 24h alone or in combination with Cisplatin. Cells were seeded at 0.25×10^6 cells / 5ml in 25cm^2 tissue culture flasks. After 24h incubation, cells were harvested and fixed in 70% Ethanol and PI cell cycle flow cytometry analysis was performed, as outlined in Section 2.7. Cells in pre G1 population represented apoptotic cells. Values are representative of mean $\pm$ SEM of at least three independent experiments.* $P < 0.05$; ** $P < 0.01$ *** $P < 0.001$ (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software). Synergism determined by two-way analysis of variance (ANOVA) of interaction effect of single agent and co-treatment response ($\ddagger P < 0.01$).

MDG 39

Cell line	Vehicle	MDG 39 [25µM]	Cisplatin [25µM]	MDG 39 + Cisplatin	Percentage Increase	Synergism (P statistic)
CRL 5915 (48h)	0.89% (±0.15%)	2.09% (±0.32%)	31.19% (±1.79%)	31.56% (±6.78%)	(n/s)	(n/s)
One 58 (48h)	8.37% (±0.94%)	5.23% (±0.34%)	10.18% (±0.96%)	12.43% (±1.27%)	(n/s)	(n/s)
Ju77 (24h)	1.17% (±0.19%)	0.61% (±0.14%)	37.87% (±1.44%)	36.06% (±1.61%)	(n/s)	(n/s)
(48h)	1.36% (±0.54%)	1.58% (±0.63%)	40.94% (±2.57%)	43.92% (±0.77%)	(n/s)	(n/s)
CRL 5820 (48h)	1.04% (±0.13%)	1.57% (±0.27%)	31.70% (±1.90%)	29.76% (±3.60%)	(n/s)	(n/s)

MDG 42

Cell line	Vehicle	MDG 42 [25µM]	Cisplatin [25µM]	MDG 42 + Cisplatin	Percentage Increase	Synergism (P statistic)
CRL 5915 (48h)	0.89% (±0.15%)	2.54% (±2.34%)	31.19% (±1.79%)	53.24% (±2.08%)	↑ 22.05%	† P<0.0001
One 58 (48h)	8.37% (±0.94%)	12.04% (±0.33%)	10.18% (±0.96%)	25.97% (±1.71%)	↑ 15.79%	† P<0.0001
Ju77 (24h)	1.17% (±0.19%)	0.93% (±0.19%)	37.87% (±1.44%)	35.74% (±3.59%)	(n/s)	(n/s)
(48h)	1.36% (±0.54%)	1.16% (±0.19%)	40.94% (±2.57%)	46.71% (±1.76%)	(n/s)	(n/s)
CRL 5820 (48h)	1.04% (±0.13%)	1.87% (±0.16%)	31.70% (±1.90%)	28.53% (±3.76%)	(n/s)	(n/s)

MDG 44

Cell line	Vehicle	MDG 44 [25µM]	Cisplatin [25µM]	MDG 44 + Cisplatin	Percentage Increase	Synergism (P statistic)
CRL 5915 (48h)	0.89% (±0.15%)	1.62% (±0.19%)	31.19% (±1.79%)	59.76% (±1.36%)	↑ 28.57%	† P<0.0001
One 58 (48h)	4.80% (±0.48%)	4.57% (±0.35%)	9.94% (±0.87%)	18.03% (±2.42%)	↑ 8.09%	† P<0.01
(72h)	4.50% (±0.57%)	4.54% (±0.29%)	9.83% (±0.89%)	22.54% (±3.30%)	↑ 12.71%	† P<0.01
Ju77 (24h)	1.17% (±0.19%)	2.34% (±0.97%)	37.87% (±1.44%)	33.53% (±2.04%)	(n/s)	(n/s)
(48h)	1.36% (±0.54%)	1.65% (±0.31%)	40.94% (±2.57%)	40.69% (±2.42%)	(n/s)	(n/s)
CRL 5820 (48h)	0.91% (±0.08%)	22.95% (±6.07%)	45.39% (±4.81%)	71.06% (±3.03%)	(n/s)	(n/s)

Table 4.3 Summary of results of MDG SMAC mimetic and cisplatin co-treatment experiments in MPM cell lines.

In order to investigate this putative mechanism of action for MDG compounds, a cIAP-1/2 “on target” assay and cellular functional assay were used for analysis in MPM cell lines. Recent publications on novel SMAC mimetic targeting cIAPs assayed cIAP-1/2 degradation directly by Western Blot as an indication of “on target” binding to cIAP-1/2 (Varfolomeev et al., 2007, Vince et al., 2007). Concurrently, a functional assay investigating the ability of MDG compounds to sensitise or synergistically increase death receptor ligands TNF- α and TRAIL induced cell death in MPM cell lines was performed using an Alamar blue cell viability assay.

4.5.1 MDG 44 does not induce proteosomal degradation of cIAPs: MDG 44 induces an increase in expression of cIAP-1 and a consequential decrease in cIAP-2.

The “on target” cIAP-1/2 degradation experiment was performed using MDG 44 on all four MPM cell lines. MDG 44 was chosen for experimentation as it was the most potent compound of the series when examined for cytotoxicity and apoptotic induction. Cells were treated with 30 μ M MDG 44 for time points between 10 min to 24h (0 min, 10 min, 30 min, 1h, 4h, 8h, 14h, & 24h). Lysates were prepared and c-IAP-1 and cIAP-2 expression examined by Western Blot, as outlined in Section 2.5.

SMAC mimetic compounds and native SMAC used in cIAP degradation experiments published by Vince et al (Vince et al., 2007) and Varfolomeev et al (Varfolomeev et al., 2007) report rapid degradation of cIAP-1/2 between 10 min – 1h post treatment. Treatment with MDG 44 in all four MPM cell lines did not induce degradation of either cIAP-1 or cIAP-2 in this time frame (Fig 4.9). MDG 44 treatment instead induced a time dependent increase in cIAP-1 protein expression between 4h and 24h (Fig 4.9.A – 4.9.D). Conversely, cIAP-2 expression was seen to decrease at late time points, a possible consequence of increased cIAP-1 expression. The cIAP-1 protein is known to be a post-transcriptional negative regulator of cellular protein levels of cIAP-2, with cIAP-2 being an ubiquitin target of the cIAP-1 RING domain E3 ligase (Conze et al., 2005, Cheung et al., 2008).

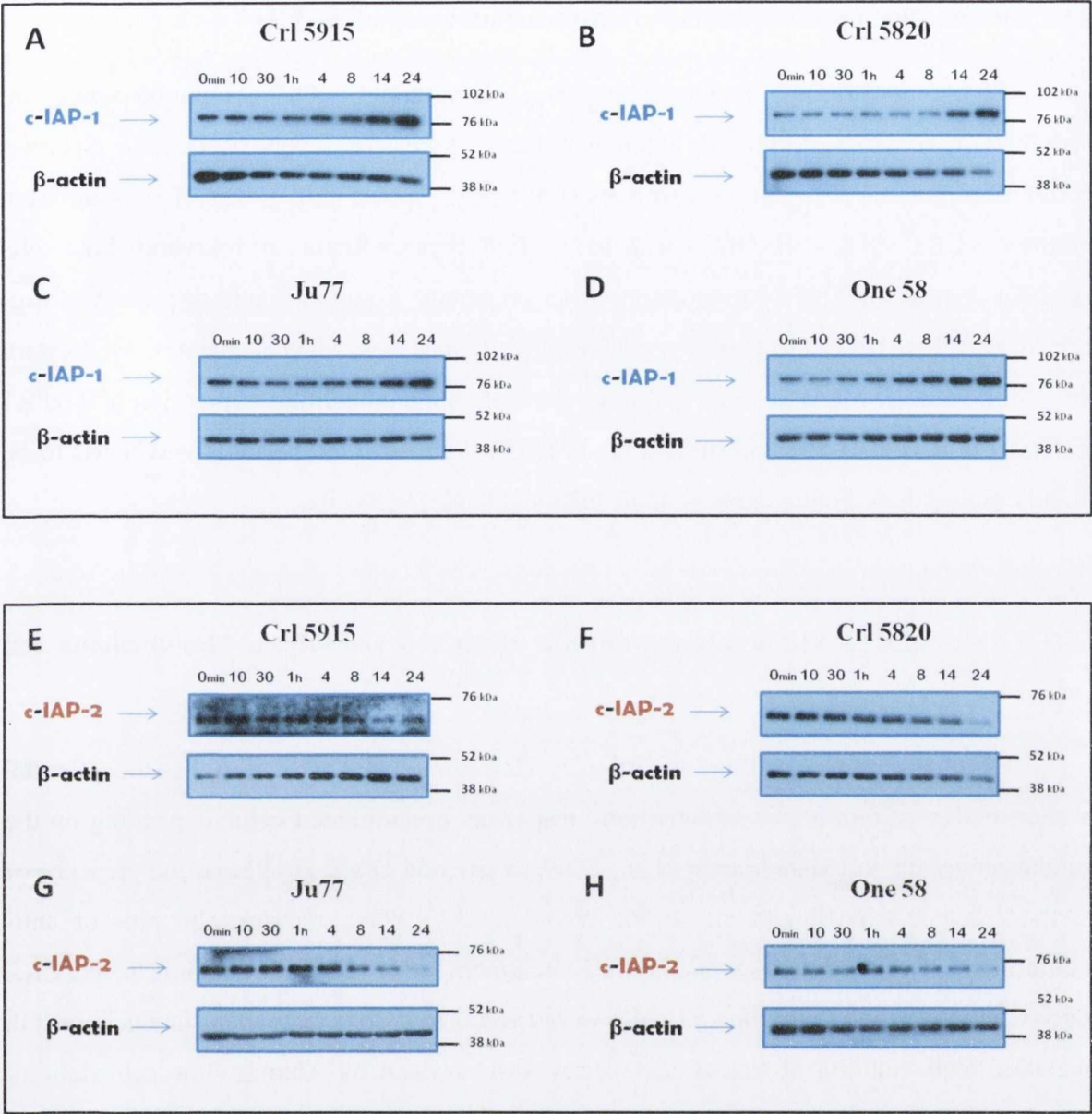


Figure 4.9: MDG 44 did not induce proteosomal degradation of cIAP-1 and cIAP-2 in MPM cell lines

(A - D) c-IAP-1 and (E - H) c-IAP-2 protein expression in response to MDG 44 treatment in CRL 5915, CRL 5820, Ju77 and One 58 cell lines. Cells were seeded at a density of 0.5×10^6 cells/5ml. Cells were treated with 30 μ M MDG 44. At indicated time points, cells were harvested and lysed, on ice, for 1h, in 1 x Ripa Buffer containing DTT and protease inhibitor cocktail. Protein (30 μ g) was resolved on 10% SDS-PAGE gel and transferred to PVDF membrane as outlined in Section 2.5. Blots were blocked in 5% Marvel TBS-Tween, probed with anti-c-IAP-1 (Primary Ab – 1:1,000) or anti-c-IAP-2 (Primary Ab – 1:1,000) and re-probed with anti- β -actin (Primary Ab – 1:10,000) as a loading control, as outlined in Section 2.5.6.

4.5.2 All Mesothelioma cell lines express abundant levels of TNFR1

Before performing the functional assay on potential MDG compound synergism with TNF- α , it was necessary to examine protein expression levels of Tumour Necrosis Factor Receptor – 1 (TNFR1) in cell lines of interest. Whole cell lysates of sub-confluent cultures of CRL 5915, CRL 5820, Ju77 and One 58 Mesothelioma cell lines and HeLa cells (positive control for TNFR1 expression) were prepared. Equal amounts of protein (30 μ g) were resolved by 10% SDS-PAGE and TNFR1 protein expression was analysed by Western blot. HeLa and all Mesothelioma cell lines showed abundant cellular expression of TNFR1 protein (Fig 4.10). Expression of TNFR1 in CRL 5820 and One 58 cells was noted to be slightly higher than protein expression in Ju77 and CRL 5915 cells.

4.5.3 TNF- α and TRAIL treatment did not affect cell viability in Mesothelioma cell lines

Death-receptor signalling, in particular TNF- α mediated activation, has the potential to elicit both proliferative or pro-apoptotic responses in stimulated cells, depending on the cellular environment (Mahalingam et al., 2009, Wertz and Dixit, 2010) and the presence of cIAP-1/2 regulation (Shu et al., 1996, Wang et al., 1998). To test the pro- or anti-proliferative effects of TNF- α and TRAIL treatment alone in Mesothelioma cells, CRL 5915, CRL 5820, Ju77 and One 58 cells were treated in a dose dependent manner for 24h and 48h. Cell viability of treated samples was determined by Alamar Blue cell viability assay. All Mesothelioma cell lines were shown to be insensitive to TNF- α and TRAIL treatment at 24h and 48h up to a high concentration of 100ng/ml (Fig 4.11). Cell viability did not deviate significantly above untreated controls, indicating no proliferative or death response of cells to TNF- α or TRAIL treatment.

4.5.4 MDG compounds in combination with death-receptor signalling did not synergistically induce cell death in One 58, Ju77 and CRL 5915 cell lines

MDG SMAC mimetics, MDG 39, MDG 42 and MDG 44, were treated (25 μ M) alone or in combination with death-receptor ligands TNF- α (10ng/ml) or TRAIL (10ng/ml) in One 58, Ju77 and CRL 5915 cell lines. Induction of cell death was analysed by Alamar

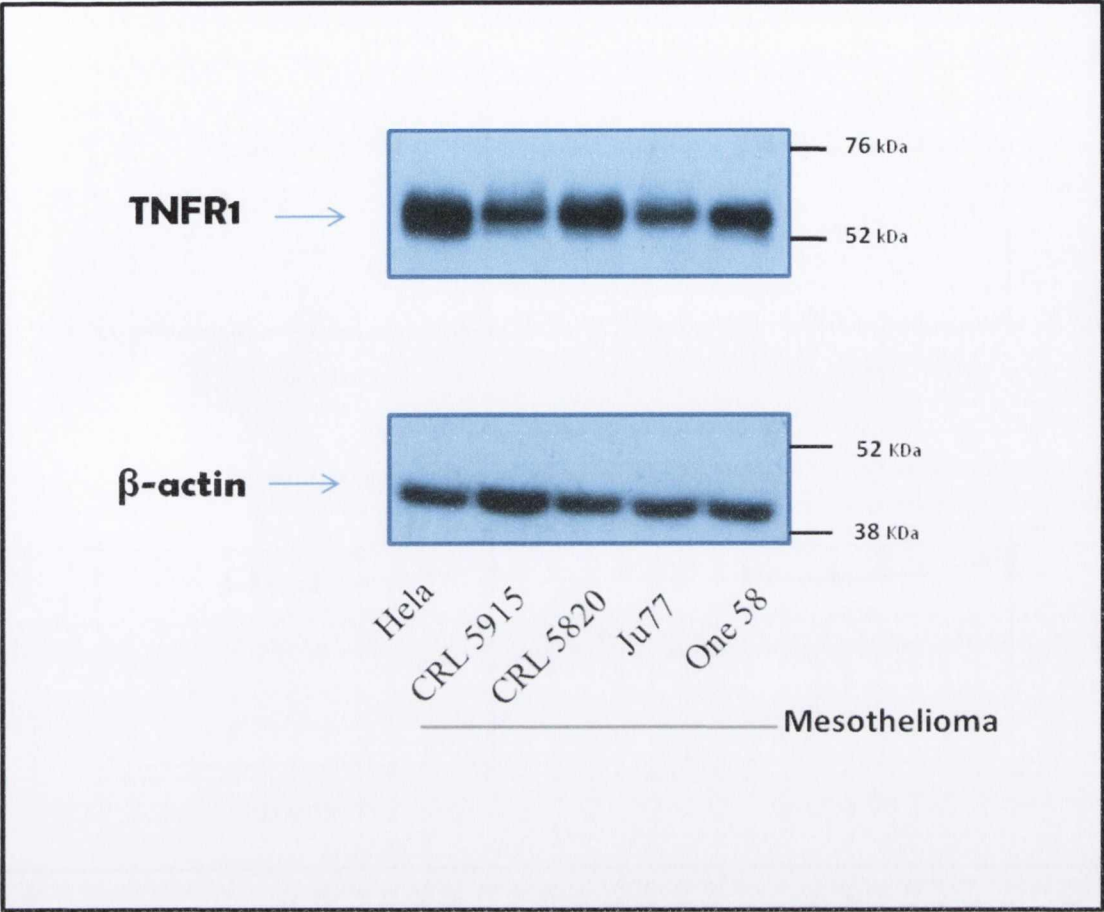


Figure 4.10: Expression of TNFR1 in Mesothelioma cell lines.

Whole cell lysates were prepared from sub confluent cultures of One 58, CRL 5820, CRL 5915, Ju77 and HeLa cell lines. Proteins (30µg) were resolved on 10% SDS-PAGE gel and transferred to PVDF membrane, as outlined in Section 2.5. Blots were blocked in 5% Marvel TBS-Tween, probed with anti-TNFR1 (Primary Ab – 1:1,000) and re-probed with anti-β-actin (Primary Ab – 1:10,000) as a loading control, as outlined in Section 2.5.6. Results are representative of three independent experiments.

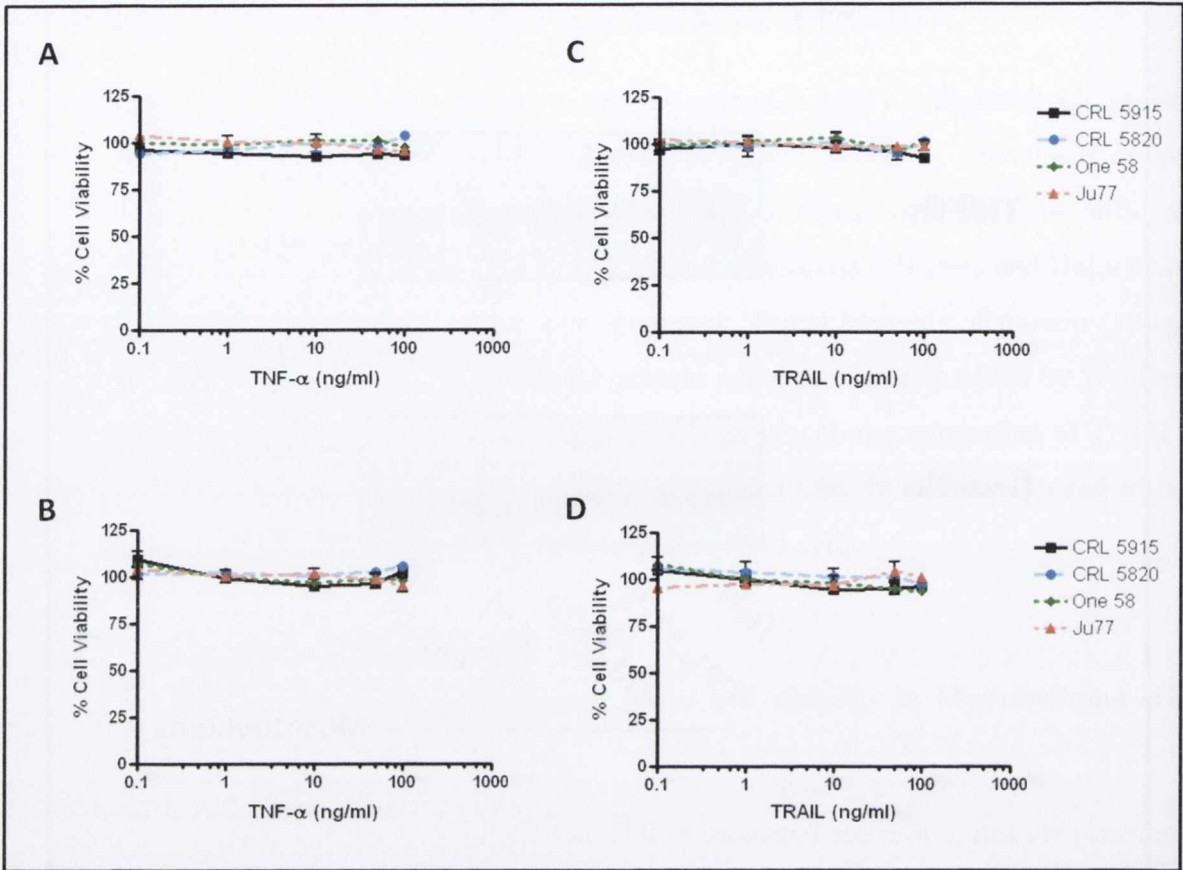


Figure 4.11: TNF- α and TRAIL did not induce cell death in Mesothelioma cell lines.

Mesothelioma cells treated with TNF- α for 24h (A) and 48h (B) or TRAIL for 24h (C) and 48h (D). CRL 5915, CRL 5820, One 58 and Ju77 cells seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200 μ l medium and left to adhere for 18h. Cells were treated with indicated concentrations of TNF- α or TRAIL and left to incubate for 24h or 48h before analysis of cell viability by way of Alamar Blue assay, outlined in Section 2.6. Results are representative of one experiment performed in triplicate.

blue cell viability assay, as outlined in Section 2.6. Results indicated that in all three cell lines, MDG compounds did not sensitise cells to either TNF- α or TRAIL induced apoptosis (Fig 4.12). MDG compound treatment alone also induced no significant increase in cell death. Etoposide treatment induced significant levels of cell death in all three cell lines and in One 58 cells, TNF- α co-treatment induced a significant, synergistic increase in cell death compared to single treatment (Fig 4.12.B). Treatment with TRAIL had no synergistic cytotoxic effect for any co-treatment regime.

4.5.5 Embelin did not synergistically induce cell death in combination with TNF- α in CRL 5915, Ju77 and One 58 cells

Although embelin has not been reported as an inhibitor of cIAPs, it was also investigated in the cIAP targeting functional assay to see if this IAP inhibitor could act through this mechanism, sensitising MPM cells to death receptor mediated apoptosis. CRL 5915, Ju77 and One 58 cells were treated with embelin in a dose-dependent manner alone or in combination with TNF- α [10ng/ml] for 24h and, cell death was analysed by Alamar Blue cell viability assay. Dose-response curves showed no increased cell death with TNF- α co-treatment compared to embelin treatment alone (Fig 4.13). Results indicate that embelin does not sensitise cells to death receptor mediated apoptosis.

4.6 TNF- α induced a protective effect against cytotoxicity in CRL 5820 cells

Although TNF- α can participate in activating caspase-8 dependent cell death mechanisms in cancer cells, its name is a slight misnomer as it also participates in promoting cell survival and proliferation in cancer cells (Silke and Brink, 2010). These protective effects are of particular interest in the study of Mesothelioma as TNF- α is proposed to participate in the aetiology of this neoplasm (Lemaire and Ouellet, 1996, Xie et al., 2000, Mossman and Churg, 1998). As well as a role in mediating cell transformation and tumorigenesis, TNF- α has been shown to be able to elicit a protective effect against induction of cell death in Mesothelioma (Yang et al., 2006), and IAP proteins are implicated in mediating this resistance. A paper by *G.J. Gordon et al* reported that TNF- α induces an increase in mRNA and protein expression levels of cIAP1/2 and, to a lesser degree, XIAP in select MPM cell lines and it is this alteration that produced a protective effect against cell

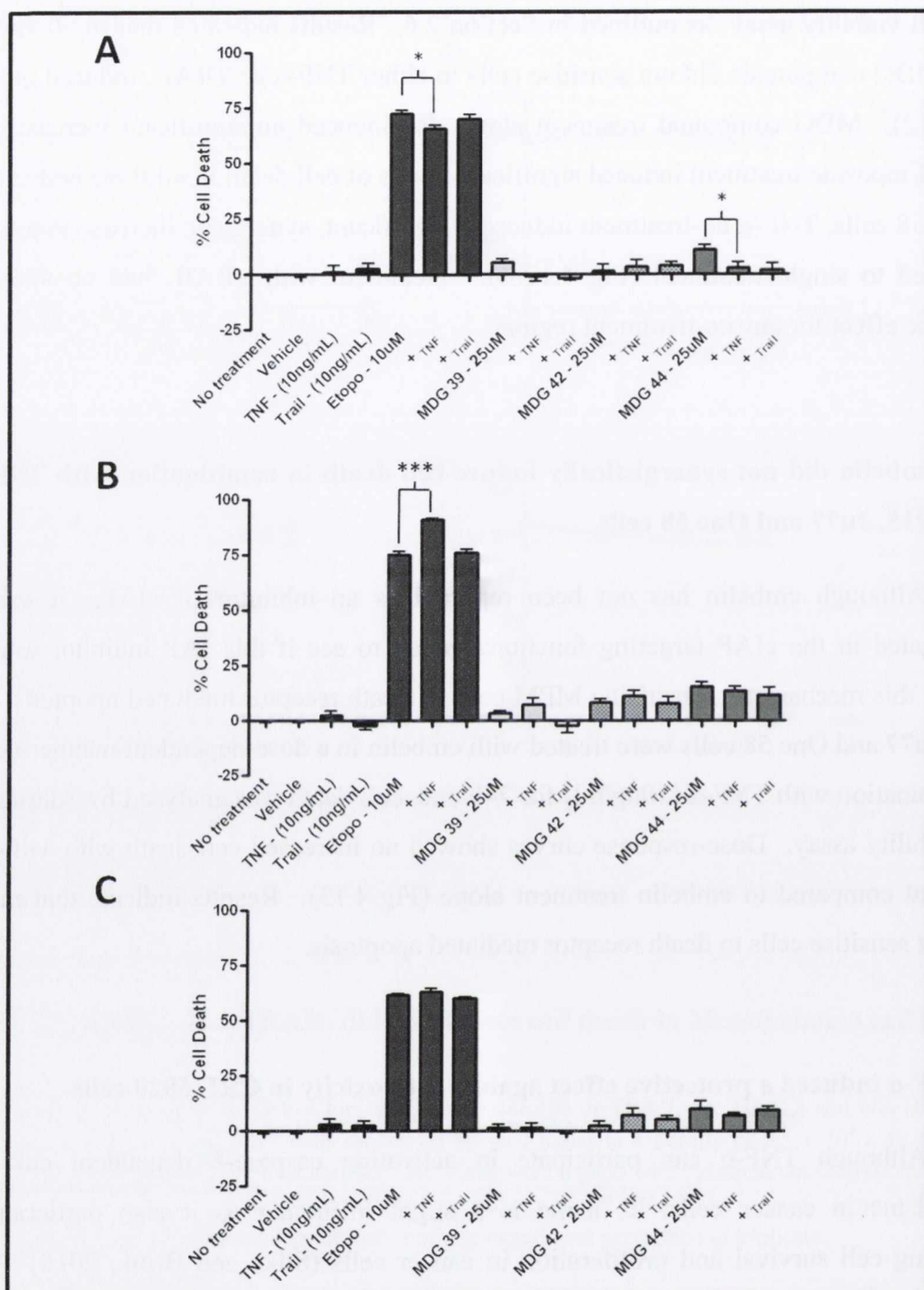


Figure 412: MDG SMAC mimetics did not sensitise CRL 5915, One 58 and Ju77 cell lines to death receptor mediated cell death.

CRL 5915(A), One 58 (B) and Ju77 (C) cells treated with Etoposide, MDG 39, MDG 42 and MDG 44 alone or in combination with TNF- α or TRAIL for 48h. Cells were seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200 μ l medium and left to adhere for 18h. Cells were treated at indicated concentrations and left to incubate for 48h before analysis of cell viability by way of Alamar Blue assay, outlined in Section 2.6. Results are representative of two independent experiments performed in triplicate. Statistical significance tested using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

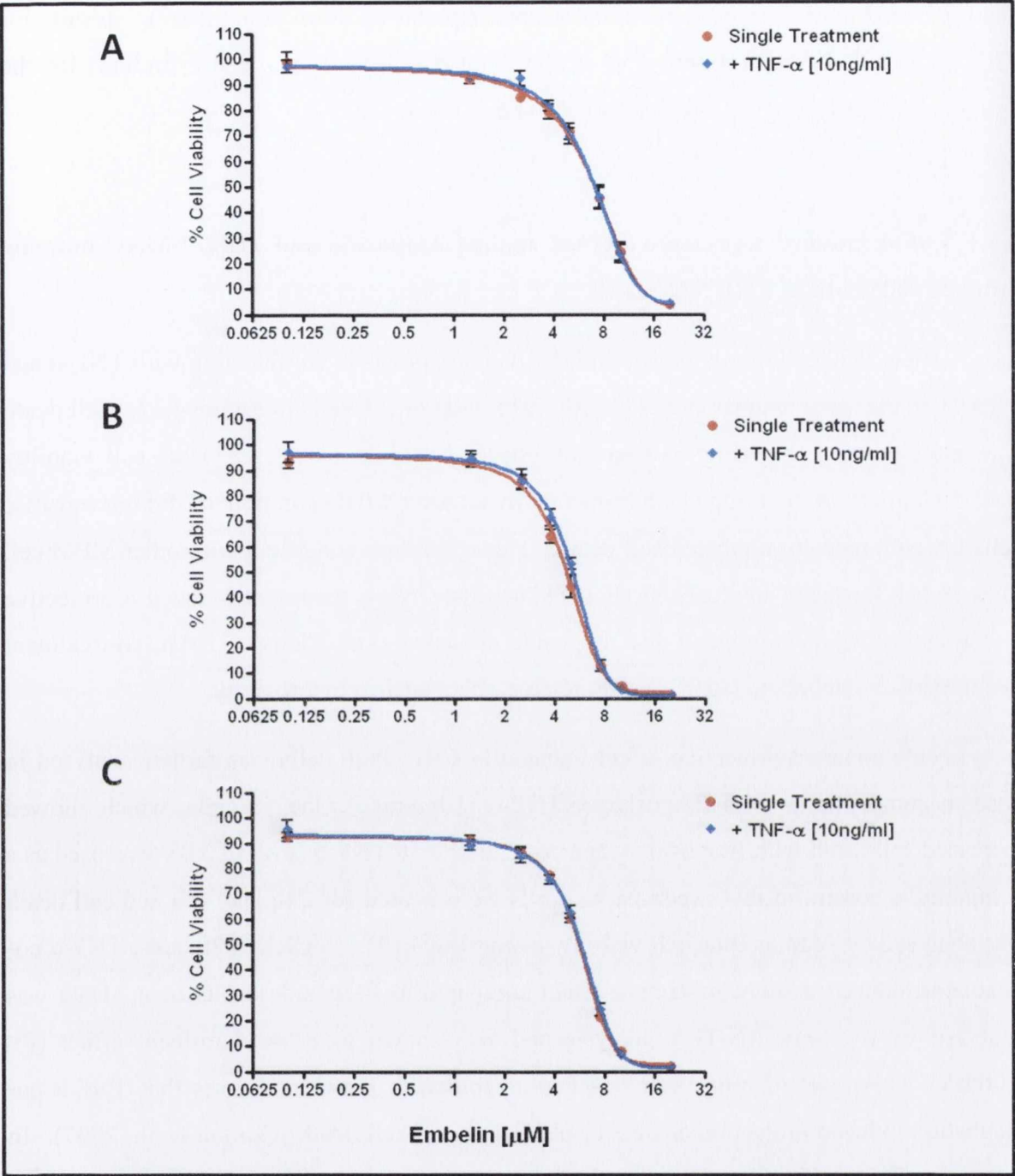


Figure 4.13: Embelin did not sensitise CRL 5915, One 58 and Ju77 cell lines to death receptor mediated cell death.

Embelin treatment alone or in combination with TNF- α [10ng/ml] for 24h in CRL 5915 (A), One 58 (B) and Ju77 (C) cell lines. Cells seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200 μl medium and left to adhere for 18h. Cells were treated at indicated concentrations of Embelin alone or in combination with TNF- α [10ng/ml] and left to incubate for 48h before analysis of cell viability by way of Alamar Blue assay, outlined in Section 2.6. Results are representative of three independent experiments performed in triplicate.

death induced by the intrinsic apoptotic agent, cisplatin (Gordon et al., 2007). Results for CRL 5820 cells co-treated with TNF- α and cytotoxic drugs mirror these findings for the cytoprotective effect of TNF- α in select MPM cell lines.

4.6.1 TNF- α induced a protective effect against Etoposide and MDG SMAC mimetic induced cell death in CRL 5820 cells

CRL 5820 cells were treated with MDG compounds in combination with TNF- α and TRAIL, in the same manner as Ju77, CRL 5915 and One 58 cells in Figure 4.12. Cell death was analysed after 24h (Fig 4.14.A) and 48h (Fig 4.14.B) by Alamar Blue cell viability assay. Contrary to the original experimental hypothesis, MDG compounds did not sensitise cells to death receptor mediated cell death. This result was consistent with other MPM cell lines tested, however, uniquely in CRL 5820 cells, TNF- α treatment induced a protective effect against MDG compound and Etoposide mediated cytotoxicity. TRAIL co-treatment in comparison elicited no significant protective effect against cytotoxicity.

This apparent protective effect induced in CRL 5820 cells was further analysed in dose response curves with Etoposide $\pm$ TNF- α (10ng/ml). One 58 cells, which showed increased cell death with Etoposide when co-treated with TNF- α (Fig 4.12.B), was used as a comparative control in this experiment. Cells were treated for 24h and 48h and cell death was analysed by Alamar Blue cell viability assay (Fig 4.15). In CRL 5820 cells, TNF- α co-treatment induced a slight protective effect compared to Etoposide on its own. Data was analysed by two-way ANOVA analysis and was shown to be a significant effect ($P < 0.0001$). This level of protection was comparable with previous reports that TNF- α co-incubation induced protection against cisplatin induced cell death (Gordon et al., 2007). In comparison, One 58 cells were sensitised to TNF- α induced cell death. Co-treatment of Etoposide with TNF- α induced a significant, dose dependent increase in cell death compared to Etoposide treatment alone. These results indicate that TNF- α treatment in CRL 5820 cells has a protective effect against induction of cell death by cytotoxic agents and this effect is specific to the CRL 5820 cell line.

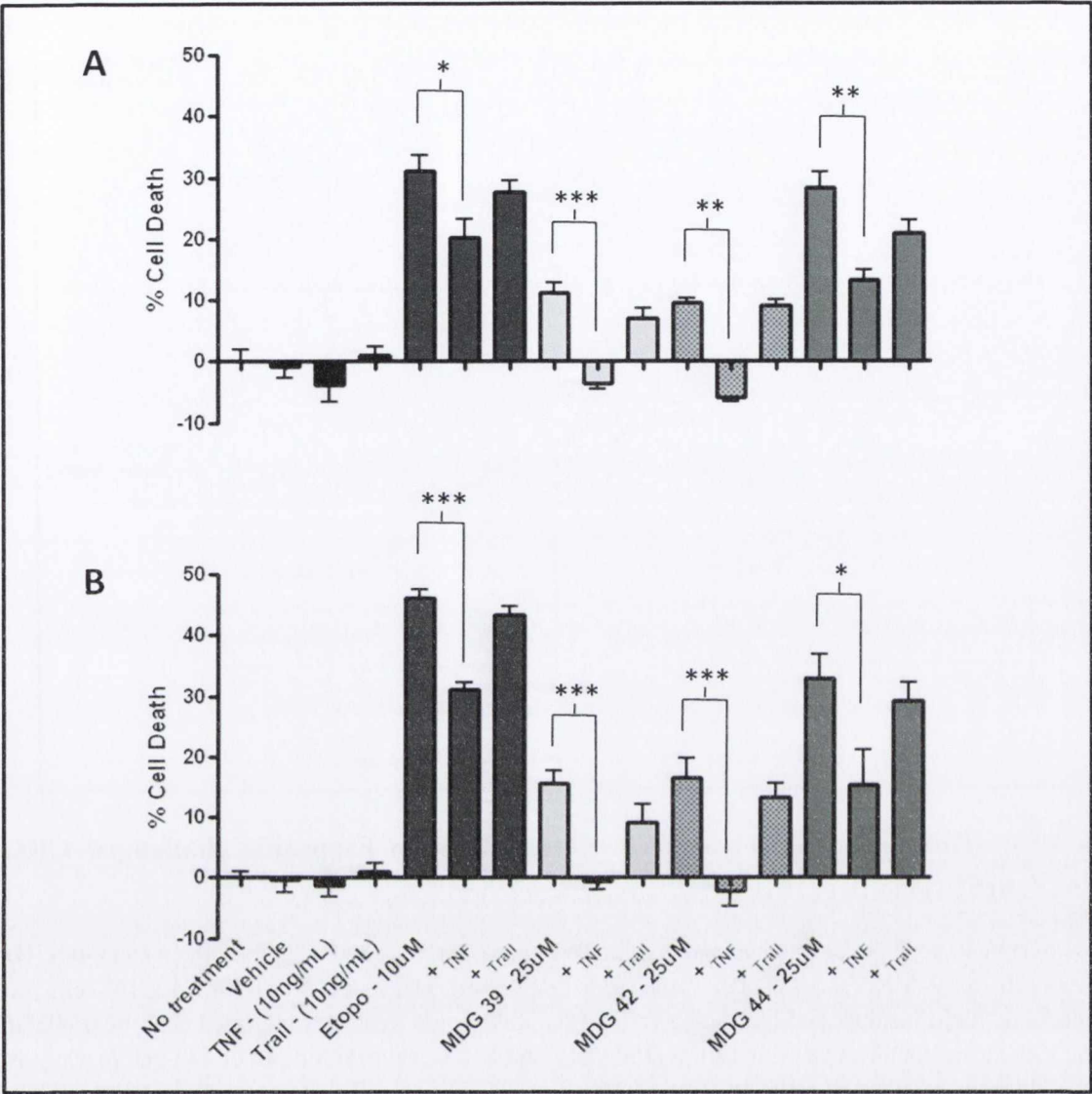


Figure 4.14: TNF- α induced a protective effect against Etoposide and MDG SMAC mimetic induced cell death in CRL 5820 cells.

CRL 5820 cells treated with Etoposide, MDG 39, MDG 42 and MDG 44 alone or in combination with TNF- α or TRAIL for 24h (A) and 48h (B). CRL 5820 cells seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200 μ l medium and left to adhere for 18h. Cells were treated at indicated concentrations and left to incubate for 24h or 48h before analysis of cell viability by way of Alamar Blue assay, outlined in Section 2.6. Results are representative of two independent experiments performed in triplicate. *P<0.05; **P<0.01; ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

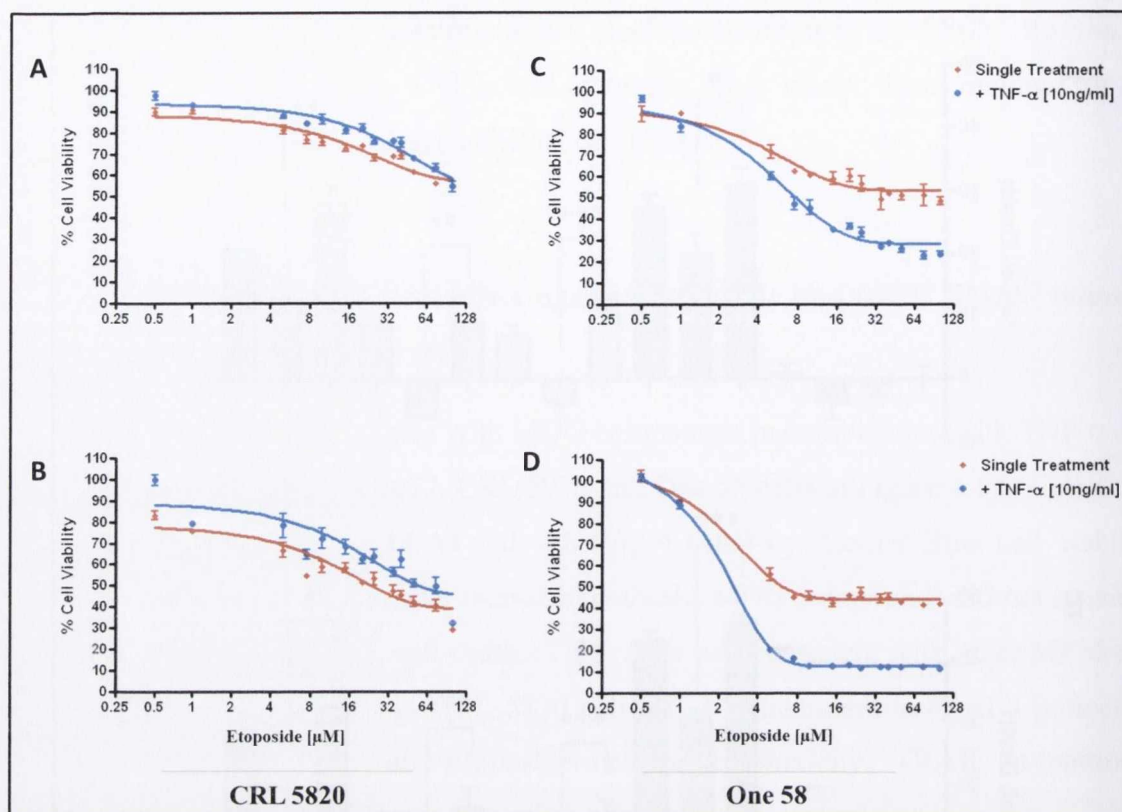


Figure 4.15: Differential effect of TNF- α treatment in Etoposide challenged CRL 5820 and One58 cells

Etoposide treatment alone (♦) or in combination with TNF- α (●) in CRL 5820 cells for 24h (A) and 48h (B) and One 58 cells for 24h (C) and 48h (D). Cells seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200μl medium and left to adhere for 18h. Cells were treated at indicated concentrations of Etoposide alone or in combination with TNF- α [10ng/ml] and left to incubate for 24h or 48h before analysis of cell viability by way of Alamar Blue assay, outlined in Section 2.6. Results are representative of two independent experiments performed in triplicate.

4.6.2 TNF- α induced a protective effect against Embelin induced cell death in CRL 5820 cells.

CRL 5820 cells were treated with embelin in combination with TNF- α , in the same manner as Ju77, CRL 5915 and One 58 cells in Fig 4.13. Cell death was analysed by Alamar Blue cell viability assay after incubation for 24h (Fig 4.16.A) and 48h (Fig 4.16.B). Again, a protective effect was induced with TNF- α co-treatment in CRL 5820 cells. This was, as with MDG compound and Etoposide co-treatments, a unique response in CRL 5820 cells, unlike the identical cell death response seen with embelin $\pm$ TNF- α in Ju77, CRL 5915 and One 58 cell lines. The hypothesised sensitisation of cells to TNF- α mediated apoptosis by cIAP inhibition of embelin did not occur and further suggests that embelin does not work through cIAP inhibition.

4.6.3 Western blot analysis of IAP expression levels in response to TNF- α treatment in MPM cell lines

Having identified that TNF- α treatment can induce a protective effect against induction of cell death specifically in CRL 5820 cells, the possible mechanism by which TNF- α mediated this protection was investigated. Previous studies reported a link between TNF- α and increased IAP expression conferring cisplatin resistance in select MPM cell lines (Gordon et al., 2007) and this mechanism of action was investigated here. CRL 5820 cells were treated with TNF- α (10ng/ml) in a time dependent manner and cIAP-1, cIAP-2 and XIAP protein expression was analysed by Western blot (Fig 4.17.A). One 58 cells were used as a comparative control cell line as these cells were not protected but rather sensitised to TNF- α treatment (Fig 4.17.B).

TNF- α treatment induced an increase in cIAP-2 expression but, contrary to previous reports (Gordon et al., 2007) had no effect on cIAP-1 or XIAP protein expression levels in CRL 5820 cells. A highly significant increase in cIAP-2 is observed after 8h treatment which is sustained at high levels until the final time point of 29h in CRL 5820 cells. Importantly however, similar increases in cIAP-2 expression are also observed with TNF- α treatment in One 58 cells (Fig 4.17.B). This casts doubt over the theory that TNF- α inducing increased expression of IAPs confers resistance in CRL 5820 cells.

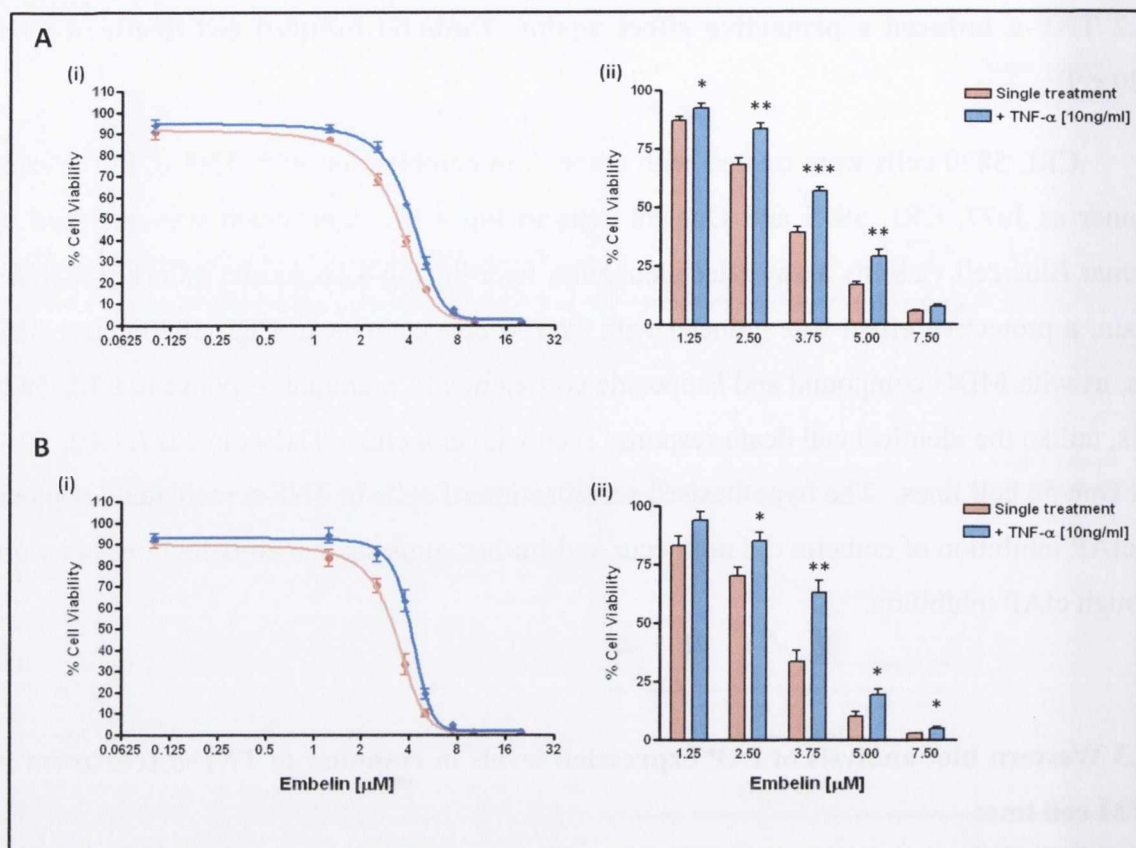


Figure 4.16: TNF- α induced a protective effect against Embelin induced cell death in CRL 5820 cells

CRL 5915 cells were treated with Embelin alone or in combination with TNF- α [10ng/ml] for 24h (A) and 48h (B) [(i) Dose response curve and (ii) Statistical analysis of data]. Cells were seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200 μl medium and left to adhere for 18h. Cells were treated at indicated concentrations of Embelin alone or in combination with TNF- α [10ng/ml] and left to incubate for 48h before analysis of cell viability by way of Alamar Blue assay, outlined in Section 2.6. Results are representative of at least two independent experiments performed in triplicate. *P<0.05; **P<0.01; ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

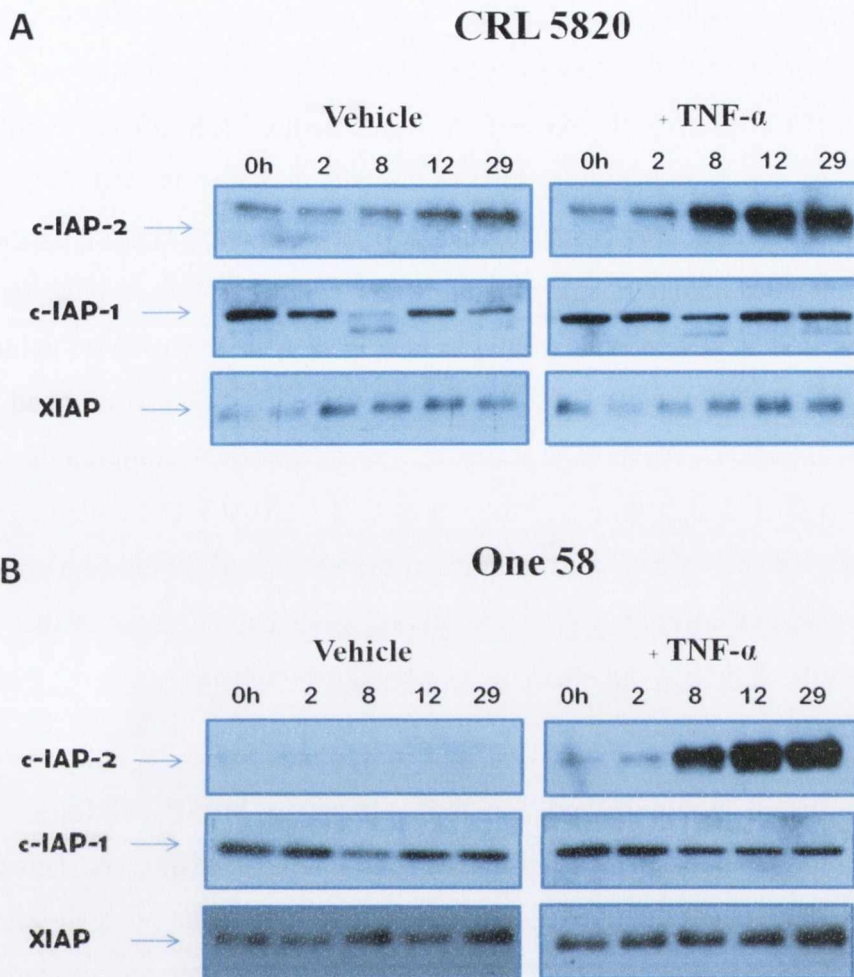


Figure 4.17: TNF- α induced highly significant increases in cIAP-2 expression in CRL 5820 and One 58 cell lines

XIAP, cIAP-1 and cIAP-2 expression in response TNF- α treatment was analysed in CRL 5820 (A) and One 58 (B) cell lines. Cells were seeded at a density of 0.17×10^6 cell / Well (3ml media) on a 96 well plate, and left to adhere for 18h. Cells were treated with Vehicle (PBS + 5% FBS) or TNF- α (10ng/ml) for five time points between 0h and 29h. Whole cell lysates were prepared and 25 μ g of protein was resolved on a 10% SDS-PAGE gel and transferred to PVDF membrane, as outlined in Section 2.5.3 – 2.5.4. Blots were blocked in 5% Marvel TBS-Tween, probed with anti-cIAP-2 (Primary Ab – 1 in 750) ; anti-cIAP-1 (Primary Ab – 1 in 1,000) and anti-XIAP (Primary Ab – 1 in 1,250), as outlined in Section 2.5.5. Results are representative of two independent experiments.

Discussion

Results in this Chapter reveal that computationally designed SMAC mimetics did not bind to XIAP or cIAP-1/2. Fluorescence polarisation results showed no competitive binding to XIAP-BIR3 of any of the series of novel compounds. Similarly in cIAP-1/2 “on target” experiments, compounds did not bind to the BIR domains of cIAP-1/2 as indicated by no induced proteosomal degradation of the proteins. In fact, cIAP-1 expression was increased in a time dependent manner in all MPM cell lines in response to MDG 44 treatment. SMAC and small-molecule mimetics of SMAC have been shown to induce rapid degradation of cIAP-1/2 in cells, not seen with the MDG test compound treatment (Varfolomeev et al., 2007, Vince et al., 2007). As these novel compounds showed no affinity for the original design target of the Caspase-9 / SMAC-AVPI binding pocket of XIAP-BIR3, or for the secondary putative target of the SMAC-AVPI binding pockets of the BIR2 and BIR3 domains of cIAP-1/2, the results of this Chapter suggests that MDG test compounds elicit effects through an alternate target and mechanism.

While compounds did not bind IAPs, lead compounds MDG 39, MDG 42 and MDG 44 did have significant cytotoxic and apoptotic effects in MPM cell lines. All three compounds were able to induce cell death in the high IAP expressing, resistant CRL 5820 cells, with this cell line being the most responsive to treatment. The most significant result was the ability of MDG 42 and MDG 44 to synergistically increase cisplatin induced apoptosis in two of the four MPM cell lines – CRL 5915 and One 58. The synergistic response in MPM did not correlate with the XIAP expression profile of cells. Sensitivity to MDG compound induced synergism with cisplatin showed no relation to XIAP protein levels in MPM cells, contrary to what was observed in embelin and cisplatin co-treatment experiments in Chapter 3. This was a further indication of XIAP not being a target for these compounds. MDG 44 was the most potent synergistic lead compound, inducing no apoptosis alone, but in combination with cisplatin, approximately doubling cisplatin induced apoptosis in CRL 5915 cells. The cellular effects of lead compound MDG 44 and MDG 42 and their synergistic capacity in combination with established chemotherapy in MPM was a result believed to be worthy of further analysis in the next Chapter.

The “on target” results for the novel computationally designed small molecule SMAC mimetic were disappointing as the pharmacophore failed to predict any positive IAP inhibiting compounds. MPM remains a very attractive model for the study of IAP

resistance and SMAC mimetic design. Numerous studies implicate increased IAP expression and TNFR signalling as a factor in MPM tumorigenesis and chemotherapeutic resistance. The results in Chapter 3 confirm the role of IAP expression in resistance in MPM and the ability of embelin to re-sensitise cells to chemotherapy indicates the promise of SMAC mimetic therapy as possible treatment strategy in this neoplasm. XIAP targeting drugs, like embelin, can directly relieve XIAP mediated resistance in MPM (Chapter 3). Targeting of cIAP-1/2 can potentiate death receptor mediated apoptotic signalling (Bockbrader et al., 2005, Li et al., 2004, Vince et al., 2007), synergise with genotoxic drugs, like cisplatin (Probst et al., 2010), and potentially subvert TNF- α mediated protection of MPM (Yang et al., 2006, Gordon et al., 2007). MDG compound design was based on a pharmacophore developed from the AVPI N-terminal tetrapeptide of SMAC. SMAC-AVPI represents a very amenable target for the design of small molecule mimetics and has been used in numerous studies for the computational design of novel SMAC mimetics (Crisostomo et al., 2009, Sun et al., 2004, Oost et al., 2004, Seneci et al., 2009). Recent publications on novel SMAC compounds have reported increased potency with the design of bivalent compounds – two SMAC mimetics linked together by a bridging complex (Varfolomeev et al., 2007). Such compounds have been shown to better mimic the dimer structure of native SMAC and, like SMAC, are able to bind both BIR2 and BIR3 domains of the same IAP protein (Cossu et al., 2009). Bivalency allows the simultaneous competitive inhibition of active caspase-3/7 (BIR2) and active caspase-9 (BIR3) binding to XIAP and single compound binding to BIR2 and 3 of cIAP-1/2, allowing potent and efficient induction of autoubiquitination and subsequent proteosomal degradation. For the future design of novel SMAC mimetics, analysis and redesign of the basic pharmacophore is required to develop true IAP targeting compounds. Small molecule design still has a greater therapeutic potential over peptides, interfering RNA and peptidic molecules in SMAC mimetic rational drug design due to fewer efficacy, delivery and pharmacokinetic limitations. Design of bivalent molecules has been shown to be a better strategy for getting more potent and effective molecules and should be employed in future development.

Another interesting result from work in this Chapter was the protective effect induced by TNF- α in CRL 5820 cells. In line with results that MDG compounds do not target cIAP-1/2, no sensitisation of cells to death receptor signalling was observed in any of the four MPM cell lines. Conversely, a protective effect against cytotoxicity from etoposide, embelin and MDG compound treatment was mediated by TNF- α co-treatment in

CRL 5820 cells. A link between TNF- α induced increase in IAP expression and resistance in susceptible MPM cell lines has been recently reported (Gordon et al., 2007) and was investigated with regard to TNF- α results in CRL 5820 cells. Highly significant increases specifically in cIAP-2 expression, not XIAP or cIAP-1, was observed in response to TNF- α treatment. However, a similar increase in cIAP-2 expression was observed in One 58 cells, a cell line not protected by TNF- α co-treatment.

In the original reference article (Gordon et al., 2007), TNF- α induced significant increase in mRNA translation and protein expression of cIAP-1 and cIAP-2 and, to a lesser degree, XIAP, which conferred resistance against cisplatin induced cell death. In other articles, increased IAP expression, including cIAP-2, has also been implicated in blocking apoptosis in an induced cisplatin resistant MPM cell line (Janson et al., 2010) and cIAP-2 was believed to be specifically responsible for cellular resistance to SMAC mimetic therapy (Petersen et al., 2010). In the later study, co-treatment of SMAC mimetic and TNF- α initially induces rapid degradation of cIAP-2 but, unlike cIAP-1, TNF- α drives recovery and increased cIAP-2 protein expression levels, a response in cells that correlates with a resistant phenotype to SMAC mimetics. While results indicate TNF- α induced cIAP-2 expression could possibly mediate resistance, its mechanism of inhibiting intrinsic apoptotic induction (e.g. etoposide, cisplatin, embelin, etc) is poorly defined. Recent publications report that, contrary to previous predictions (Eckelman et al., 2006), cIAPs can directly affect caspase activation and stability. The cIAP-BIR2 & BIR-3 domains (cIAP-1; cIAP-2 not used in experimentation) can directly bind effector caspases in intermediate activation states and induce ubiquitination and degradation of caspase-3/7 (Choi et al., 2009), while another study reports that the same cIAP domains are essential for preventing effector caspase activation via binding and inhibiting the apoptosome (Burke et al., 2010). Defining the role of TNF- α inducing increased cIAP-2 expression and mediating apoptotic resistance in CRL 5820 cells is speculative at best, due to the similar response in One 58 cells. Further study into this phenomenon would be required and may be outside the scope of this study.

In the next Chapter, the apoptotic potential of lead compound MDG 44 will be investigated in MPM cell lines. While MDG 44 and its series of compounds have been proven not to bind IAPs, its ability to synergise with cisplatin in susceptible cell lines was a result of particular interest. Cisplatin is the primary clinical option for chemotherapeutic treatment of MPM in advanced, unresectable stage III and IV disease (Bertino et al., 2009). The current FDA approved therapy employs combinational treatment regimens of cisplatin

with the anti-folate compound, pemetrexed. In a first and largest clinical trial on this regime, co-treatment of cisplatin / pemetrexed significantly increased median progression times, median survival times, and response rates compared to cisplatin alone (Vogelzang et al., 2003). However, the increase in median survival time was just 2.8 months with combinational treatment (12.1 vs. 9.3) and overall patient response rates were less than 50%, indicating the necessity for further improvement in MPM therapy. While the target for MDG 44 was unknown, its ability to cause cell death in resistant MPM and synergise with cisplatin treatment is a result of possible clinical significance and deemed worthy for further study.

Investigations in Chapter 5 looked to examine the mechanism of PCD induced by MDG 44, hoping to confirm a regulated programme of cellular disassembly and potentially shed light on possible cellular targets for MDG 44. Classical indicators of an apoptotic mechanism of PCD were examined including caspase dependence, PARP cleavage and effects on cellular morphology.

Chapter 5

***Investigating the mechanism of MDG 44 induced cell death in Malignant
Pleural Mesothelioma***

Introduction

From investigations in Chapter 3 and 4, MDG 44 and MDG 42 have been identified as lead compounds from a series of novel, computationally-designed SMAC mimetic compounds. “On target” analysis of the computationally-designed, SMAC mimetics revealed these compounds did not bind to their proposed sites on XIAP and cIAP-1/2, but these lead compounds did elicit significant cytotoxic effects when screened in MPM cell lines, independent of IAP targeting. MDG 44 and MDG 42 were able to induce single agent cell death and synergistically increase cisplatin induced apoptosis at low micro-molar concentrations in two out of four MPM cell lines tested. While the initial goal of the study was to develop novel SMAC mimetic therapies for the treatment of MPM, a cancer model shown to be IAP resistant and sensitive to SMAC mimetic therapy (Chapter 3), the efficacy of these drugs observed in MPM was deemed significant enough to be taken forward for further analysis. Of particular interest was the capacity of MDG 44 and MDG 42 to combine with Cisplatin synergistically in combinational treatment regimens; Cisplatin being part of the prescribed clinical treatment regimen for MPM patients – Cisplatin in combination with anti-folate therapy (pemetrexed) (Belli et al., 2009). In Chapter 4, experimentation produced preliminary results with regard to the single agent and synergistic capacity of MDG compounds to induce and enhance an apoptotic form of PCD in MPM. In the current Chapter, the most potent compound of the series, MDG 44, was taken forward for comprehensive analysis of its cellular potency in MPM and investigation into the mechanisms by which it induces PCD.

Programmed cell death (PCD) refers to the final fate of the cell occurring through an orchestrated disassembly of cellular components, which allows the recycling of base material and preventing chaotic lysis, toxicity to adjacent tissue and induction of an inflammatory response (Edinger and Thompson, 2004). A number of types and pathways of PCD exist including classical type I apoptosis (extrinsic, intrinsic, caspase-independent), and type II autophagy (“Self-eating” of the cell), differentiated by the conserved protein pathways contributing in their induction and the distinct morphological features in their execution (Yang and Klionsky, 2010). Recently, necrotic-like cell death pathways have been identified as non-passive processes; an organised, alternate form of cell death in which induction of an inflammatory response is beneficial (Edinger and Thompson, 2004). Within these programmes of cell death much cellular crosstalk can occur between them when responding to diverse stimuli (Zhivotovsky and Orrenius, 2010). Induction of classical PCD

processes, like apoptosis, is dependent on expression of the required cellular machinery to carry them out and the type of cellular stress, imbalance or insult that is induced. For cancer drug development, engagement of a classical programmed form of cell death is preferred to aid cancer specificity and reduce toxicity to normal tissue.

The cellular machinery required for induction of an apoptotic PCD response includes abundant ATP levels and a functional network of apoptosis proteins. These networks of proteins regulate, initiate and execute the apoptotic programme and include the death-receptor, IAP, Bcl-2 and caspase protein families. The death-domain receptor family and related accessory proteins mediate extrinsic activation of apoptosis via death receptor ligation (TNF- α , TRAIL, FasL) (Schneider and Tschopp, 2000). Bcl-2 family proteins are both regulators and initiators of the intrinsic / mitochondrial apoptotic pathway with pro-apoptotic inducers considered essential for induction of apoptosis (Buytaert et al., 2006). Bcl-2 proteins are activated by genotoxic agents or cellular stress, mediating MOMP and release of apoptotic activators. Intrinsic and extrinsic stimuli converge at the activation of caspases; the central executioners of the apoptotic programme (Shiozaki and Shi, 2004). Extrinsic stimulus drives initiator caspase-8 activation while intrinsic apoptotic stimulus is mediated by initiator caspase-9 (Jin and El-Deiry, 2005). Both initiator caspases activate executioner caspases-3/7 which implements the proteolytic activation of numerous proteins necessary to execute apoptosis. Caspase-3/7 participate in the cleavage of nuclear protein poly-(ADP-ribose) polymerase (PARP) and proteolytic activation of endonucleases, including DFF40/CAD (DNA fragmentation factor 40 / caspase-activated DNase), which induce DNA double strand breaks and DNA fragmentation characteristic of apoptosis (Widlak and Garrard, 2005, Duriez and Shah, 1997). No step in the apoptotic programme is known to be truly committal to apoptotic execution, however, caspase dependence and activation in response to drug treatment is considered a strong indication of induction of a classical apoptotic pathway.

The activation of these protein cascades culminate in execution of the PCD programme and specific changes in cellular morphology. Idiosyncratic morphological changes occur in a stepwise manner during execution of apoptosis, starting with chromatin condensation (pyknosis) and followed by cell shrinkage, plasma membrane blebbing, DNA fragmentation (karyorrhexis) and formation of apoptotic bodies (Hacker, 2000). Alternate morphological features are also indicative of a Type II, autophagic form of cell death. The

formation of double membraned, cytoplasmic vesicles, termed autophagosomes, is an indication of autophagy (Yang and Klionsky, 2010).

Formation of autophagosomes in autophagy, constitutes a survival response to a multitude of cellular stresses including, hypoxia, growth factor deprivation and misfolded protein aggregate (aggresome) formation (Kroemer et al., 2010). Autophagosomes sequester cytosolic components into a membrane bound vesicle and fuse with lysosomes, forming autolysosomes, which facilitates the degradation of trafficked material (Yang and Klionsky, 2010). Autophagosomes can non-specifically take up and process cytoplasmic proteins, lipids and organelles to attempt to maintain cellular energy and nutrient homeostasis during times of nutrient deprivation (Macroautophagy) or specifically sequester and degrade defected / misfolded protein aggregates to prevent damage to the cell (Chaperone-mediated Autophagy). Prolonged cellular stress, coupled with excessive autophagic flux, culminates in cell death – Type II autophagic PCD – as the cell is overwhelmed by perpetual nutrient cycling and is said to digest itself through autophagy (“self-eating”) (Mizushima et al., 2008).

The wide range of mechanisms and cellular stresses by which chemotherapeutic drugs can induce PCD (in particular apoptosis) in MPM cells, was of interest in this Chapter. Activation of the extrinsic apoptotic pathway is primarily mediated through the ligation of death-domain receptors, while intrinsic pathway activation can occur in response to a wide variety of stimulus. With conventional chemotherapy, intrinsic apoptotic induction requires drug internalisation and binding to its specific cellular target, which triggers a form of cellular stress that activates apoptosis. Cellular stress mechanisms that culminate into induction of an apoptotic response include DNA damage, nutrient / growth factor deprivation, ER stress / unfolded protein response (UPR), cell cycle / mitotic arrest and oxidative stress (Creagh and Martin, 2003). Examples of this include the chemotherapeutic agent cisplatin which binds to DNA forming cross-linked adducts, damaging DNA and inhibiting replication. Detection of damage by DNA repair mechanisms and p53 induce an intrinsic apoptotic response (Basu and Krishnamurthy, 2010). The genotoxic agent doxorubicin, as well as intercalating between nucleic acid bases, induces the generation of excess reactive oxygen species (ROS), both activating pathways of apoptotic induction (Fu et al., 2010). ROS are oxygen radicals (H_2O_2 ; $OH^\bullet$) generated by the partial reduction of O_2 in the electron transport chain. A delicate balance exists between ROS generation and the cells detoxification mechanisms of glutathione,

catalase and superoxide dismutase. Excess ROS generation can cause damage to DNA, proteins and lipids, causing cell cycle arrest and apoptosis (Ott et al., 2007).

Cell cycle arrest in response to drug treatment is another cellular effect indicative of a particular class of drug, affecting specific cellular targets. A well-defined class of arresting drugs include MTAs, which cause arrest in the G2/M phase of the cell cycle due to disruption of microtubule polymerisation and depolymerisation dynamics, preventing mitotic spindle assembly and cell division (Wilson and Jordan, 2004). S phase arrest can be induced by drugs that inhibit DNA replication, cause DNA damage (cisplatin) (Jain et al., 2010) or affect cyclin or cyclin dependent kinase (Cdk) expression (resveratrol) (Joe et al., 2002). Regulation of expression levels and timing of Cyclin/Cdk complexes can also contribute to a blockade in G1-S phase transition, causing G1 phase arrest. Histone deacetylase inhibitors, proteasome inhibitors and molecular chaperone inhibitors can alter transcriptional expression, degradation and stability of G1-S phase transition Cyclin / Cdk complexes (Cyclin D1/Cdk4) and regulation proteins (p27) to induce G1 arrest (Owa et al., 2001, Toyoshima and Hunter, 1994).

Experimentation in Chapter 5 looked to investigate the potency of lead compound MDG 44 and confirm induction of a type I, apoptotic form of PCD within MPM cells. Apoptotic induction was monitored by PI cell cycle analysis, detection of classical morphological features indicative of apoptosis (chromatin condensation, DNA fragmentation) and evidence for caspase activation and dependence in response to treatment. Alternate assays for apoptotic analysis were performed including Annexin V staining and detection of DNA damage by single-cell gel electrophoresis (Comet assay). These experiments were performed to classify both the single-agent apoptotic capacity of MDG 44 and its synergistic potential in enhancing Cisplatin induced apoptosis in MPM.

Experiments were also performed to examine the mechanism or cellular stress pathway by which lead compound MDG 44 induces PCD. The CRL 5915 MPM cancer model was used to profile the cellular effects induced in cells in response to MDG 44 treatment. Experiments were performed which investigated the contribution of extrinsic (caspase-8) and intrinsic (caspase-9) apoptotic pathway signalling, oxidative stress (ROS), cell cycle arrest and alterations in cellular morphology in response to MDG 44 treatment in MPM. As MDG compounds were shown not to bind to their computationally predicted target of IAP family proteins, a secondary aim of these experiments was to give insight into

putative cellular targets through which MDG lead compounds may induce and synergistically enhance apoptosis in select MPM cell lines. Target identification would provide a model framework for further analysis of MDG 44 anti-cancer activity and inform their possible efficacy as a therapeutic in MPM.

Results

5.1 MPM cell lines expressed abundant levels of pro-apoptotic Bcl-2 family proteins

To investigate the mechanism of PCD employed by MDG 44, it was first necessary to confirm the capacity of MPM cell lines to induce apoptosis. Bcl-2 family proteins consist of both pro- and anti-apoptotic regulators and inducers of the intrinsic apoptotic pathway. This protein family control mitochondrial outer membrane permeabilisation and the release of pro-apoptotic factors (cyto c; SMAC, HtrA2) which drive apoptosome formation and caspase-9 mediated cell death (Cory and Adams, 2002). Oligomerisation of pro-apoptotic Bax and Bak proteins in the MOM in response to intrinsic apoptotic stimulus allows release of apoptotic inducers and expression of Bax and Bak proteins are believed to be essential for induction of the intrinsic apoptosis pathway in many cell types (Buytaert et al., 2006). Protein expression of Bax and Bak were investigated in all four MPM cell lines. All four lines showed abundant basal expression levels of both Bax (Fig 5.1.A) and Bak (Fig 5.1.B). Differential expression was observed between cell lines – decreased Bax in CRL 5820 cell and decreased Bak in CRL 5915 cells – but, all showed detectable levels indicating a capacity for induction of apoptosis.

Bcl-2 expression was also investigated in MPM and HeLa cell lines. Bcl-2 is an anti-apoptotic regulator of Bax and Bak, forming heterodimers with these proteins, preventing oligomerisation and mitochondrial membrane permeabilisation. Bcl-2 null cells have been shown to have deficient regulation of apoptotic induction (Cory and Adams, 2002). HeLa cells and MPM cell lines all expressed abundant levels of Bcl-2 protein (Fig 5.1.C), indicating the competent regulatory capacity of apoptosis in cells.

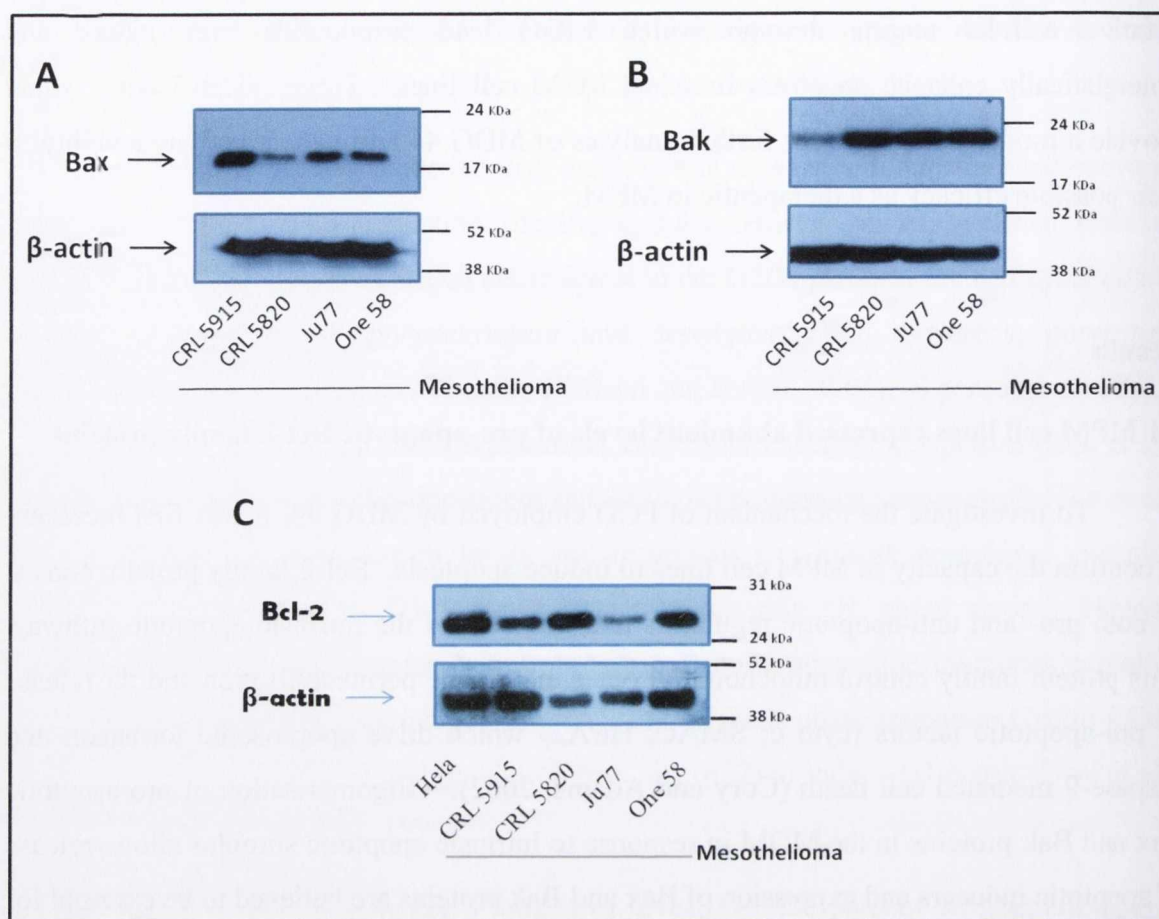


Figure 5.1 Pro-apoptotic and anti-apoptotic Bcl-2 family protein expression in Mesothelioma cell lines.

CRL 5915, CRL 5820, Ju77 and One 58 cell lines protein expression of pro-apoptotic Bax (**A**) and Bak (**B**); HeLa and MPM cell line protein expression of anti-apoptotic Bcl-2 (**C**). Sub-confluent cultures of each cell line were taken for preparation of whole cell lysates. (**A**) Proteins (20μg) were resolved on 12% SDS-PAGE gel and transferred to PVDF membrane as outlined in Section 2.5. Blots were blocked in 5% Marvel TBS-Tween and probed with anti-Bax. (**B**) Proteins (20μg) were resolved on 10% SDS-PAGE gel and transferred to PVDF membrane. Blots were blocked in 5% Marvel TES-Tween and probed with anti-Bak. (**C**) Proteins (25μg) were resolved on 12% SDS-PAGE gel and transferred to PVDF membrane. Blots were blocked in 5% Marvel TBS-Tween and probed with anti-Bcl-2. All experiments were re-probed with anti-β-actin as a loading control. Results are representative of three independent experiments.

5.2 Characterisation of apoptotic response induced by MDG 44 in MPM cell lines

The lead compound of the computationally designed series, MDG 44, has been shown to induce cytotoxicity and synergistically induce apoptosis in MPM cell lines. The ability and potency of MDG 44 to induce apoptosis as a single agent in MPM cell lines was examined by PI cell-cycle flow cytometry analysis. In order to further confirm induction of an apoptotic program of cell death by MDG 44, PARP cleavage analysis was performed on MDG 44 single agent treatments. Poly-(ADP-ribose) polymerase, or PARP, is a nuclear enzyme which catalyses the poly-(ADP)-ribosylation of nuclear proteins (Boulares et al., 1999). PARP is known to participate in apoptosis through binding to DNA strand breaks occurring during the apoptotic programme and deplete cells of substrates NAD^+ and ATP through hyperactive poly-(ADP)-ribosylation (Berger, 1985). PARP is also a proteolytic substrate of caspase-3, and its cleavage from an 116kDa full length protein to 89 kDa and 27 kDa fractions was used as an indication of caspase dependent apoptosis.

5.2.1 MDG 44 single agent treatment induced pre -G1 apoptosis in MPM cells in a dose dependent manner

All four MPM cell lines were treated with increasing concentrations of MDG 44, up to a concentration of 50 μM , and left to incubate for 24h (Fig 5.2). Cells were fixed in 70% Ethanol, followed by PI cell cycle analysis, as outlined in Section 2.7. In all four cell lines single agent treatment of MDG 44 induced significant levels of apoptosis in the range of 30 μM - 50 μM . CRL 5915 and Ju77 cells were the most susceptible cell lines to MDG 44 apoptotic induction, inducing 58.9% ($\pm$ 2.6) and 41.4% ($\pm$ 1.9) apoptosis and 40 μM incubations respectively.

5.2.2 MDG 44 single agent treatment induced PARP cleavage in MPM cells in a dose dependent manner

Similarly to the PI flow cytometry apoptotic analysis, PARP cleavage was investigated in response to MDG 44 dose-response treatment in all four MPM cell lines. Cells were treated with various concentrations of MDG 44 and incubated for 12h. At the experimental endpoint, whole cell lysates were prepared and PARP cleavage was assessed by Western blot analysis, as outlined in Section 2.5. Full length (116 kDa) and the 89 kDa

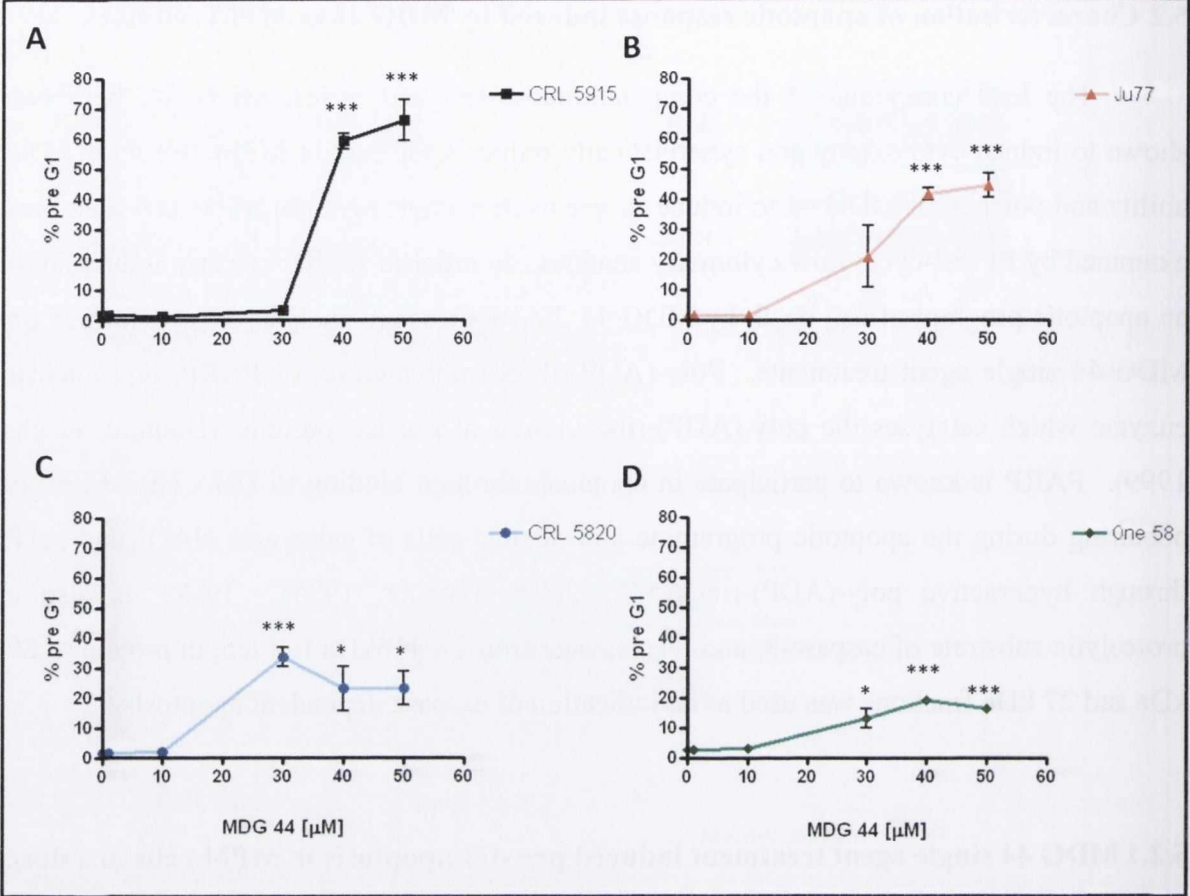


Figure 5.2: Single agent MDG 44 treatment induced apoptosis in Mesothelioma cell lines.

Mesothelioma cell lines, CRL 5915 (A), Ju77 (B), CRL 5820 (C) and One 58 (D) treated with MDG 44 in a dose dependent manner for 24h. Cells were seeded at a density of 0.25×10^6 cells/5ml. After 24h incubation, cells were harvested and fixed in 70% Ethanol. Cell cycle flow cytometry analysis was performed, as outlined in Section 2.7. Values are representative of mean $\pm$ SEM of three independent experiments. * $P < 0.05$; ** $P < 0.01$ *** $P < 0.001$ (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

cleaved fragment of PARP were detected using an anti-PARP antibody. In all four MPM cell lines a cleaved 89 kDa fragment was observed with MDG44 treatment of 40 - 50 μ M (Fig 5.3). This result further confirmed the ability of MDG 44 to specifically induce apoptosis in MPM cell lines.

5.3 Investigation into the time-dependence and potency of MDG 44 treatment in the CRL 5915 MPM cell line

Further analysis of the apoptotic effects of MDG 44 was performed in a single cell line. The CRL 5915 cell line was chosen as a test model for MDG 44 due to its greater sensitivity to single agent and co-treatment induced cell death, compared to other MPM cell lines. The apoptotic and mechanistic pathways through which MDG 44 elicits its cellular effects were profiled in this MPM cancer cell model. Initial experiments investigated dose and time dependent effects of MDG 44 treatment in CRL 5915 cells. These experiments looked to define drug potency, cell viability IC_{50} values and the time scale for induction of apoptosis in cells. The IC_{50} value of a drug refers to the concentration at which drug induces 50% cell death in a particular cell line and is used to define potency of a compound, an important question with regard to its efficacy as a therapy.

5.3.1 MDG 44 induced dose and time dependent cell death in CRL 5915 cells

Dose response curves of MDG 44 treatment were performed in CRL 5915 cells and cell viability was analysed by Alamar blue assay, as outlined in Section 2.6. Cells were treated with various concentrations of MDG 44, up to a concentration of 50 μ M, for 24h and 48h incubations (Fig 5.4.A). Results indicate that at both time points MDG 44 treatment induces a dose dependent decrease in cell viability in CRL 5915 cells. After 24h incubation, MDG 44 treatment had an IC_{50} value of 33.6 μ M (± 0.5). Similar potency was observed with longer treatment, with a lower EC_{50} value of 31.3 μ M (± 0.6) after 48h treatment. Results show that MDG 44 was a compound with low micromolar efficacy in a susceptible MPM cell line.

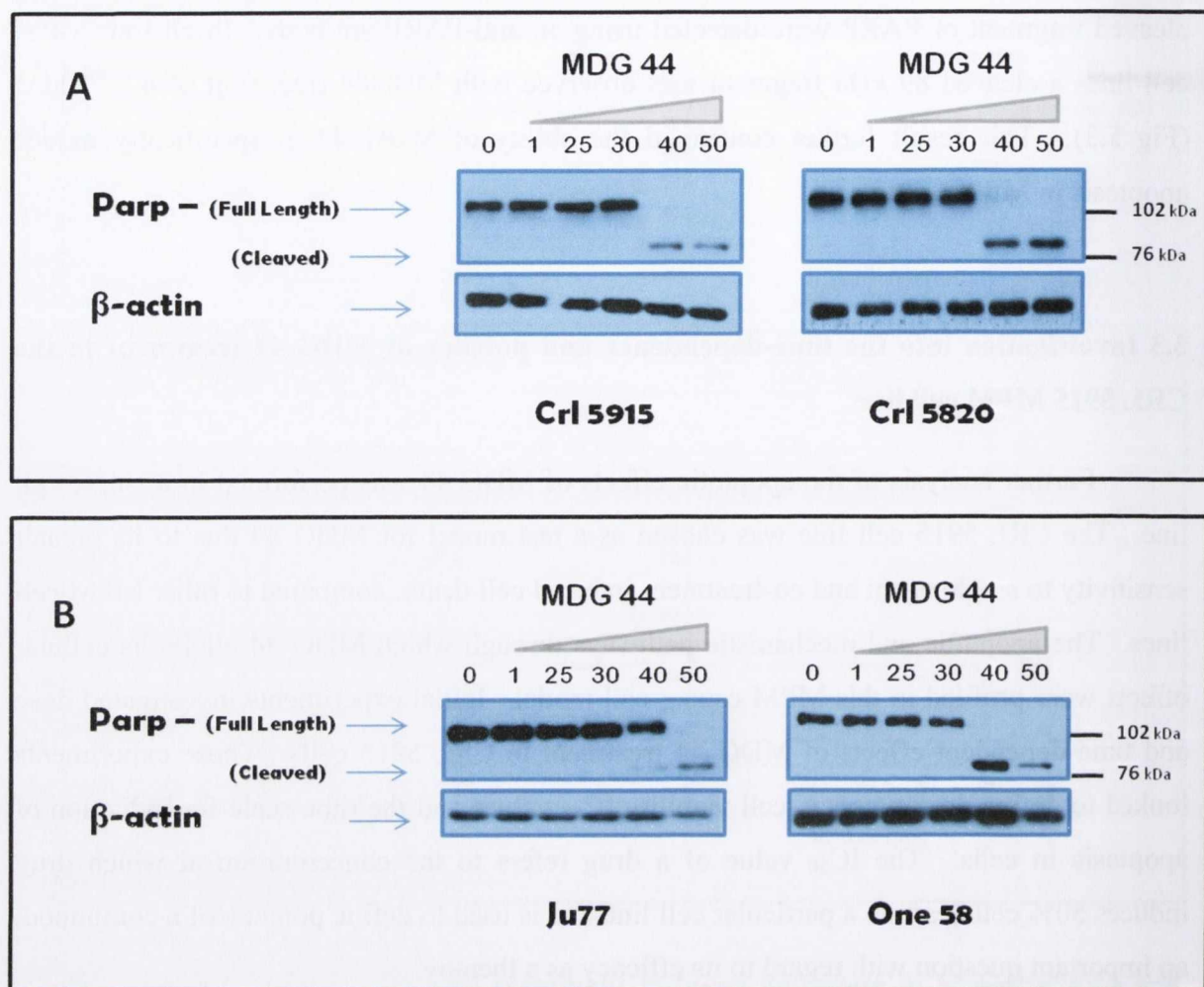


Figure 5.3: Single agent MDG 44 treatment induced PARP cleavage in Mesothelioma cell lines.

Mesothelioma cell lines, CRL 5915 & CRL 5820 (**A**) and Ju77 & One 58 (**B**) treated with vehicle (0 μ M) or MDG 44 (1 - 50 μ M) for 12h. Cells were seeded at a density of 0.5×10^6 cells/5ml. After 12h treatment with MDG 44, cells were harvested and lysed, on ice, for 1h, in 1 x Ripa Buffer containing DTT and protease inhibitor cocktail. Protein (30 μ g) was resolved on 8% SDS-PAGE gel and transferred to PVDF membrane as outlined in Section 2.5. Blots were blocked in 5% Marvel TBS-Tween, probed with anti-PARP (Primary Ab – 1:1,000) and re-probed with anti- β -actin (Primary Ab – 1:10,000) as a loading control, as outlined in Section 2.5.6. Results are representative of three independent experiments.

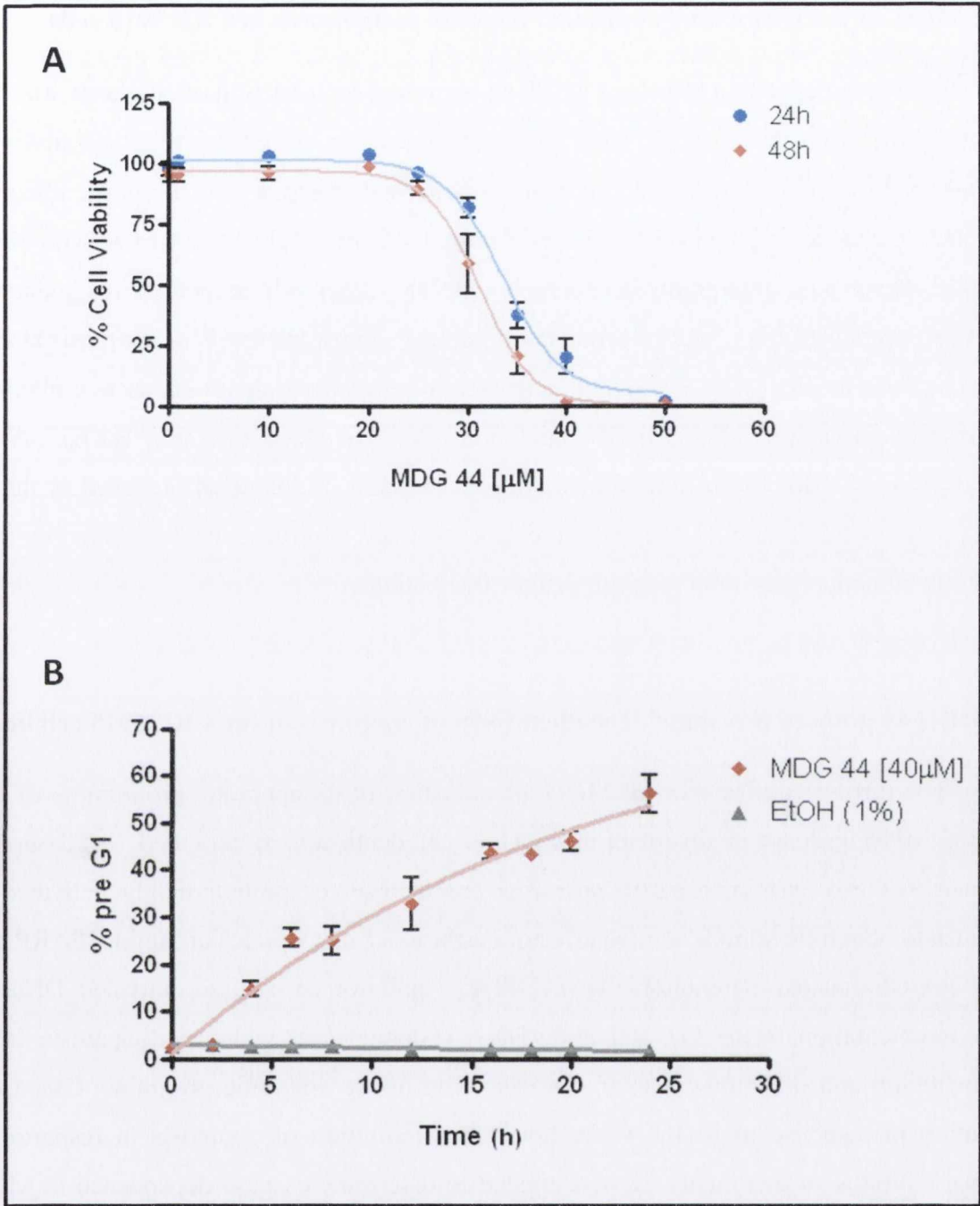


Figure 5.4: MDG 44 induced dose and time dependent cell death and apoptosis in the CRL 5915 cell line.

(A) MDG 44 induces dose and time dependent cell death in CRL 5915 cell line. Cells seeded on 96 well plates at a density of 0.5×10^4 cells per well, containing 200 μl media and left to adhere for 18h. Cells were treated at indicated concentrations of MDG 44 for 24h (●) and 48h (◆). Cell death was analysed by Alamar Blue cell viability assay, as outlined in Section 2.6. EC_{50} values generated for MDG44 were 33.6 μM (± 0.5) (24h) and 31.3 μM (± 0.6) (48h).

(B) MDG 44 induces time dependent apoptosis in CRL 5915 cell line. Cells were seeded at a density of 0.25×10^6 cells / T25 (5ml) and left to adhere for 18h prior to treatment with EtOH [1%] (▲) and MDG 44 [40 μM] (◆) for indicated time points. Apoptosis was measured by PI cell cycle analysis, as outlined in Section 2.7. Statistical analysis was performed using Graphpad Prism 4 statistical software.

5.3.2 MDG 44 induced a time dependent increase in apoptosis in CRL 5915 cells

The time dependent effects of MDG 44 treatment on induction of apoptosis was also investigated in CRL 5915 cells. Time dependent induction of cell death is indicative of a programmed or regulated form of cell death, as opposed to rapid loss of cellular integrity, lysis and death associated with necrotic cell death. CRL 5915 cells were treated with 40 μ M of MDG 44 for nine time points between 2h and 24h. Apoptotic induction was quantified by PI cell cycle analysis. MDG 44 treatment induced significant levels of apoptosis at time points as short as 4h (14.7% $\pm$ 1.5) and increased in a time dependent manner to a maximal response of 56.1% ($\pm$ 4.0%) pre-G1 population after 24h incubation (Fig 5.4.B). A lag period occurred prior to 4h time points, with no increase in apoptosis observed at the 2h time point. Vehicle (EtOH 1%) was used as an assay control at each time point. No apoptotic induction was observed with vehicle incubations.

5.4 MDG 44 induced a caspase dependent form of apoptosis in the CRL 5915 cell line

For further confirmation of MDG 44 induction of an apoptotic programme of cell death in MPM, caspase involvement in MDG 44 cell death was investigated. As described previously, these cysteine-aspartic proteases are themselves proteolytically activated in response to apoptotic stimuli. Active effector caspase-3/7 cleave nuclear lamins, PARP and the DNA endonuclease inhibitors ICAD/DFF45 (inhibitor of caspase activated DNase / DNA fragmentation factor 45), activating DNA endonucleases which participate in DNA fragmentation and other processes in the execution of the apoptotic program. Caspase-3 activation in response to MDG 44 treatment and inhibition of apoptosis in response to caspase inhibitor co-treatment was investigated to determine caspase dependence in MDG 44 treatment.

The pathway of apoptotic induction (extrinsic or intrinsic) was also examined in CRL 5915 cells. Chemotherapeutics that induce DNA damage, oxidative stress, nutrient deprivation and numerous other forms of cellular stress, cause signal cascades that culminate in the release of mitochondrial intermembrane space proteins (Cyto c) and proteolytic activation of pro-caspase-9. Extrinsic ligation of death receptors by TNF- α , TRAIL, FasL and other associated factors can induce FADD death receptor complex activation of Caspase-8 (Jin and El-Deiry, 2005). Caspase-9 and caspase-8 activation was

assayed to determine the pathway of apoptosis employed with MDG 44 treatment. Caspase dependence and the profile of the caspase cascade induced in response to MDG 44 is a confirmation of the drug inducing a classical apoptotic response in cells and a better indication of the drug's mechanism of action and possible cellular target.

5.4.1 MDG 44 induced cell death is inhibited by pan-caspase inhibitor, z-VAD-FMK

Induction of a caspase dependent form of apoptosis was assayed using the pan-caspase inhibitor z-VAD-FMK. CRL 5195 cells were treated with Vehicle or MDG 44 [40 μ M], with or without a 1h pre-treatment of z-VAD-FMK [40 μ M]. Cells were left to incubate for 6h or 24h before PI cell cycle flow cytometry analysis was performed. The increase in the apoptotic pre-G1 population of cells was analysed in treated samples. Representative histograms of 6h (Fig 5.5.A) and 24h (Fig. 5.5.B) experiments showed distinct pre-G1 (M1 region) peaks in MDG 44 treated samples (overlay), indicating apoptotic induction. In z-VAD-FMK pre-treated samples however, apoptosis was completely abrogated. No pre-G1 peak was observed and instead a normal profile of cell cycle distribution was observed. These results were confirmed in triplicate experiments (Fig 5.5.C). Apoptotic induction of 26.7% ($\pm$ 3.9) and 42.9% ($\pm$ 5.9) for 6h and 24h incubation of MDG 44 was completely abolished by pre-treatment with z-VAD-FMK.

Effector caspase-3 activation was also examined in response MDG 44 treatment. Caspase activation was assayed using a fluorogenic substrate for caspase-3 (Ac-DEVD-AMC). Fold increases in fluorescence intensity (FI) of cleaved 7-amino-4-methylcoumarin (AMC) was used to measure caspase activity, as outlined in Section 2.8.2. Cells treated with MDG 44 for 6h showed a highly significant (5.1 ± 0.4 fold) increase in caspase-3 activity (Fig 5.5.D). Pre-treatment with z-VAD-FMK however completely inhibited MDG 44 induced caspase-3 activation, reducing activation back to normal control levels (fold induction of 1.0 ± 0.1). These results indicated that MDG 44 induced apoptosis in CRL 5915 cells is a caspase-dependent process.

5.4.2 MDG 44 induced cell death occurs through the intrinsic apoptotic pathway

Time dependent caspase activation experiments were performed to confirm the role of caspase signalling in MDG 44 mediated apoptosis in CRL 5915 cells. Caspase activity

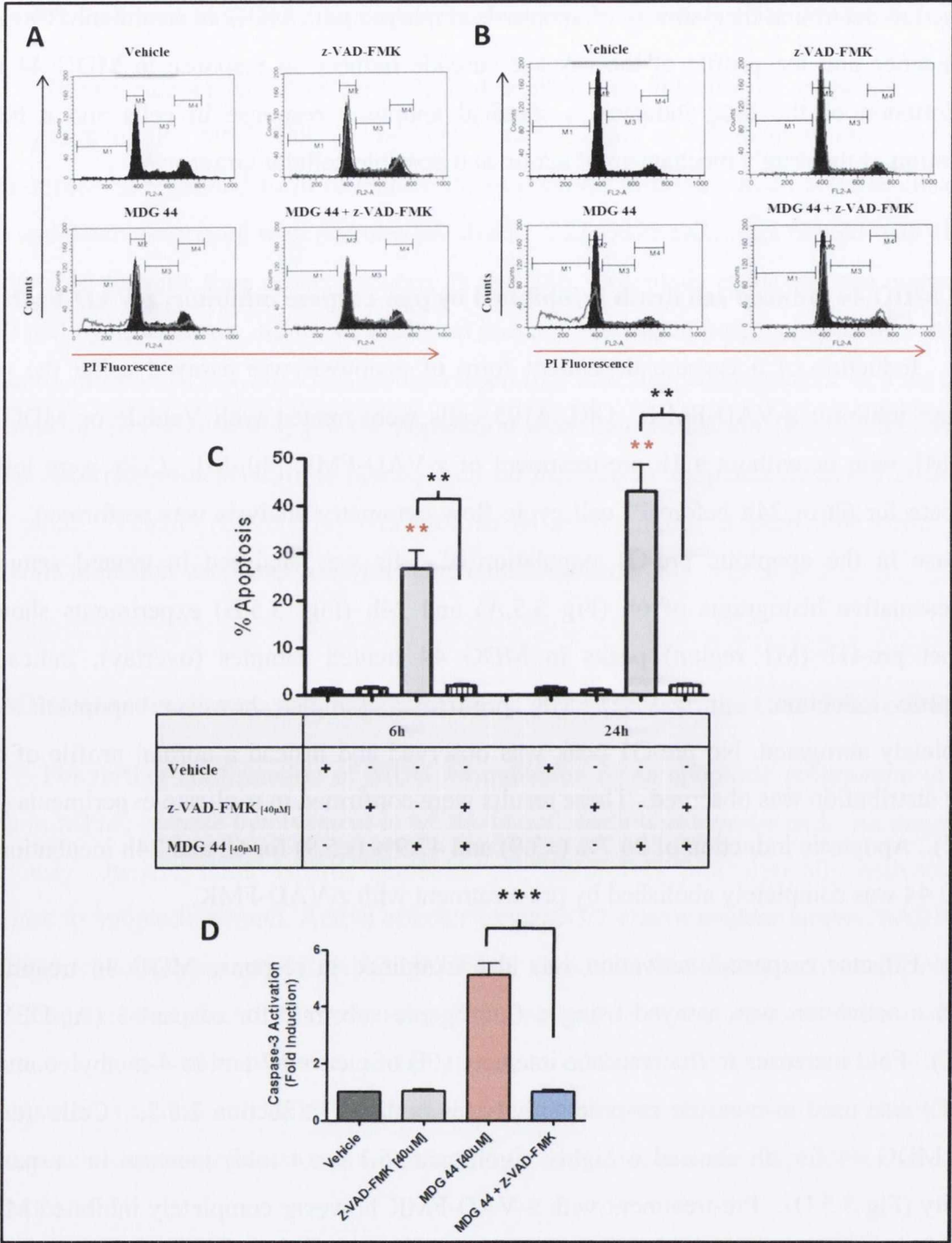


Figure 5.5: MDG 44 induced cell death was inhibited by pan-caspase inhibitor, z-VAD-FMK.

PI cell-cycle flow cytometry histograms of cells treated with MDG 44 [40μM] +/- z-VAD-FMK [40μM] for 6h (A) and 24h (B) time points. Triplicate data analysis for (A) and (B) treatments in CRL 5915 cells (C). Caspase-3 activation assay of CRL 5915 cells treated with MDG 44 +/- z-VAD-FMK for 6h (D). CRL 5915 cells were seeded at a density of 0.1×10^6 cells per well containing 2.5ml culture media. Cells were pre-treated for 1 h with z-VAD-fmk prior to treatment with test compounds for indicated time points. Apoptotic induction was analysed by PI staining and cell-cycle Flow Cytometry analysis, as outlined in Section 2.7. Caspase-3 activation was analysed as outlined in Section 2.8.3. Values are representative of mean $\pm$ SEM of three independent experiments. **P<0.01 ***P<0.001(Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

was assayed using fluorogenic substrates for active caspases, as outlined in Section 2.8.2. Time dependent effects of MDG 44 treatment were examined on the activity of effector caspase-3 and intrinsic and extrinsic pathway initiator caspases, caspase-9 and caspase-8. Etoposide, a topoisomerase II inhibitor that induces DNA strand breaks and consequential intrinsic (caspase-9) apoptotic pathway activation, was used as a positive assay control.

CRL 5915 cells were treated with MDG 44 [40 μ M] or vehicle (EtOH 1%) for seven time points between 0h and 10h incubations. This assay time frame encompassed the initiation to execution phase of MDG 44 induced apoptosis, as observed in time dependent apoptotic experimentation with MDG 44 (Fig 5.4.B). A significant level of caspase-3 activation was detected after 3h of incubation, preceded by a lag period of 1h and 2h (Fig 5.6.A). Fold activity of caspase-3 increases in a time dependent manner reaching a saturating level of approximately a 14 fold increase in active caspase-3 after 6h of incubation (13.7 $\pm$ 1.9 fold increase). Vehicle treated samples did not induce caspase-3 activation, with no deviation from normalised control levels (Fold induction of “1”). The time frame of caspase activation coincides with the time dependence of apoptotic induction observed in Fig 5.5.B. Caspase-3 activation was seen to prior to induction of apoptosis, detected by the increase in the apoptotic / pre-G1 population of cells.

Caspase-9 and Caspase-8 activation was detected using fluorogenic substrates Ac-LEHD-AMC and Ac-IETD-AMC, respectively. Significant time dependent increases in caspase-9 activation were detected (Fig 5.6.B). Significant levels of caspase-9 activity were detected after 3h time points, reaching a maximum between 4 – 6h, before dropping with longer incubations. No caspase-8 activity was detected in response to MDG 44 incubation at any time point. Caspase-8 activity did not deviate from untreated (zero hour) or vehicle treated controls (Fig 5.6.C). Cells treated with the positive control, Etoposide (10 μ M for 48h), produced comparable levels of caspase-3 and caspase-9 activity and no induction of caspase-8 activation (Fig 5.6.D).

One experimental caveat is the difference in magnitude of induction of caspase-3 vs. caspase-9 activity. The fold increase in caspase-3 activation is significantly higher than that of caspase-9 (15 vs. 2.5 fold increase). A possible explanation for this is the difference in abundance of initiator and effector caspase protein in cells and the biological amplification of the apoptotic signal as it is transduced down the caspase cascade. Once activated initiator caspase-9 should activate much more effector caspase-3 in order to amplify the apoptotic

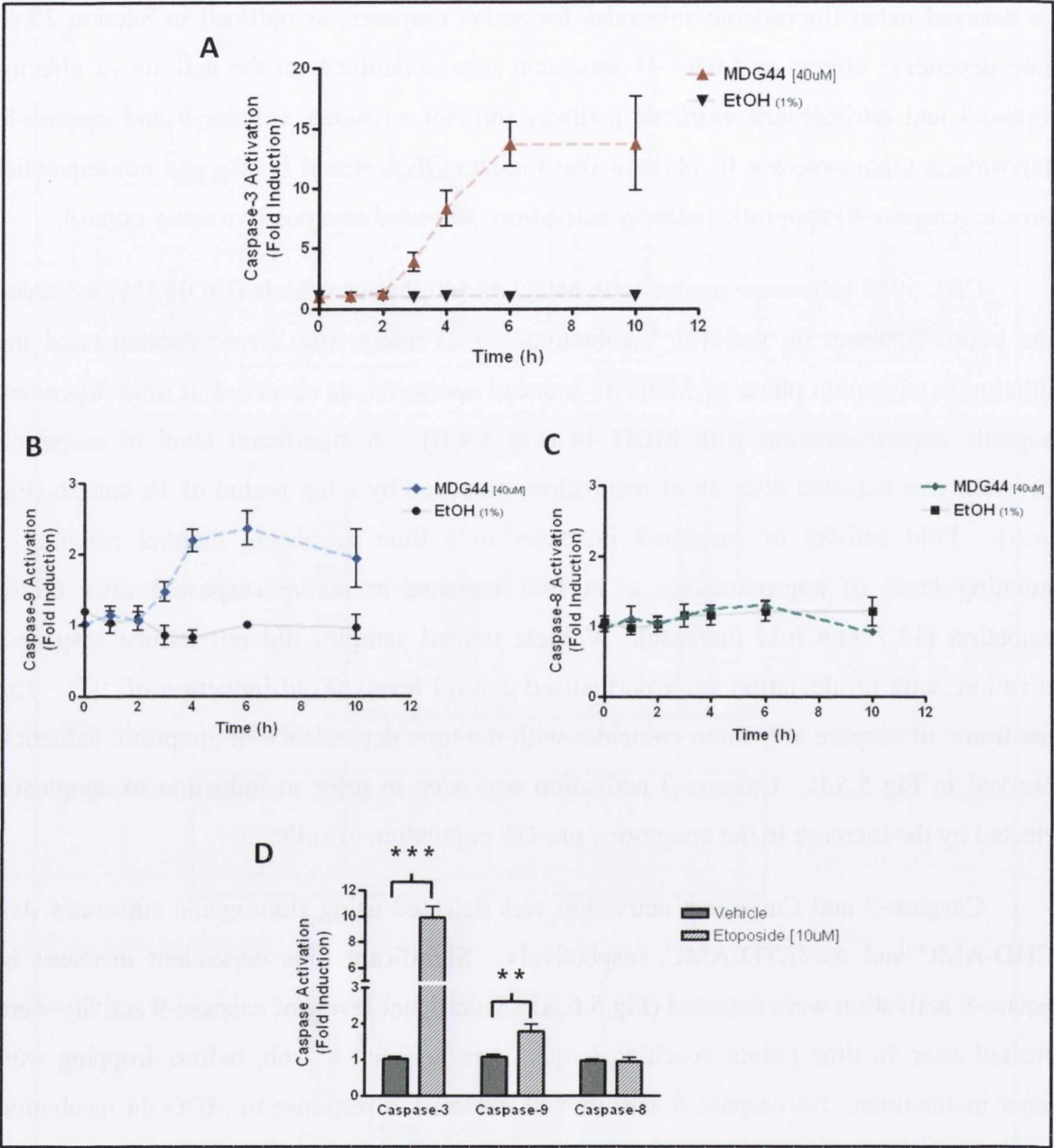


Figure 5.6: MDG 44 induced activation of caspase -3 & -9, but not caspase – 8 in CRL 5915 cells.

CRL 5915 cells treated with MDG 44 were tested for induction of caspase-3 (A), caspase-9 (B) and caspase-8 (C) activation, in a time dependent manner. Etoposide treatment (48h) was used as a positive control (D). CRL 5915 cells were seeded at a density of 0.325×10^6 cells /T25 (5ml). Cells were treated with MDG 44 [40uM] and caspase activation was determined, as outlined in Section 2.8.3. Two way ANOVA and student t test was used to test significance of data (Graphpad Prism version 4.0 software package). **P<0.01 ***P<0.001.

signal. Results for MDG 44 are also directly comparable to the effect observed with the positive control, Etoposide, and with previous reports investigating initiator and effector caspase activation (Yang et al., 2003).

5.4.3 MDG 44 induced cell death was inhibited by the caspase-9 specific inhibitor, z-LEHD-FMK

To confirm previous caspase-9 activation data that MDG 44 induces an intrinsic apoptotic pathway response in cells, experiments were performed with MDG 44 treatment in combination with the caspase-9 specific inhibitor, z-LEHD-FMK. CRL 5915 cells were treated with MDG 44, with or without pre-treatment with z-LEHD-FMK for 8h and 48h time points. MDG 44 induced significant levels of apoptosis of 34.7% (± 2.0) and 48.6% (± 6.5) after 8h (MDG 44 – 50 μ M) and 48h (MDG 44 - 35 μ M) incubations respectively (Fig 5.7.A). Pre-treatment with z-LEHD-FMK however resulted in significant inhibition of MDG 44 induced apoptosis. This dependence on caspase-9 signalling for full execution of MDG 44 induced apoptosis is further confirmation that single agent treatment of the compound induces activation of the intrinsic apoptotic pathway.

Cisplatin treatment in combination with z-LEHD-FMK was used as a positive control for an apoptotic agent inducing cell death via caspase-9 activation and the intrinsic apoptotic pathway. Cisplatin induced apoptosis in CRL 5915 cells after incubation with drug for 48h was significantly inhibited by pre-treatment with z-LEHD-FMK (Fig 5.7.B). Cisplatin treatment caused 47.8% (± 1.0) apoptosis, which dropped to 23.4% (± 1.1) in conjunction with z-LEHD-FMK.

5.4.4 MDG 44 treatment of CRL 5915 cells induced the proteolytic cleavage of pro-caspase-9

The induction of caspase-9 activation was also detected by Western blot analysis of MDG 44 treated cells (Fig 5.8). Cells were treated with MDG 44 [40 μ M] for seven time points between 0h and 10h incubations. Pro-caspase-9 exists as a 47kDa zymogen until cyto c release from the mitochondria and apoptosome formation induces auto-proteolytic cleavage of pro-caspase-9 into the proteolytically active 35kDa fragment and 10kDa

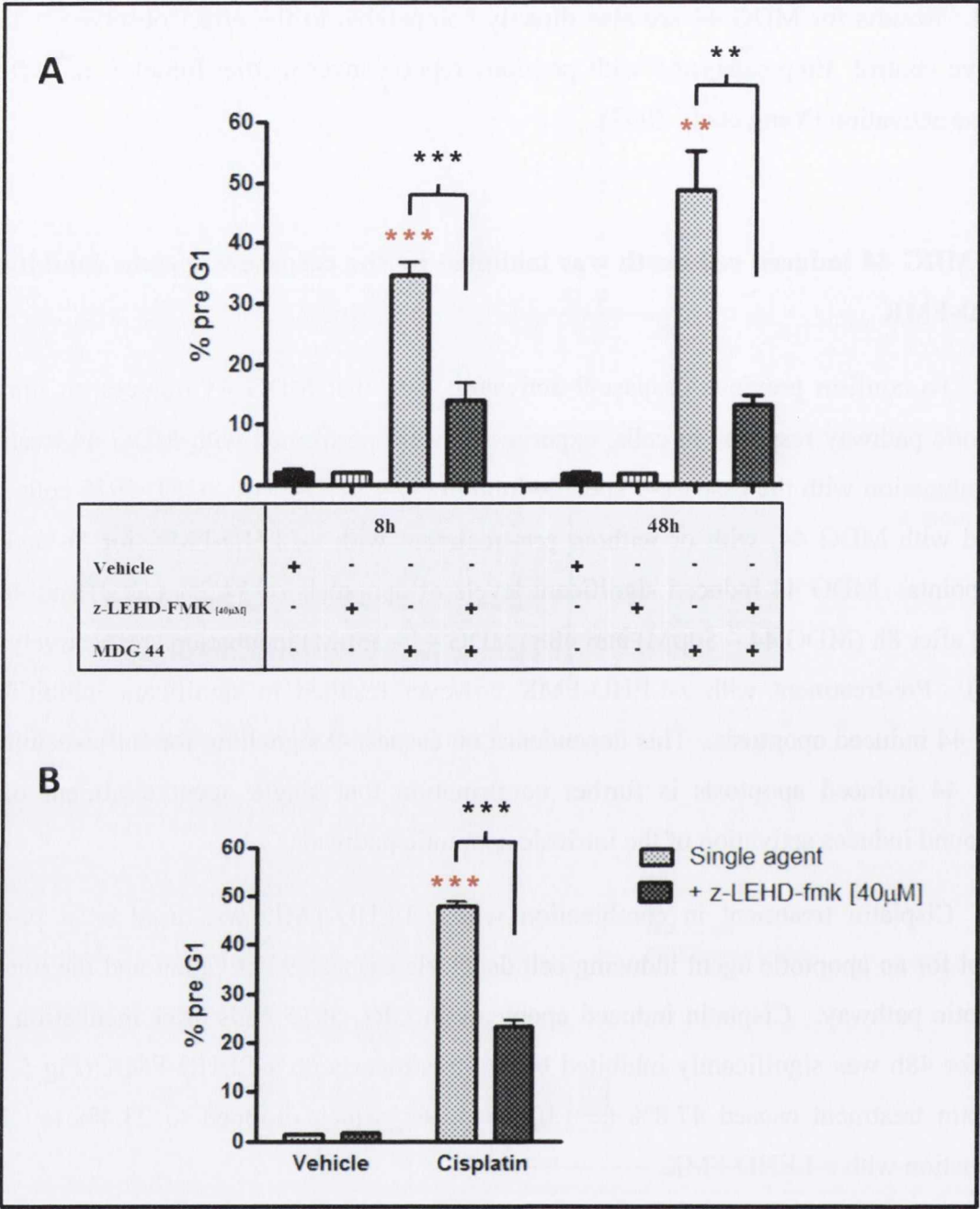


Figure 5.7: MDG 44 induced cell death was inhibited by the caspase-9 specific inhibitor, z-LEHD-FMK

CRL 5915 cells were treated with MDG 44 +/- caspase-9 specific inhibitor (z-LEHD-fmk) for 8h or 48h (A). Cisplatin treatment in CRL 5915 cells (48h), with or without z-LEHD-fmk pre-treatment, was used as a positive control for caspase-9 dependant apoptotic induction (B). CRL 5915 cells were seeded at a density of 0.1×10^6 cells per well containing 2.5ml culture media. Cells were pre-treated for 1 h with z-LEHD-fmk [40μM] prior to treatment with test compounds for indicated time points. Apoptotic induction was analysed by PI staining and cell-cycle Flow Cytometry analysis. Values are representative of mean $\pm$ SEM of three independent experiments. ** $P < 0.01$ *** $P < 0.001$ (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

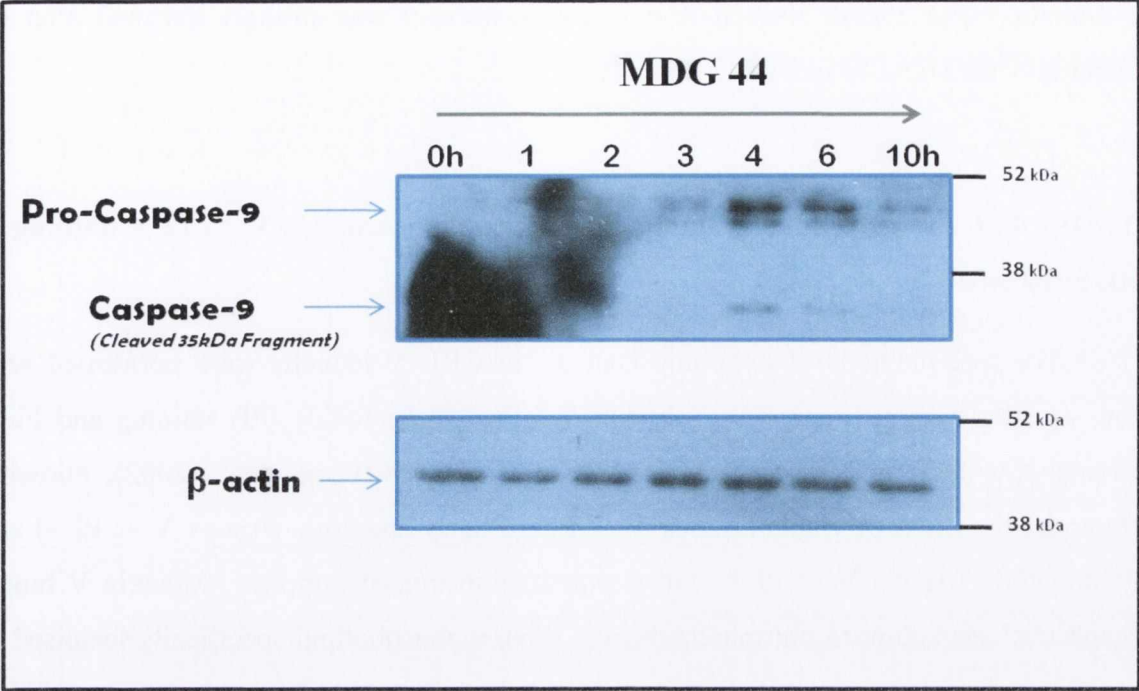


Figure 5.8: MDG 44 induced pro-caspase-9 proteolytic activation, detected by Western Blot.

CRL 5915 cells were seeded at a density of 0.325×10^6 cells / T25 (5ml) and treated with $40\mu\text{M}$ MDG 44 for indicated time points. Whole cell lysates were generated, $30\mu\text{g}$ of protein was resolved on a 12% gel and the induction of caspase-9 cleavage was investigated by Western Blot (Section 2.5). PVDF membrane probed with anti-cleaved caspase-9 (Primary Ab – 1:1,000) and re-probed for β -actin (Primary Ab – 1:10,000) as a loading control.

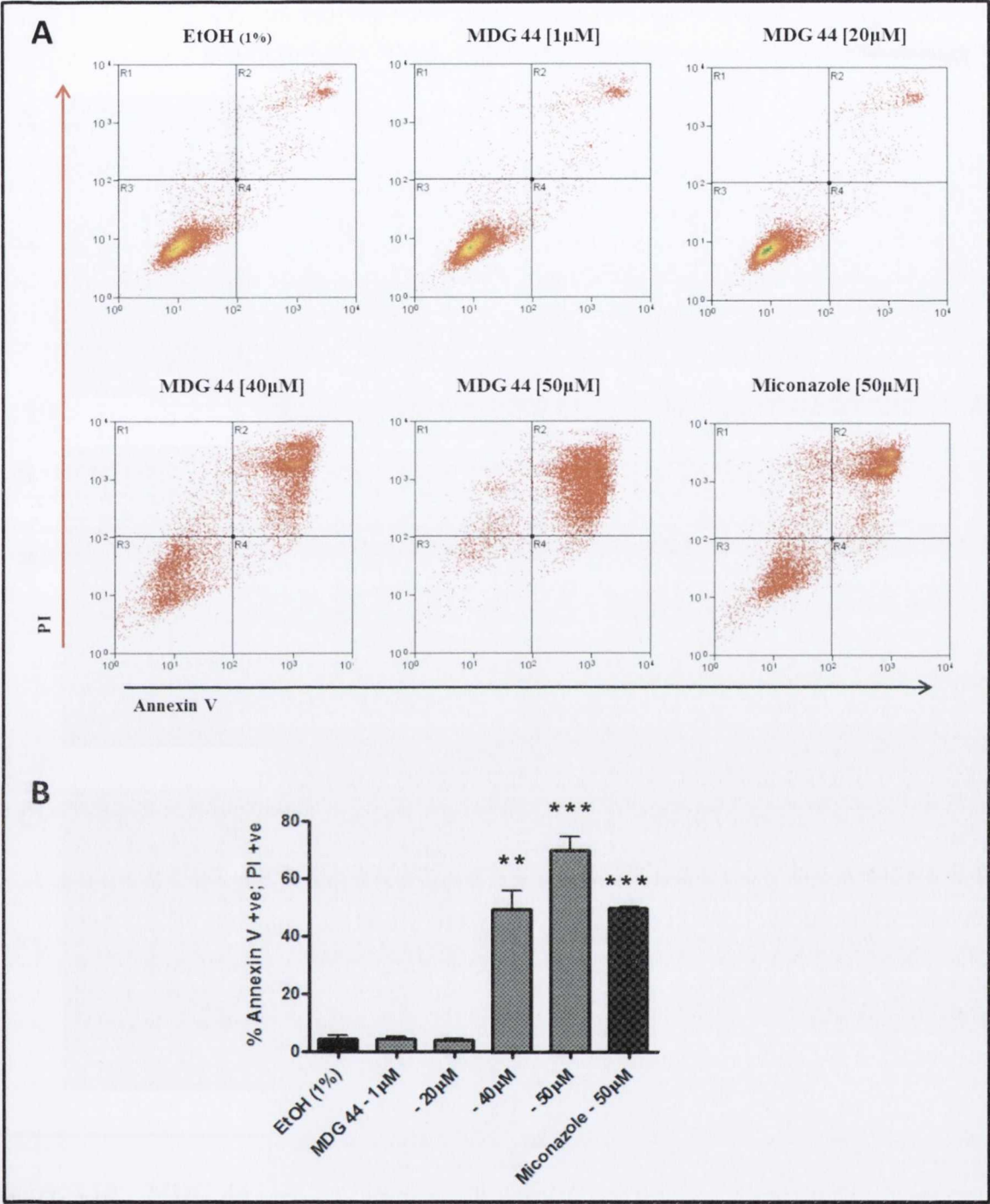
pro-domain. The 35kDa cleaved fragment of caspase-9 was strongly detected after 4h incubation with MDG 44 in CRL 5915 cells.

5.5 MDG 44 treatment induced significant increases in Annexin V + / PI + staining in CRL 5915 cells

Alternate methods of apoptotic analysis in CRL 5915 cells were performed with MDG 44 single agent treatment. Annexin V / Propidium Iodide (PI) staining and Flow Cytometry analysis is a commonly used apoptosis assay (Peng et al., 2002), allowing differential detection of viable (Annex V –; PI –), Early-apoptotic (Annex V +; PI –) and Late-apoptotic (Annex V +; PI +) cell populations in treated samples. Annexin V binds, through Ca^{2+} chelation, to phosphatidylserine (PS), a phospholipid specifically localised to the outer leaflet of the plasma membrane during the initial steps of the apoptotic program (Fadok et al., 1992). PI alternately can enter and stain Late-apoptotic cells by binding to DNA in the nucleus. CRL 5915 cell were treated with increasing concentrations of MDG 44 [1 μM – 50 μM] for 24h and the Annexin V / PI assay was performed as described in Section 2.9. Representative dot plots for Annexin V / PI dual staining analysis of MDG 44 treatment of CRL 5915 cells are shown (Fig 5.9.A). Incubations with MDG 44 at 40 μM and 50 μM were shown to significantly increase the percentage population of Annexin V + / PI + apoptotic cells in CRL 5915 cells – 49.07% (± 6.3) and 69.30% (± 4.8) apoptotic cells respectively (Fig 5.9.B). Miconazole, an anti-fungal azole apoptotic inducer and test compound in Chapter 8, was used as a positive control and induced significant increases in Annexin V + / PI + cells (49.65% ± 0.2 Late-apoptotic cells).

5.6 MDG 44 induced DNA damage indicative of apoptosis, determined by Single-Cell Gel Electrophoresis analysis (Comet assay)

The effect of MDG 44 treatment on nuclear integrity and induction of DNA damage was analysed by Comet assay. CRL 5915 cells were treated with Vehicle or MDG 44 for 5h and then subjected to embedding in agarose and electrophoresis, as outlined in Section 2.10. PI staining of agarose slides and fluorescent imaging revealed that MDG 44 treatment induced the formation of a DNA smear or “Comet Tail” spreading from the nucleus of cells (Fig 5.10). This formation of a Comet tail is indicative of DNA damage and apoptosis.



9

Figure 5.9: MDG 44 single agent treatment induces an increase in Annexin V + / PI + apoptotic cells

Representative dot plot analysis of Annexin V / PI dual stained CRL 5915 cells treated with vehicle (EtOH 1%), increasing concentrations of MDG 44 [1 - 50 μM] or Miconazole [50 μM] for 24h (A). Experiments were performed in triplicate and the percentage induction of Annexin V + / PI + apoptotic cells (Region 2 “R2”; Upper right quadrant of dot plots in A) was analysed for statistical significance (B). CRL 5915 cells were seeded at a density of 0.225 x 10⁶ cells / T25 (5ml). Cells were treated with vehicle or indicated concentrations of test compounds for 24h. Annexin V / PI staining and flow cytometry analysis is outlined in Section 2.9. Values are representative of mean ± SEM of three independent experiments. **P<0.01; ***P<0.001 (Determined using unpaired Student’s t-test, part of Graphpad Prism 4 statistical software).

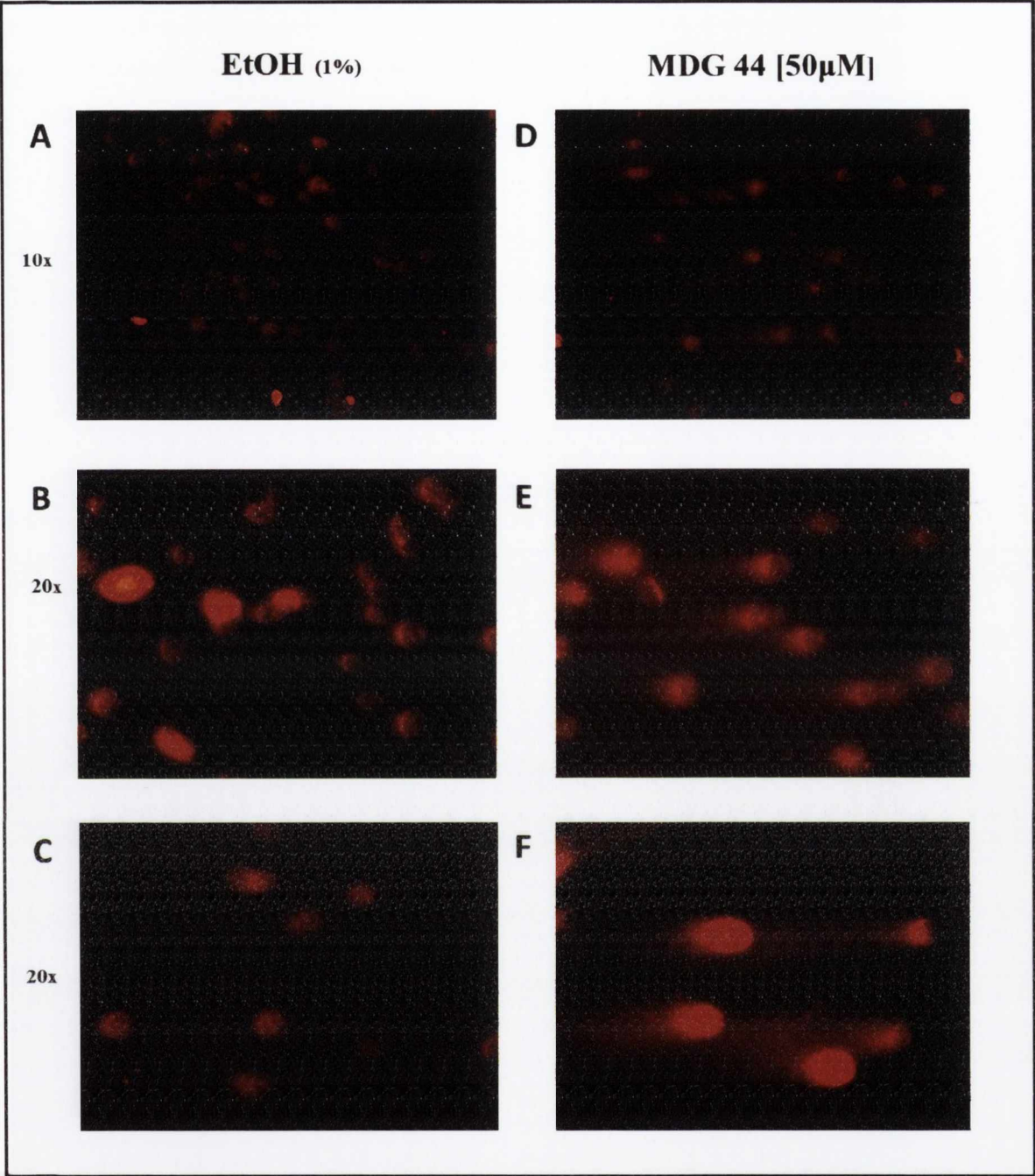


Figure 5.10: MDG 44 treatment induced DNA fragmentation indicative of apoptosis, as indicated by the formation of DNA “Comet Tails” in a single cell gel electrophoresis Comet assay

CRL 5915 cells were treated with Vehicle (EtOH 1%) or MDG 44 [40 μM] for 5h. CRL 5915 cells were seeded at a density of 0.3125 x 10⁶ cells / T25 (5ml). Cells were treated with Vehicle (EtOH 1%) or MDG 44 for 5h. The Comet assay was performed as outlined in section 2.10. Cells and DNA “Comet Tails” were viewed and pictures taken using an Olympus IX81-long focal length fluorescent microscope (20X magnification) and F-View II digital camera. Three independent experiments were performed and representative images of Vehicle and MDG 44 treated cells are shown in figures A – C and D – E, respectively.

During single-cell gel electrophoresis (Comet assay), endonuclease fragmented DNA in apoptotic cells is subjected to a large electrophoretic shift through the agarose gel, forming a Comet tail (Choucroun et al., 2001).

In contrast to MDG 44 incubations, vehicle treatment in CRL 5915 cells showed no formation of Comet tails (Fig 5.10), indicative of an undamaged, functioning nucleus. Vehicle treated samples with intact DNA and Chromatin structures of the nucleus, due to their size, display little electrophoretic shift through agarose during electrophoresis and no Comet tail formation.

5.7 Examination of synergistic induction of Programmed Cell Death with MDG 44 and Cisplatin combinational treatments in CRL 5915 cells

Having investigated the mechanism of MDG 44, single-agent induced PCD in MPM cell lines, the synergistic capacity of MDG 44 in combination with Cisplatin was next examined. Experiments were performed to confirm the capacity of MDG 44 to synergistically increase Cisplatin induced apoptosis, specifically. Initial screening in Chapter 4 revealed that MDG 44 could synergise with Cisplatin at a sub-lethal concentration of MDG 44 [25µM], in PI cell-cycle Flow Cytometry assays. Experimentation was continued in an attempt to classify and confirm that an apoptotic program of cell death is induced with MDG 44 and Cisplatin combinational treatments in MPM. The CRL 5915 cell line was chosen as the MPM cell model for this analysis – CRL 5915 cells were the most responsive in pre-G1 apoptotic analysis of MDG 44 synergism with cisplatin (Table 4.3) and MDG 44 single agent dose response experiments (Fig 5.2).

5.7.1 MDG 44 synergistically increased cisplatin induced PARP cleavage in CRL 5915 cells

To confirm synergistic apoptosis induction of MDG 44 in combination with cisplatin, PARP cleavage analysis of combination treatments was performed. Cells were treated in a similar manner as PI cell cycle experiments performed in Chapter 4. The experiment consisted of CRL 5915 cells incubated with MDG 44 [1µM & 25µM] and Cisplatin [25µM], alone or in combination for 12h (Fig 5.11.A), 24h (Fig 5.11.B) and 36h (Fig 5.11.C) time points. At each endpoint, whole cell lysates were prepared and PARP

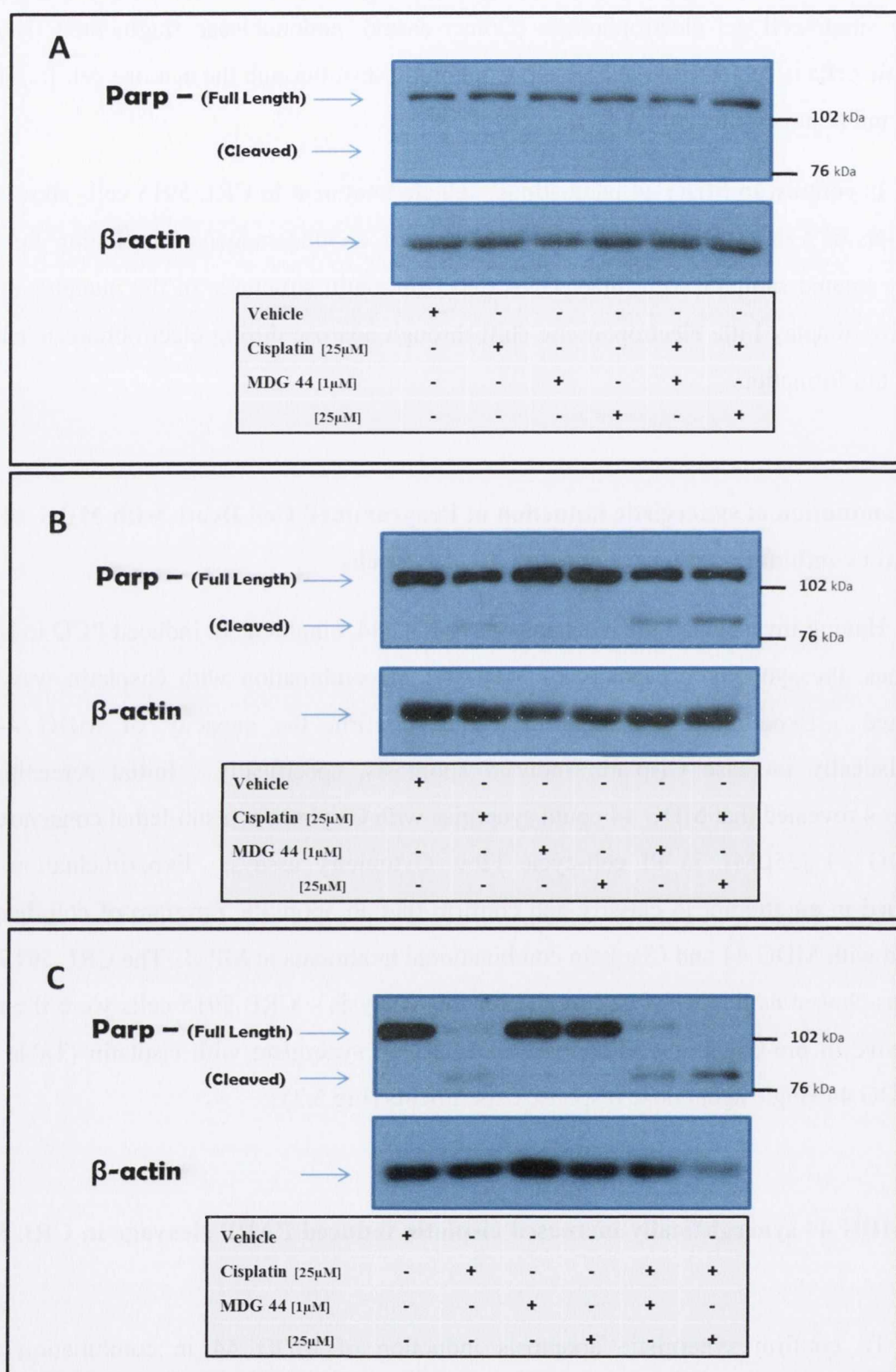


Figure 5.11: MDG 44 synergistically increased Cisplatin induced PARP cleavage.

CRL 5915 cells were seeded at a density of 0.4×10^6 cells / T25 (5ml) and treated with Cisplatin +/- MDG 44 for 12h (A), 24h (B) and 36h (C). Whole cell lysates were generated, 30μg of protein was resolved on an 8% gel and the induction of PARP cleavage was investigated using anti-PARP (Primary Ab – 1:1,000) (Section 2.5). PVDF membrane was re-probed with anti-β-actin (Primary Ab – 1:10,000) as a loading control.

cleavage was analysed by Western blot analysis, as outlined in Section 2.5. The full length 116 kDa protein and the 89 kDa cleaved fragment of PARP were both detected. Results indicate that after 12h incubation, no PARP cleavage was detected with either single or co-administration treatment regimes. After 24h incubation, a small level of cleaved PARP is observed with cisplatin treatment; however, treatment in combination with MDG 44 resulted in a significant increase in the cleavage of PARP (Fig 5.11.B). This result was mirrored in 36h assays (Fig 5.11.C). Significant degradation of full length PARP was observed in all cisplatin treated samples, however, cisplatin in combination with MDG 44 [25µM] induced complete degradation of full length PARP and consequently, a significant increase in the amount of cleaved PARP compared to cisplatin treatment alone.

Treatment with MDG 44 alone in this assay, at 1µM and 25µM, did not induce PARP cleavage at any incubation time point tested (12h – 36h). These results indicate that at non-toxic concentrations (25µM), MDG 44 did not induce apoptosis alone (no cleaved Parp), but was able to synergise with cisplatin, significantly enhancing cisplatin induced apoptosis in the susceptible CRL 5915, MPM cell line.

5.7.2 MDG 44 synergistically increased Cisplatin induced apoptosis in a dose dependent manner

CRL 5915 cells were treated with increasing concentrations of MDG 44 [1µM – 30µM], with or without co-treatment with Cisplatin for 48h. PI cell cycle analysis revealed no significant increase in pre G1 apoptosis with incubations of MDG 44 alone (30µM – 2.4% ±0.6) (Fig 5.12.A). However, treatment with MDG 44 in combination with Cisplatin induced dose dependent, synergistic increases in Cisplatin induced apoptosis. Cisplatin treatment alone induced 19.3% (± 0.8) apoptosis. A synergistic increase in apoptosis was observed with co-treatment of 15µM MDG 44 (27.6% apoptosis ± 1.3), increasing in dose dependent manner to the highest co-treatment concentration of 30µM MDG 44 (61.1% apoptosis ±3.4).

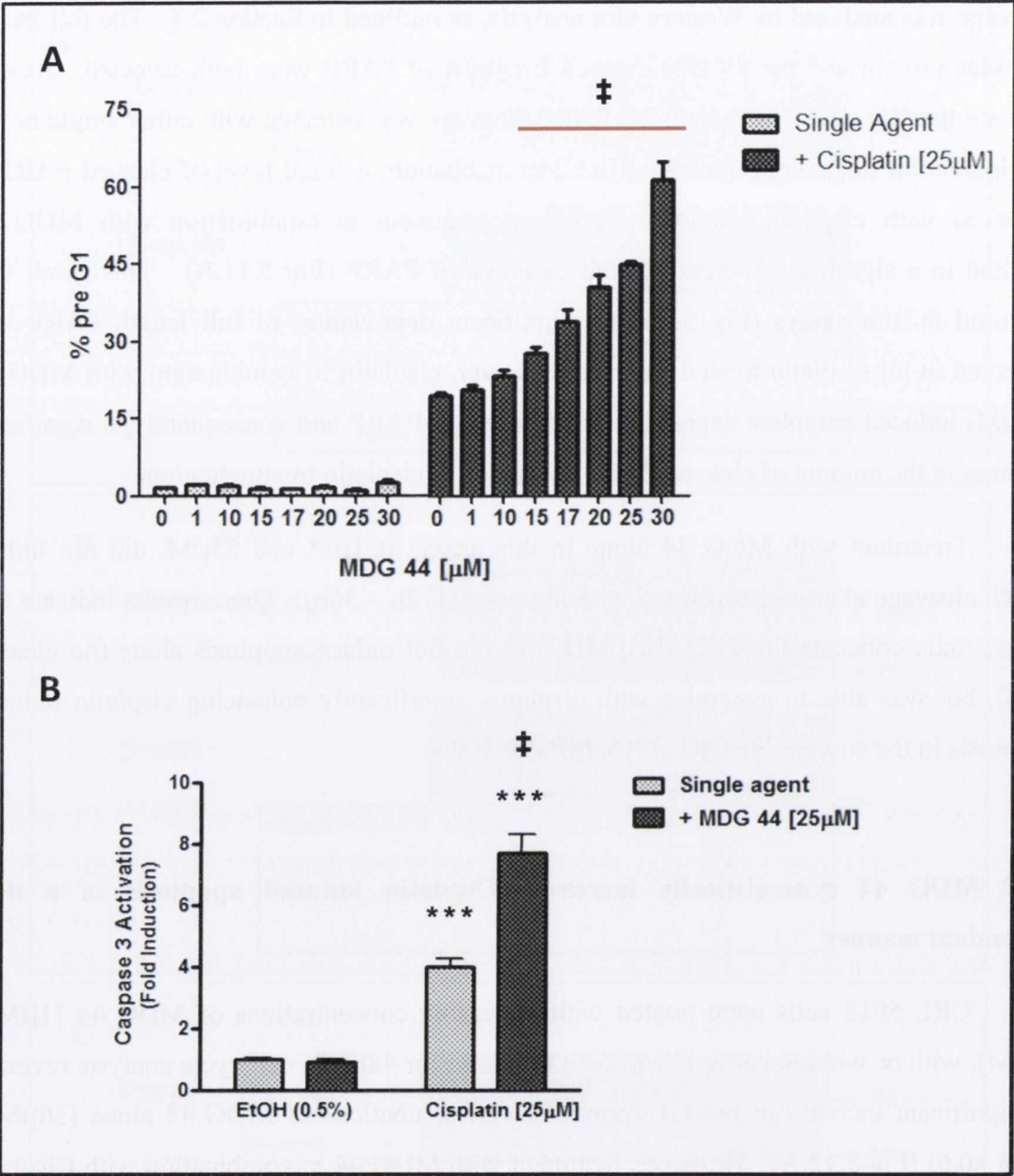


Figure 5.12: MDG 44 in combination with Cisplatin synergistically increases Cisplatin induced apoptosis in CRL 5915 cells

(A) CRL 5915 cells treated in a dose dependant manner with MDG 44 [1 - 30 μ M] alone or in combination with Cisplatin [25 μ M]. CRL 5915 cells were seeded at a density of 0.225×10^6 cells / 5ml media. Cells were treated with increasing concentrations of MDG 44 +/- Cisplatin for 48h. Apoptotic induction was analysed by PI staining and cell-cycle Flow Cytometry analysis.

(B) Cisplatin [25 μ M] in combination with MDG 44 [25 μ M] induces a synergistic increase in caspase-3 activation in CRL 5915 treated cells. CRL 5915 cells were seeded at a density of 0.12×10^6 cells / well (3ml media). Cells were treated with MDG 44 [25 μ M] alone or in combination with Cisplatin [25 μ M] for 26h and caspase activation was determined, as outlined in Section 2.8.3.

Values are representative of mean $\pm$ SEM of three independent experiments. ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software). Synergism was determined by two-way analysis of variance (ANOVA) of the interaction effect of single agent and co-treatment response ($\ddagger$ P<0.01).

5.7.3 MDG 44 synergistically increased Cisplatin induced Caspase-3 activation in CRL 5915 cells

The effect of co-treatment on the proteolytic activation of caspase was also investigated in CRL 5915 cells. Cells were incubated with Cisplatin alone or in combination with MDG 44 for 26h and caspase-3 activation was monitored using the caspase-3 specific fluorogenic substrate (Ac-DEVD-AMC). Cisplatin treatment alone for 26h induced a 4.0 ($\pm$ 0.3) fold increase in caspase activation (Fig 5.12.B). MDG 44 treatment alone had no effect on induction of caspase activation, but when incubated in combination with Cisplatin, MDG 44 synergistically increased activation of caspase to 7.7 ($\pm$ 0.7) fold levels above untreated controls.

5.7.4 MDG 44 in combination with Cisplatin induced a synergistic increase in Annexin V + / PI + apoptotic cells

Annexin V / PI dual staining analysis was performed as an alternate assay for confirmation of specific engagement of an apoptotic program of cell death in MDG 44 / Cisplatin combinational treatments. CRL 5915 cells were treated with MDG 44 and Cisplatin, alone or in combination for 48h. Representative dot plot results for Dual staining analysis are illustrated in Figure 5.13.A. The percentage population of Annexin V + / PI +, late-apoptotic cells detected with Vehicle and MDG 44 single agent treatment was 2.9% ($\pm$ 0.1) and 3.0 ($\pm$ 0.8) respectively (Fig 5.13.B). Treatment with Cisplatin caused an increase in the Annexin V + / PI + cell population to 13.6 % ($\pm$ 2.1), however, incubation in combination with MDG 44 synergistically increased the Annexin V + / PI +, apoptotic population of cells to 37.9% ($\pm$ 1.7).

5.8 MDG 44 induces single agent apoptosis in Chronic Lymphocytic Leukaemia (CLL) in vitro cell models

The ability of MDG 44 to induce apoptosis in alternate cancer cell models was investigated. As the cellular target of MDG 44 was not known, its specificity for apoptotic induction only in MPM could not be assumed. Single agent treatments with MDG 44 were performed in both resistant (HG-3) and non-resistant (PGA-1) Chronic Lymphocytic Leukaemia (CLL) cell lines. Cells were treated with Vehicle or drug for 48h and apoptotic

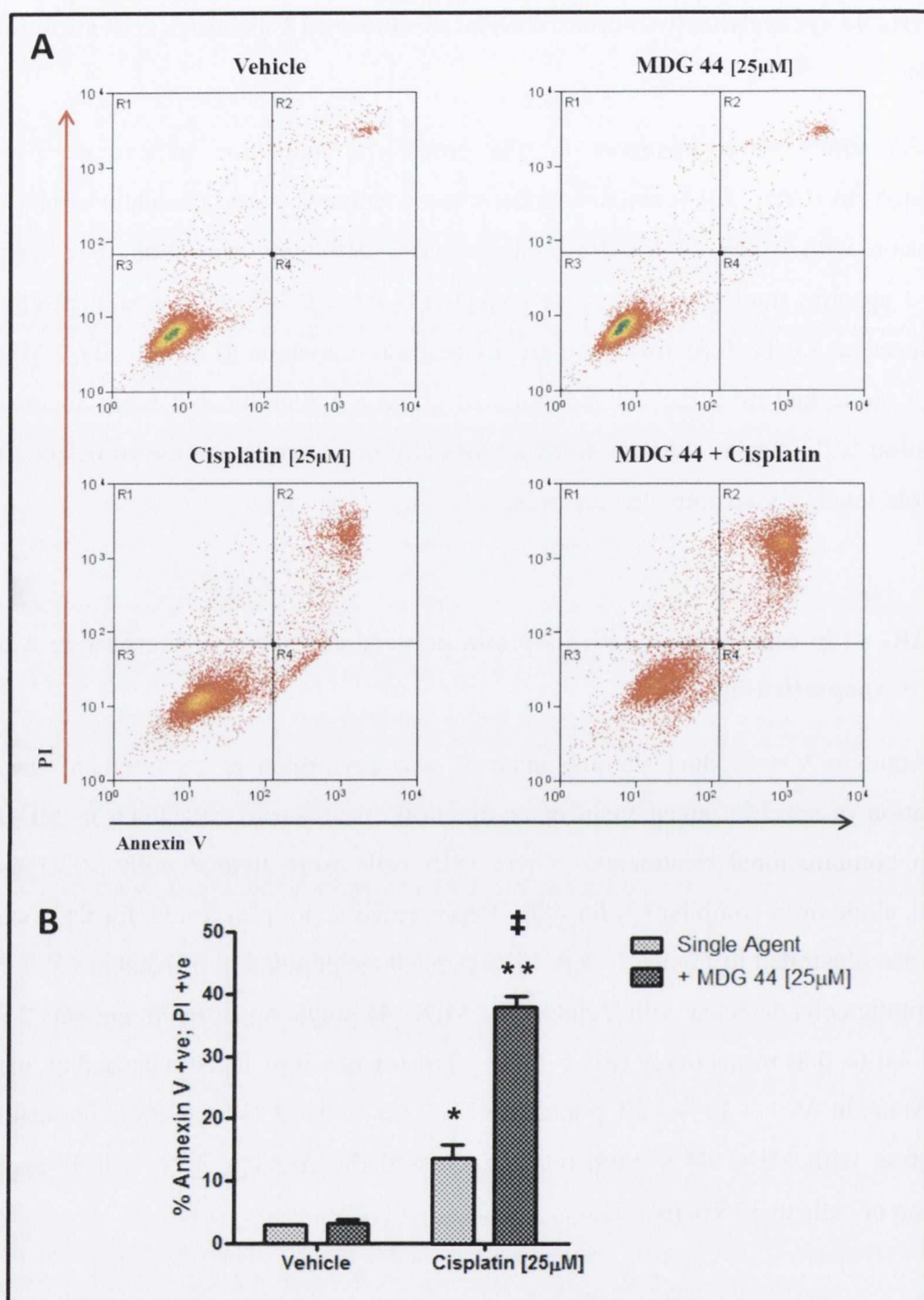


Figure 5.13: MDG 44 treatment in combination with Cisplatin induced a synergistic increase in Annexin V + / PI + apoptotic cells

Representative dot plot analysis of Annexin V / PI dual stained CRL 5915 cells treated with MDG 44 [25µM] + / - Cisplatin [25µM] for 48h (A). Experiments were performed in triplicate and the percentage induction of Annexin V + / PI + apoptotic cells (Region 2 “R2”; Upper right quadrant of dot plots in A) was analysed for statistical significance (B). CRL 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml). Cells were treated with vehicle or indicated concentrations of test compounds for 48h. Annexin V / PI staining and flow cytometry analysis is outlined in Section 2.9. Values are representative of mean $\pm$ SEM of three independent experiments. * $P < 0.05$; ** $P < 0.01$ (Determined using unpaired Student’s t-test, part of Graphpad Prism 4 statistical software). Synergism was determined by two-way analysis of variance (ANOVA) of the interaction effect of single agent and co-treatment response (‡ $P < 0.01$).

induction was analysed by PI cell cycle analysis. Results revealed that MDG 44 was able to induce significant levels of apoptosis in both resistant HG-3 ($54.6\% \pm 4.7$) and non-resistant PGA-1 ($42.8\% \pm 5.2$) cell lines with $40\mu\text{M}$ incubations with drug (Fig 5.14). (CLL experimentation was performed by Dr. Sandra Bright of the Williams' lab, School of Biochemistry and Immunology, Trinity College Dublin).

5.9 Investigation into the mechanism of cell death induced by MDG 44 treatments in the CRL 5915 cell line

Having confirmed the induction of apoptosis and signalling via the intrinsic / mitochondrial apoptotic pathway in response to MDG 44 single agent and cisplatin co-treatment in CRL 5915 cells, experiments were performed to investigate the mechanism by which apoptosis is induced. Attempts were made to profile the cellular effects induced by MDG 44, examining the contributions of cell stress mechanisms in the induction of apoptosis. As the target for MDG lead compounds was not known, mechanistic analysis was hoped to indicate or rule out putative cellular targets for further study. Experiments were performed to examine the effect of MDG 44 on cell cycle arrest, the role of oxidative stress / ROS in MDG 44 apoptotic induction and changes in cellular morphology induced by MDG 44 during apoptosis.

5.9.1 MDG SMAC mimetics did not induce S phase arrest in Mesothelioma cell lines

Arrest of cells in S phase of the cell cycle was induced in MPM cells in response to embelin single agent treatment, reported in Chapter 3 (Fig 3.9). This effect of embelin on S phase arrest has been previously proposed to be mediated independently of XIAP inhibition, but rather embelin activity through an alternate target (Joy et al., 2010). In all four MPM cell lines, lead compound MDG 44, as well as analogues MDG 39 and MDG 42, were analysed for induction of S phase arrest. Cells were treated at 1, 10 and $25\mu\text{M}$ of MDG compound for 48h and analysed by PI cell cycle analysis, outlined in Section 2.7. As opposed to embelin treatment, MDG compounds did not induce any significant level of S phase arrest in MPM cells (Fig 5.15.A).

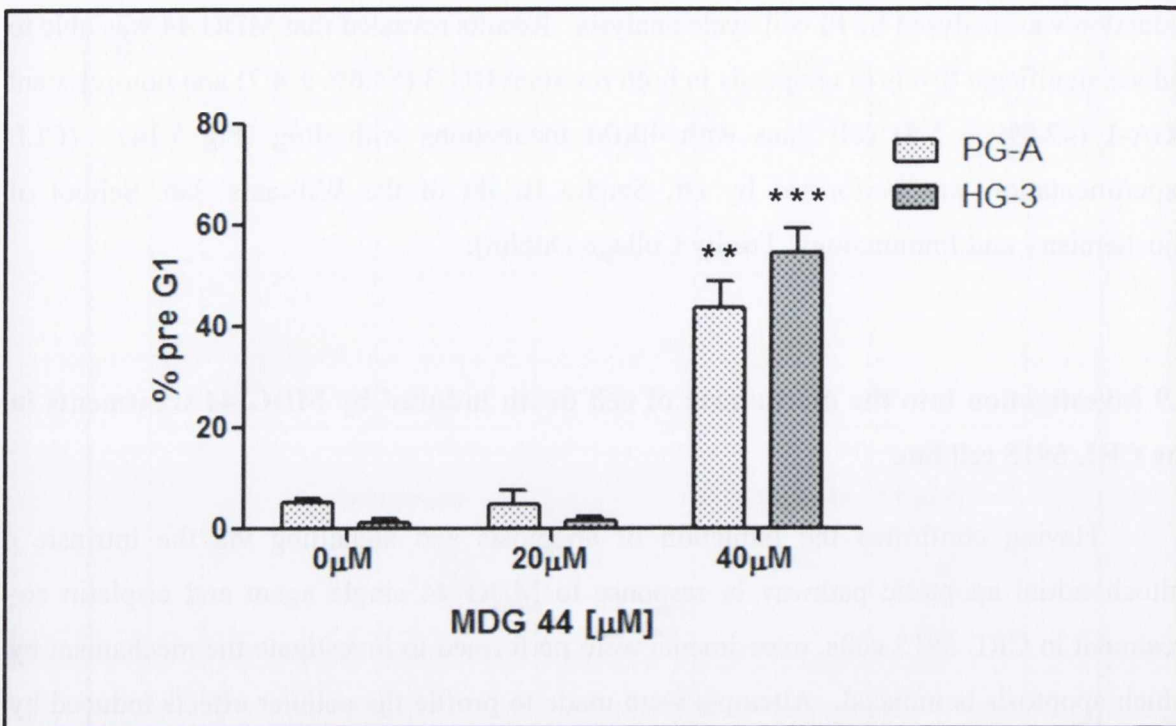


Figure 5.14: MDG 44 induced apoptosis in Chronic Lymphocytic Leukemia (CLL) cell lines PG-A and HG-3

PG-A and HG-3 cells were treated with vehicle (EtOH 1%) or MDG 44 [20μM & 40μM] for 48h. Cells were seeded at a density of 0.2×10^6 cells / ml (12-Well plate). Apoptotic induction was analysed by PI staining and cell-cycle Flow Cytometry analysis, as outlined in section 2.7. Results represent the mean $\pm$ SEM of three independent experiments. **P<0.01; ***P<0.001 (Determined using Graphpad Prism 4 statistical software). [This experiment was performed by Dr. Sandra Bright, Williams Lab, School of Biochemistry and Immunology, Trinity College Dublin].

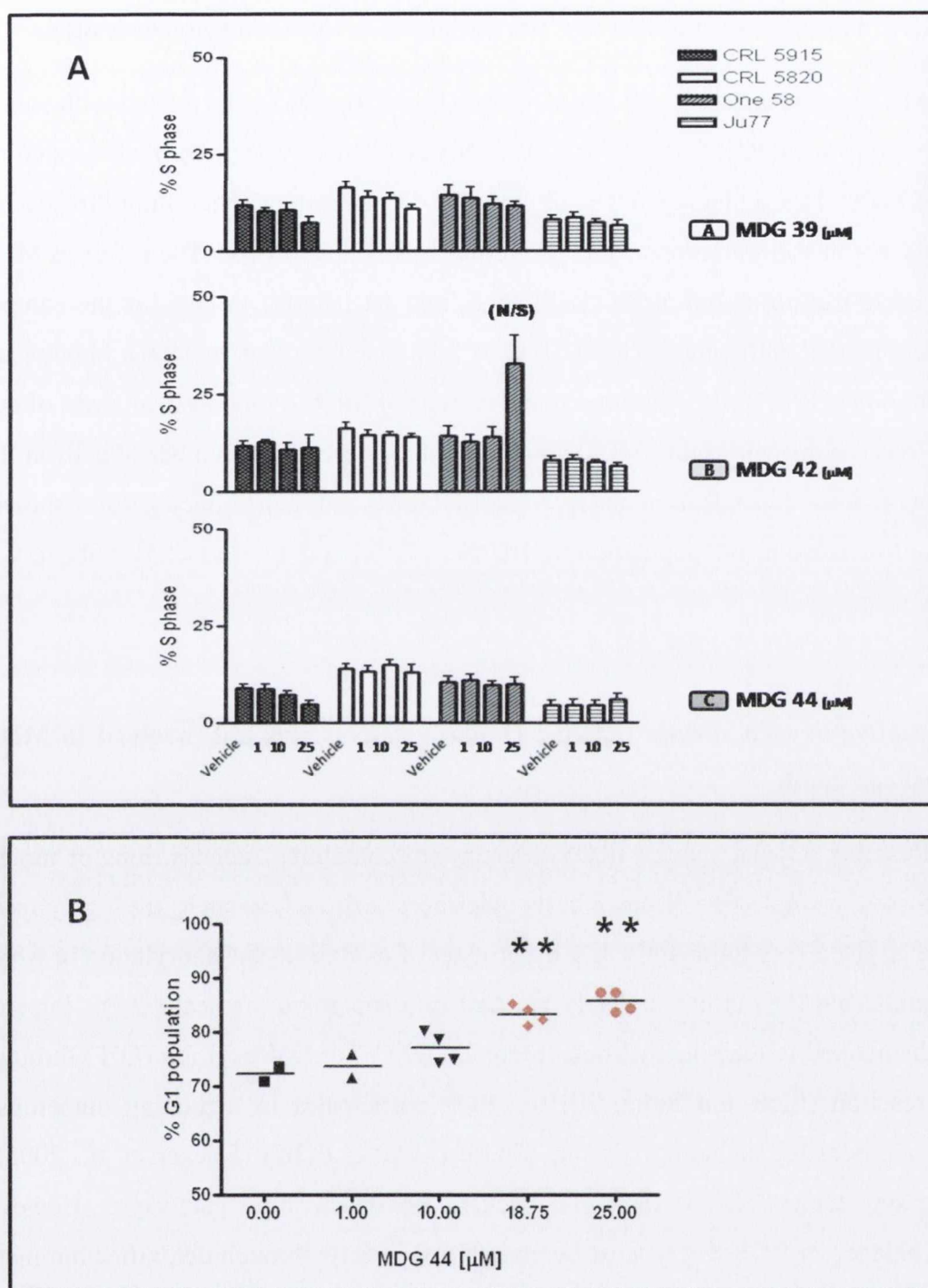


Figure 5.15: MDG 44 induces G1 cell cycle arrest at sub lethal concentrations in CRL 5915 cells.

(A) Lead MDG compounds did not induce S phase arrest in MPM cell lines. (B) MDG 44 treatment induces G1 phase cell cycle arrest in CRL 5915 cells. Cells were seeded at a density of 0.25×10^6 cells / T25 (5ml) and left to adhere for 18h prior to treatment. Proportion of cells per cell cycle stage were analysed by PI cell cycle analysis, as outlined in Section 2.7. Values are representative of mean $\pm$ SEM of three independent experiments. ** $P < 0.01$; [(N/S) – Not significant]. (Statistical analysis (unpaired Student's t-test) was performed using Graphpad Prism 4 statistical software).

5.9.2 MDG 44 induced G1 arrest in CRL 5915 cells at sub-lethal concentrations

PI cell cycle analysis was also performed on CRL 5915 cells treated with sub-lethal concentrations of MDG 44 (0 μ M - 25 μ M). This experiment was performed to examine the effect of MDG 44 at inducing cell cycle arrest at concentrations that do not directly induce apoptosis, but at concentrations which can elicit synergistic effects. The effect of MDG 44 on cell cycle transition and arrest could shed light on putative targets for the compound. Cells were treated with vehicle (EtOH 1%) or four concentrations of MDG 44 between 1 – 25 μ M for 48h (Fig 5.15.B). Various concentrations of MDG 44 induced an arrest of cells in the G1 phase of the cell cycle. MDG 44 increased the percentage number of cells in the G1 phase in a dose dependent manner, with 18.75 μ M and 25 μ M incubations showing a significant increase in G1 population of 10.9% ($\pm$ 2.2%) and 13.3% ($\pm$ 2.3%) above vehicle controls, respectively.

5.9.3 Reactive oxygen species (ROS) / Oxidative stress was not involved in MDG 44 mediated cell death

Reactive oxygen species (ROS) are oxygen containing radicals, ions or molecules which possess a single free electron in its outermost shell and, as such, are highly reactive. ROS are produced predominantly as a by-product of oxidative phosphorylation in which an oxygen molecule (O_2) is incompletely reduced to a superoxide radical ($O_2^{\cdot-}$). Superoxide can be dismutated to reactive hydrogen peroxide (H_2O_2) or hydroxyl ion ($OH^{\cdot}$) through the Fenton reaction (Liou and Storz, 2010). ROS participates in regulating numerous cell signalling processes, including tyrosine kinase (MAPK; PI3K) (Fischer et al., 2005) and transcription factors (NF κ B) (Sen and Packer, 1996) activation pathways. However, a delicate balance in ROS levels must be maintained in cells through detoxification methods mediated by catalase, glutathione and superoxide dismutase, in order to prevent oxidative stress (Percy, 1984). Excess levels of ROS are able to cause cell cycle arrest or death by damaging nuclear DNA, oxidative damage of proteins and lipids and damage within the mitochondria (mtDNA) causing apoptosis by release of cyto c and other pro-apoptotic inducers (Ott et al., 2007). Promoting increase in ROS and induction of oxidative stress is a mechanism employed by numerous chemotherapeutics in cancer including, anthracycline (Conklin, 2004), photo-dynamic therapy (PDT) agents (Xue et al., 2010), and radiotherapy (Gao et al., 2008).

The potential role of oxidative stress / ROS in MDG 44 mediated cell death was investigated using N-acetyl cysteine (NAC), an anti-oxidant that increases levels of free radical scavenger, glutathione, and prevents oxidative stress through detoxification of ROS (van Zandwijk, 1995). CRL 5915 cells were pre-treated for 1h with 5mM NAC prior to treatment with MDG 44 for 4h, 8h and 24h (Fig 5.16.A). Induction of apoptosis was determined by PI staining and Flow cytometry analysis. Comparable apoptotic effects were observed between cells treated with MDG 44 alone or cells pre-treated with NAC. Two-way ANOVA analysis confirmed NAC pre-treatment caused no significant reduction of MDG 44 induced apoptosis.

The chemotherapeutic Doxorubicin and the ROS agent Hydrogen Peroxide (H_2O_2) were used as positive controls for ROS mediated cell death in this assay. Incubation with increasing concentrations of H_2O_2 caused dose dependent induction of apoptosis, which was significantly inhibited by pre-treatment of cells with NAC (Fig 5.16.C). Doxorubicin is an anthracycline chemotherapeutic agent with pleiotropic mechanisms of inducing cell death that includes DNA damage, as well as, excess ROS generation / oxidative stress (Tsang et al., 2003). Pre-incubation of NAC was able to reduce apoptotic induction of apoptosis induced by doxorubicin, inhibiting the proportion of cell death in CRL 5915 cells mediated by ROS (Fig 5.16.B).

Results indicate that MDG 44 does not cause the generation of excess ROS and induction of oxidative stress is not the mechanism MDG 44 uses to induce apoptosis in MPM cells.

5.9.4 MDG 44 treatment induced an increase in cellular vesicles and morphological changes indicative of apoptosis in CRL 5915 cells

CRL 5915 cells incubated with MDG 44 were observed to display a distinct cellular morphology in culture. Under light microscopy, cells appeared to become more granular and induced formation of vesicles within the cytoplasm of the cell was observed. Cytospin and RapiDiff staining of MDG 44 treated cells was performed to detect the presence of morphological features indicative of apoptosis, investigate the formation of cytoplasmic vesicles within treated cells and gain a broader picture of the effect of MDG 44 treatment on cellular morphology.

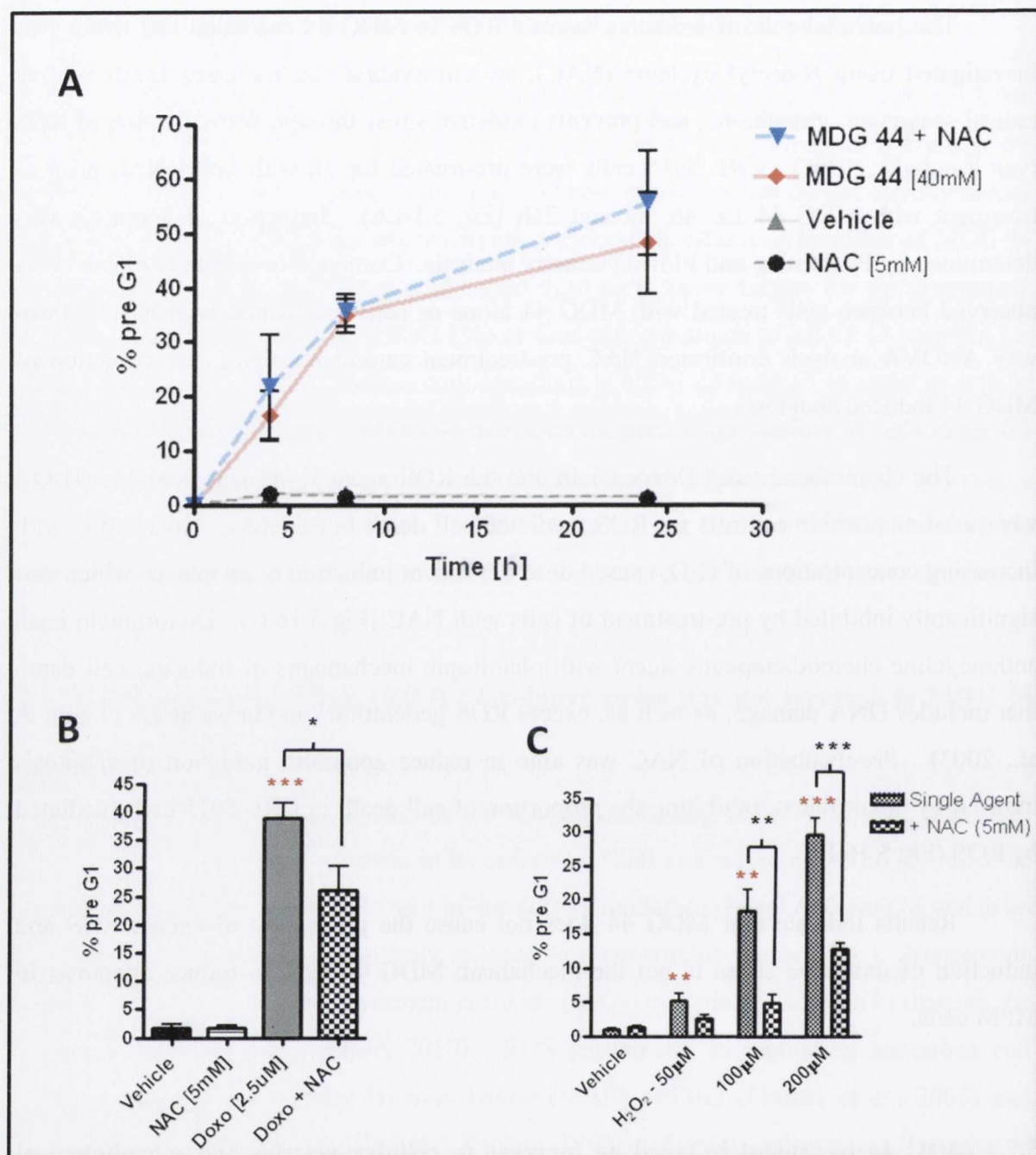


Figure 5.16: Reactive oxygen species (ROS) are not involved in MDG 44 induced cell death.

CRL 5915 cells were treated with indicated concentrations of MDG 44 +/- N-Acetyl Cysteine (NAC) [5mM] for 24h (A). Cells were treated with Doxorubicin (B) or Hydrogen Peroxide (H₂O₂) (C) +/- NAC for 48h as positives controls for ROS mediated apoptotic induction. CRL 5915 cells were seeded at a density of 0.25×10^6 cells / T25 (5ml) and left to adhere for 18h. Cells were pre-treated for 1h with 5mM N-acetyl cysteine (NAC), prior to treatment with test compounds for indicated time points. Apoptotic induction was analysed by PI staining and cell-cycle flow cytometry analysis, as outlined in section 2.7. Values are representative of mean $\pm$ SEM of three independent experiments. In (A), two way ANOVA analysis confirmed no significant difference between treatments ($P = 0.4994$). * $P < 0.05$; ** $P < 0.01$ (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

CRL 5915 cells were treated with Vehicle (EtOH 1%) and MDG 44 [40 μ M] for 3h – 8h time points. This time span represented the period of early apoptotic induction in CRL 5915 cells, as determined by previous time-dependent apoptosis experiments (Fig 5.4 and Fig 5.6). Cells were also treated with the Microtubule Targeting agent (MTA), vinblastine, as an alternate apoptotic control. Cytospin preparation, fixing and nuclear and cytoplasmic staining of cells was performed as outlined in Section 2.11.

In vehicle treatments, cells were observed to have intact plasma membranes, non-condensed nuclei, clear, evenly stained cytoplasm and evidence for active DNA replication and cell growth – Cells undergoing anaphase of Mitosis (Fig 5.17 – Fig 5.18); all morphological features of healthy, viable cells. In contrast, MDG 44 treatments induced distinct alterations in cell morphology. Classical, morphological features of apoptosis – DNA condensation and fragmentation (β) – were observed in cells between 3h – 6h incubations (Fig 5.17.a), and at late incubation time points (8h), complete cell shrinkage and death was observed (π) (Fig 5.17.b). As well as this, the presence of large cytoplasmic vesicles (α) was observed in MDG 44 treated cells, confirming observations from light microscopy.

Treatment with the MTA drug Vinblastine showed indications of mitotic arrest (δ) in CRL 5915 cells, as expected. However, no large cytoplasmic vesicles were detected in response to vinblastine incubations at any time point (Fig 5.18), suggesting this effect was specific to MDG 44 treatment and a possible indication of its cellular target.

Discussion

The original hypothesis of this study proposed the computational design of novel SMAC mimetic compounds able to sensitise IAP resistant MPM cells to conventional chemotherapy. Results revealed the series of novel compounds did not bind their designed target but lead compounds did elicit potent single agent and, importantly, synergistic apoptotic effects in MPM cells through an alternate mechanism. In this Chapter, we report that lead compound MDG 44 specifically induced a Type I, apoptotic form of PCD. Induction of apoptosis was observed in all four MPM cell lines tested, detected by an increase in the apoptotic pre-G1 population and cleavage of the caspase target and nuclear protein, PARP. Apoptosis was detected at concentrations of approximately 40 μ M MDG 44

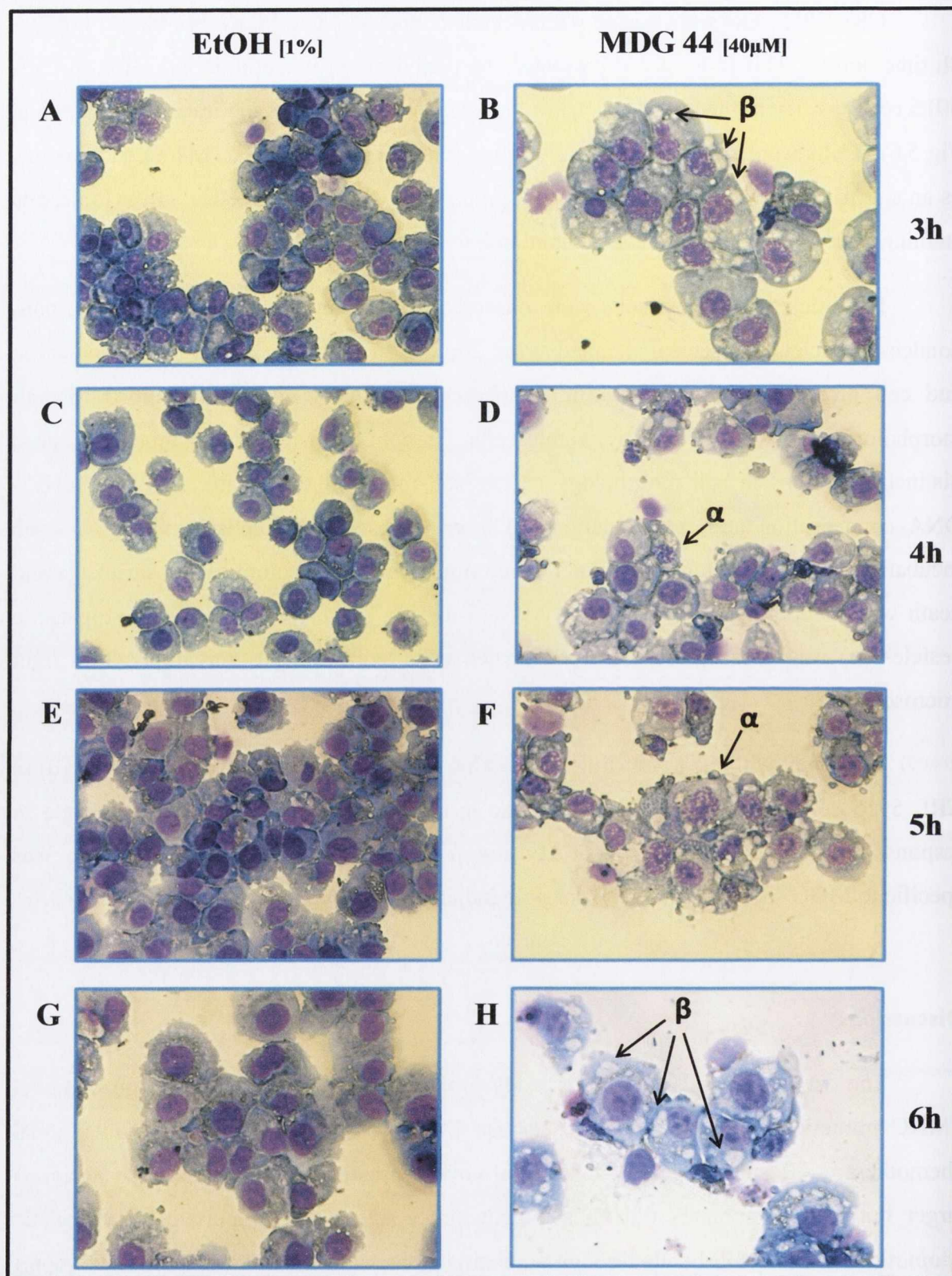


Figure 5.17.a: MDG 44 treatment induced an increase in large cytoplasmic vesicles and cellular morphology indicative of apoptosis.

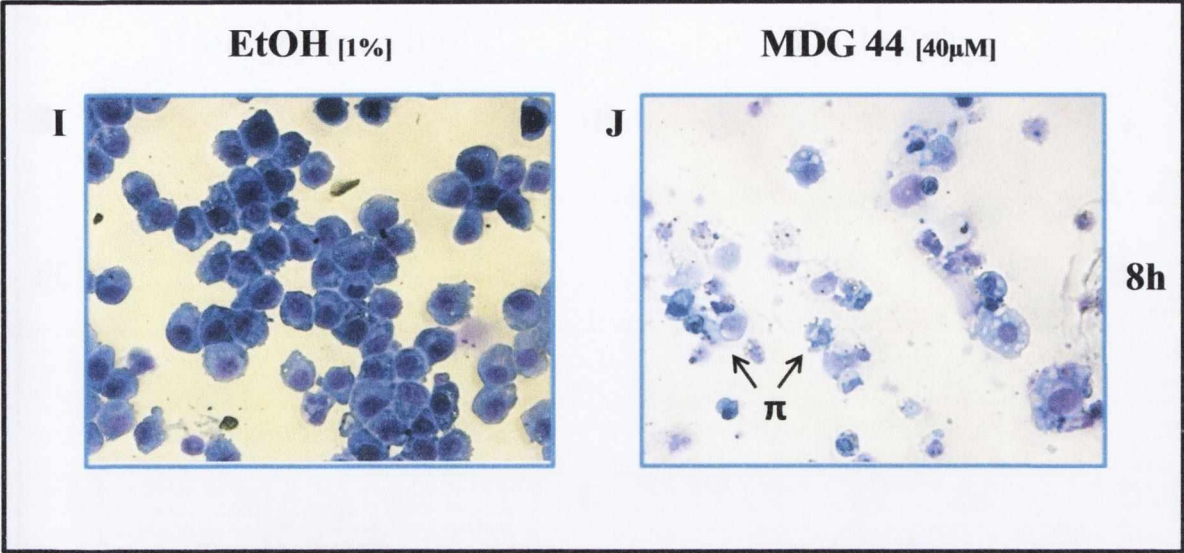


Figure 5.17.b: MDG 44 treatment induced an increase in large cytoplasmic vesicles and cellular morphology indicative of apoptosis.

CRL 5915 cells were seeded at a density of 0.25×10^6 cell / T25 (5ml) and treated with EtOH [1%] Vehicle or MDG 44 [40μM] for 3h (A & B), 4h (C & D), 5h (E & F), 6h (G & H), and 8h (I & J). Cytospins were prepared and stained as outlined in Section 2.11. Slides were viewed and photographed on a Zeiss Axiovert microscope, under 20X magnification, and changes in cellular morphology with MDG 44 treatment were indicated, including DNA fragmentation (α), large cellular vacuoles (β) and cell death (π).

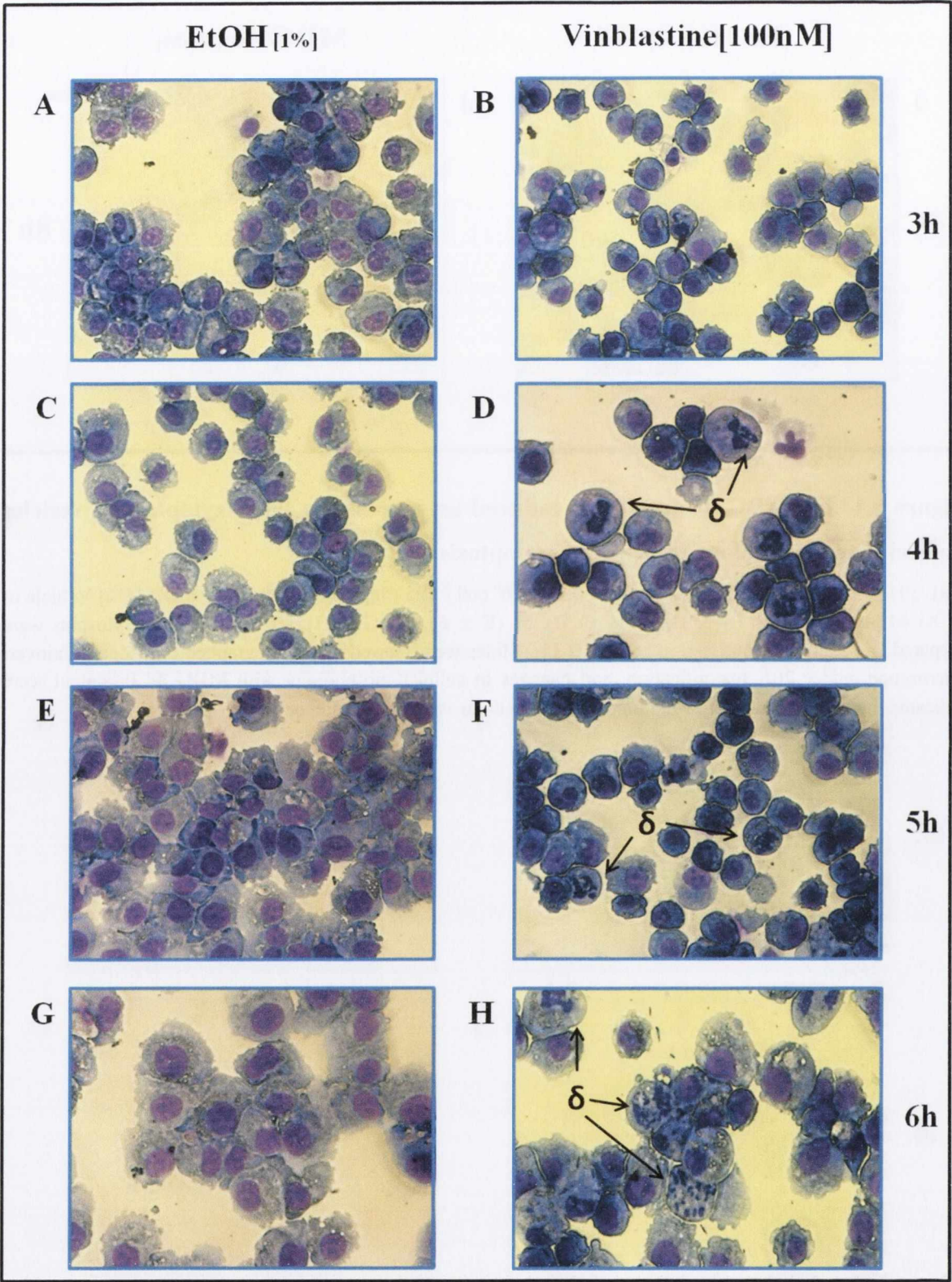


Figure 5.18.a: Vinblastine treatment induced the cellular morphology indicative of mitotic arrest.

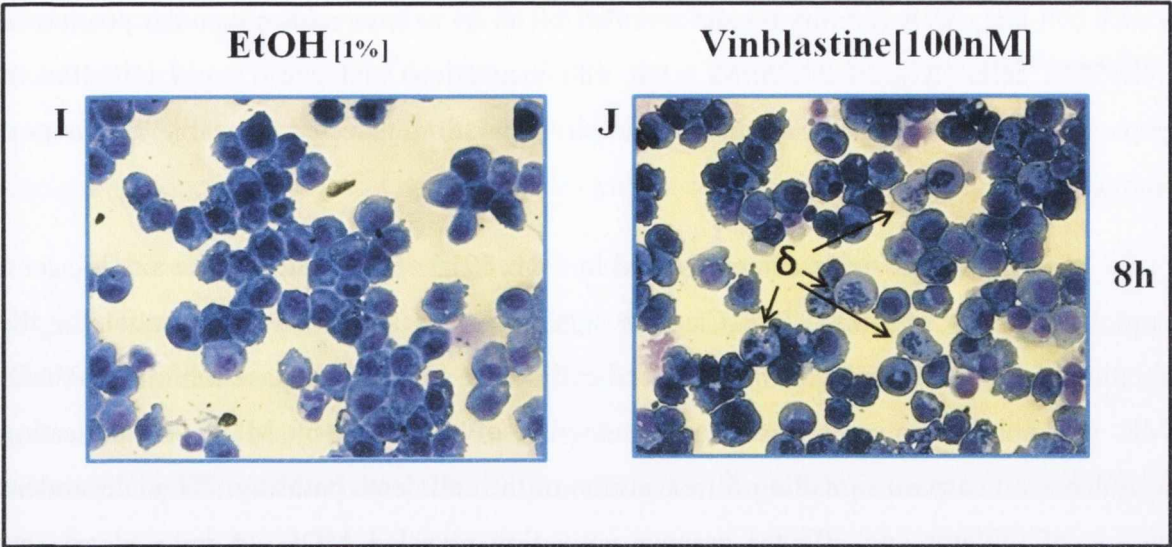


Figure 5.18.b: Vinblastine treatment induced the cellular morphology indicative of mitotic arrest.

CRL 5915 cells were seeded at a density of 0.25×10^6 cell / T25 (5ml) and treated with EtOH [1%] Vehicle or Vinblastine [100nM] for 3h (A & B), 4h (C & D), 5h (E & F), 6h (G & H), and 8h (I & J). Cytospins were prepared and stained as outlined in Section 2.11. Slides were viewed and photographed on a Zeiss Axiovert microscope, under 20X magnification, and changes in cellular morphology with Vinblastine treatment were indicated; Mitotic (G2/M) arrest (δ),

in each cell line. Cell viability results revealed MDG 44 to have mid-micomolar potency in CRL 5915 cells (IC_{50} of $31.25\mu M \pm 0.4$, 48h incubation) and cause rapid initiation of apoptosis, with an increase in apoptotic cell population detected as early as 4h post treatment.

Multiple apoptosis assays performed in CRL 5915 cells confirmed the single-agent, apoptotic capacity of MDG 44. Caspase signalling was shown to be essential for the initiation and execution of MDG 44 induced cell death. The pan-caspase inhibitor z-VAD-FMK was able to completely abrogate induction of apoptosis by MDG 44, indicating dependence on caspase signalling for execution of the cell death pathway. Time dependent analysis of initiator and effector caspase activation revealed MDG 44 induced caspase signalling via the intrinsic apoptotic pathway. Using caspase specific fluorogenic substrates, activation of caspase-9 and caspase-3, but not caspase-8, was detected after only 3h incubation with MDG 44 and increased in a time dependent manner. The involvement of caspase-9 and the intrinsic apoptotic pathway was confirmed by the dependence of MDG 44 induced cell death on caspase-9 signalling (z-LEHD-FMK inhibitory assay) and the detection of proteolytically cleaved, active caspase-9 in MDG 44 treated samples (Western Blot analysis). Importantly, caspase activity also correlated with the time dependent induction of apoptosis – significant levels of apoptosis after 4h incubation ($14.7\% \pm 1.5$) –, indicating that caspase activation and signalling via the intrinsic apoptotic pathway was participatory in the induction of apoptosis and not activated non-specifically during cell death.

Apoptotic induction was also confirmed by assaying alternate markers for type I PCD. Annexin V / PI staining revealed MDG 44 induced an increase in “late-apoptotic” cells, positively stained for plasma membrane translocated phosphatidylserine. The effect of MDG 44 on DNA damage was detected by Comet assay and Cytospin RapiDiff nuclear staining. The formation of comet tails and observation of fragmented DNA present in MDG 44 treated cells are classical indicators of apoptotic cell death.

A significant difference in single-agent and synergistic apoptotic effects for MDG 44 were detected. Analysis of MDG 44 and Cisplatin combinational treatments confirmed that MDG 44, at sub-lethal concentrations, synergistically increased Cisplatin induced apoptosis. Synergistic increases in Parp cleavage, pre G1 population, Caspase activation and Annexin V + staining were all observed with MDG 44 co-treatment. Synergistic effects were

observed at concentrations as low as 15 μ M MDG 44, compared to single agent apoptosis and PARP cleavage observed in CRL 5915 cells at concentrations of 40 μ M and above. Importantly this shows that MDG 44 can synergistically increase apoptosis induced by cisplatin at concentrations that do not induce cytotoxicity on their own.

The confirmation of a synergistic apoptotic effect with MDG 44 in combination with Cisplatin was of particular interest for MPM therapeutic development. Current, clinical treatment regimens and numerous phase trials in MPM employ Cisplatin as the co-treatment option of choice (Belli et al., 2009). The discovery of a compound with the ability to enhance Cisplatin induced apoptosis is promising for its development as a therapeutic in MPM.

Mechanistic analysis of how MDG 44 induces caspase dependent apoptosis revealed a number of interesting cellular effects induced by MDG 44 in MPM cells. Classical cell stress mechanisms employed by conventional chemotherapeutics to induce an intrinsic apoptotic pathway response were investigated. Profiling the cellular effects of MDG 44 was hoped to elucidate possible targets for further in-depth examination. Cell cycle analysis revealed that all MDG lead compounds (MDG 39, 42 & 44) did not inhibit S – G2/M phase transition in any of the four MPM cell lines tested, unlike the SMAC mimetic embelin. Recent reports have shown that embelin can induce cell cycle arrest (S phase) and apoptosis through mechanisms other than XIAP targeting, including inhibition of NF κ B activation and downregulation of cFLIP (Joy et al., 2010, Ahn et al., 2007, Siegelin et al., 2009). The lack of S phase arrest with MDG compound treatment suggests engagement of a target by MDG 44 that is distinct from SMAC mimetic-like activity. MDG 44 treatment was able to induce G1 phase arrest in CRL 5915 cells, at sub-lethal concentrations. A dose dependent increase in the G1 population was observed, with significant increases at 18.75 μ M and 25 μ M, concentrations of MDG 44 shown to synergise with cisplatin.

Analysis of ROS activity revealed that oxidative stress was not involved in MDG 44 mediated cell death. Co-incubation with the free radical scavenger N-acetyl cysteine (NAC) had no effect on MDG 44 induced apoptosis.

Cytospin cell morphology analysis gave further confirmation that MDG 44 treatment specifically induced an apoptotic programme of cell death, with classical features of apoptosis, like chromatin condensation and DNA fragmentation, evident in treated samples. Alterations in cytoplasmic morphology were also detected in response to treatment. MDG

44 incubation resulted in the formation of large cytoplasmic vesicles at time points as short as 3h post treatment. These cytoplasmic vesicles were not observed in vehicle control or vinblastine treated cells, indicating this effect was specific to MDG 44. It was suspected that these large cytoplasmic vesicles could be autophagosomes; double membraned vesicles formed during autophagy (Kroemer et al., 2010).

Macroautophagy is a highly regulated method of lysosomal degradation of cellular components, including damaged organelles, long lived proteins and protein aggregates, and acts as a complementary protein degradation process to the ubiquitin-proteasome system, which regulates short lived proteins involved in the cell cycle (cyclin dependent kinases), signal transduction (I κ B; regulator of NF κ B) and apoptosis (IAPs) (Wu et al., 2010). Macroautophagy can be activated in response to multiple cellular stress factors including nutrient starvation (mTORC1 signalling), hypoxia and ER stress / uncoupled protein response (UPR) (Kroemer et al., 2010). Induced activation of numerous autophagy related gene (ATG) protein complexes drives the nucleation and elongations of a double lipid membrane structure (phagophore) which can selectively uptake protein aggregates or non-selectively sequester organelles, protein aggregates and portions of the cytoplasm into mature autophagosomes. These cytoplasmic vesicles fuse with lysosomes to degrade sequestered components in order to remove harmful misfolded protein aggregates or recycle cellular material to maintain nutrient and energy homeostasis (Yang and Klionsky, 2010, Mizushima et al., 2008). The cytoplasmic structures detected in response to MDG 44 treatment strongly resemble autophagosome vesicles observed in previous studies (O'Sullivan-Coyne et al., 2009, Edinger and Thompson, 2004, Zhu et al., 2010).

In conclusion, investigations in Chapter 5 revealed that MDG 44 specifically induces Type I, apoptotic PCD as a single-agent and can synergistically enhance Cisplatin induced apoptosis in MPM. Analysis of cellular effects induced by MDG 44 treatment revealed induction of G1 arrest and the formation of large cytoplasmic vesicles, suspected to be autophagosomes. In the next chapter, putative targets and mechanisms of action for MDG 44 activity were investigated. The potential role of autophagy and the cause for the accumulation of autophagosomes in response to MDG 44 treatment in MPM was also investigated.

Chapter 6

Examination of alternate molecular targets and mechanisms of action for MDG 44 induced apoptosis in Malignant Pleural Mesothelioma

Introduction

Experimentation in Chapter 6 proposed to investigate further the molecular target and mechanism of action of MDG “hit” compound, MDG 44. Results in Chapter 5 revealed that MDG 44 could potently (mid-micromolar concentrations) induce an apoptotic program of cell death as a single-agent chemotherapeutic and at sub-lethal concentrations, synergistically potentiate Cisplatin induced apoptosis in an *in vitro* MPM model. However, while potent anti-cancer activities as single-agent and combinational therapeutics in this highly resistant cancer cell model were established, the molecular target to which MDG 44 binds and through which it elicits its anti-cancer activity was unknown. Investigations in Chapter 4 assayed MDG 44 and its analogues for activity as SMAC mimetic compounds, assessing their affinity for the computationally predicted target of XIAP-BIR3 and their secondary putative target, cIAP-1/2. This “On Target” experimentation revealed that, while a ligand-based design approach based on the SMAC N-terminal tetrapeptide (AVPI) proved a successful model in previous published reports (Seneci et al., 2009, Sun et al., 2004, Crisostomo et al., 2009), in-house pharmacophore design and virtual screening of compound databases failed to identify novel SMAC mimetic hits with affinity for IAPs.

The anti-cancer activity of “hit” compound MDG 44, in particular its capacity to synergise with Cisplatin in MPM made it an interesting novel structure with activity deemed significant enough for further investigation, irrespective of the lack of target identification. As outlined in Chapter 1, development of effective new therapies for resistant MPM has been largely unsuccessful (Jakobsen and Sorensen, 2011). Identification of a novel compound effective in combinational treatments with a current clinical treatment partner in MPM (Cisplatin) may represent a promising novel strategy for drug development. As the molecular target of MDG 44 was not known, this interrupted the framework for the rational drug design process originally proposed. The lack of structural knowledge of the target binding pocket impeded the computational approach to rational design of a second generation of MDG 44 / 2-indoloinone scaffold class of compounds. The lack of target information also impedes our ability to comprehend the cellular mechanism through which MDG 44 can synergise with the genotoxic activity of Cisplatin. Whether MDG 44 represents a novel structure for a known pathway or a novel pathway for apoptotic synergism with Cisplatin and other genotoxic agents could not be inferred. For this reason, target identification for MDG 44 was a priority in order to aid the future development of this class of compound as an anti-cancer agent.

Experimentation in Chapter 4 and 5 revealed a number of interesting cellular responses to MDG 44 treatment in MPM cell lines. Considering the cellular effects observed with MDG 44 incubation in MPM would help propose rational putative targets of MDG 44 for further investigation. Apoptotic analysis confirmed MDG 44 caused activation of the intrinsic apoptotic pathway in MPM. Analysis of cell stress mechanisms in MPM revealed MDG 44 treatment did not provoke oxidative stress but did induce G0/G1 arrest of cells at sub-lethal concentrations. Morphological analysis of cells confirmed the accumulation of large cytoplasmic vesicles within MPM cells in response to MDG 44 incubation. Initial analysis suggested these vesicles were autophagosomes. In Chapter 4, an "On Target" cIAP-1/2 degradation assay revealed that, contrary to initial expectations, MDG 44 incubation increased protein levels of the proteasome-regulated protein, cIAP-1 in all four MPM cell lines. The absence of cIAP degradation ruled out targeting of cIAP-1/2 as a mechanism of action for MDG 44, however the induced accumulation of cIAP-1 protein represented an interesting cellular response to this compound.

In this study, three separate modes of investigation were performed in an attempt to elucidate the molecular target of MDG 44 in MPM. The first method employed a compound patent search. Filed patents for molecules similar in structure to that of MDG 44 (i.e.: containing the 2-indolinone basic scaffold present in MDG compounds) were searched for in patent databases. Considering the targets and mechanisms of action reported for patent protected 2-indolinone scaffold compounds, was hoped would propose putative targets for investigation with MDG 44. The stated activity of similar classes of compounds to MDG 44 could represent rational mechanisms of action to describe the reported activity of this test compound.

The second mode of target investigation in this study identified the peroxisome proliferator activated receptor- γ (PPAR- γ) as a potential target of MDG 44. A serendipitous discovery revealed that compound MDG 588, an experimental PPAR- γ modulator under investigation with collaborators in the Molecular Design Group, Trinity College Dublin, contained the same 2-indolinone scaffold present in MDG 44. The structural similarities and alterations at key points of substitution (R1 and R2) between MDG 588 and MDG 44 were illustrated in Figure 6.1. At position R1, MDG 588 contains a similar hydrophobic propan-2-ylbenzene substitution to the tetra-butylbenzene moiety present on MDG 44. MDG 588 contains a methylmorpholine substitution at R2, compared to a 1, 2, 4- methylbenzene substitution present at the same position on MDG 44. MDG 588 was a computationally

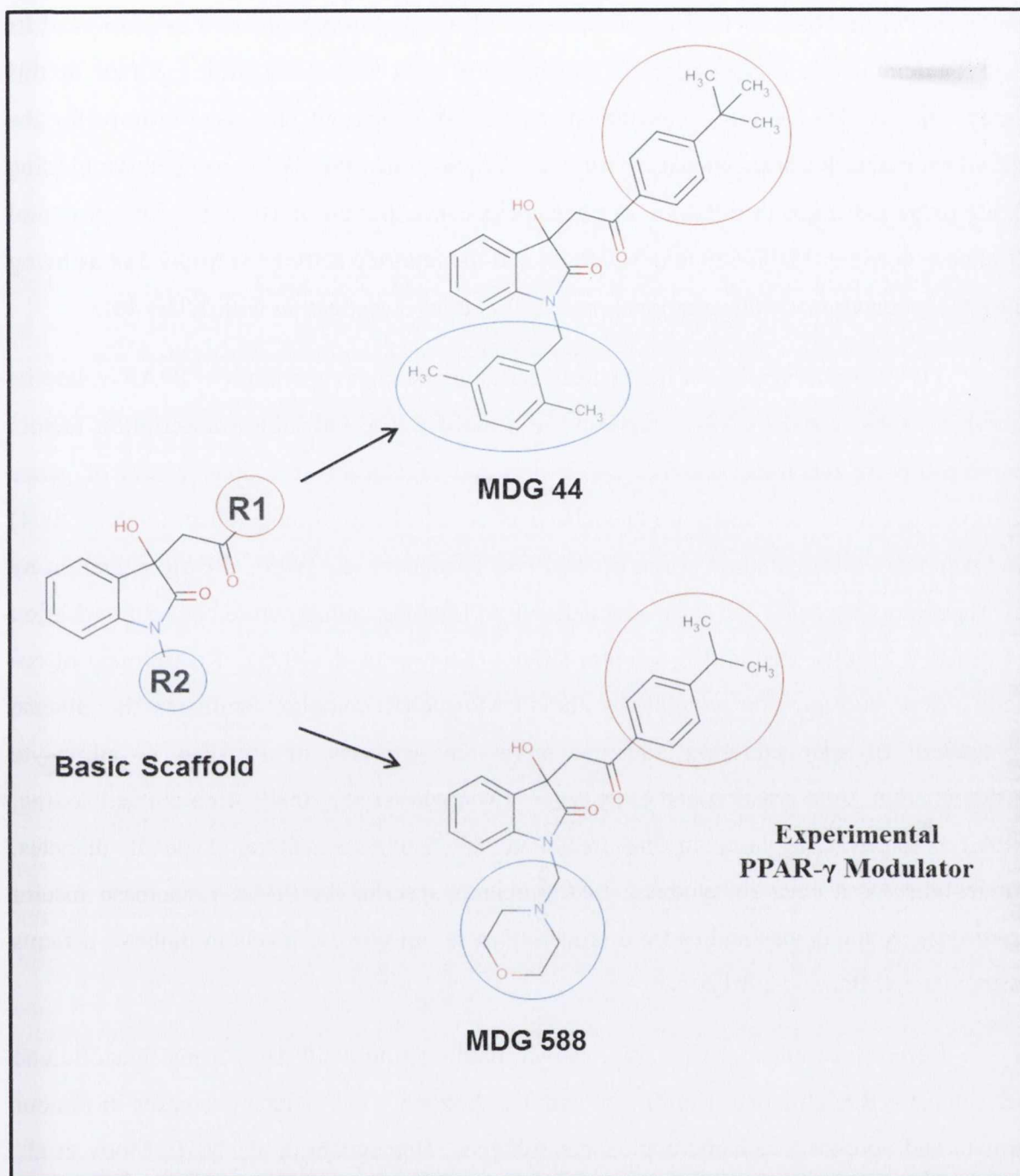


Figure 6.1: Chemical structures and structural similarities of test compound MDG 44 and experimental PPAR- γ modulator MDG 588

The basic MDG SMAC mimetic scaffold with substitution points R1 and R2 are indicated. The alternate structural substitutions in MDG 44 and MDG 588 at addition points R1 and R2 on the basic indolinone scaffold are shown.

predicted “virtual hit” for the nuclear receptor, PPAR- γ , and experimental evaluation of its activity as a PPAR- γ modulator ran concurrently with experimentation reported in this study. Initial “On Target” evaluation of MDG 588 showed it possessed affinity for the PPAR- γ binding pocket, assayed by way of a single-point FP-PPAR- γ competitive binding assay (60% reduction in mP units at a screening concentration of 100 μ M). The structural similarity between MDG 588 and MDG 44, and the reported activity of MDG 588 as being a PPAR- γ ligand, made this a rational protein for target investigation with MDG 44.

PPAR- γ is a member of the nuclear hormone receptor superfamily. PPAR- γ , and its isotypes PPAR- α and PPAR- β , represent a class of ligand-inducible transcription factors participatory in the transcriptional expression and regulation of a diverse sets of genes involved in cell cycle transition (Theocharis et al., 2004), lipid metabolism (Nahle, 2004) and adipocyte differentiation (Brun et al., 1996, Forman et al., 1995). PPAR- γ ligation by hydrophobic fatty acids and fatty acid derivatives (Prostaglandins) drive heterodimerisation of PPAR- γ with the Retinoid X receptor (RXR) (Kliwer et al., 1999). Recruitment of co-activator or co-repressor proteins by the PPAR- γ :RXR complex facilitates the diverse framework of transactivation and transrepression activates of PPAR- γ in adipocyte differentiation, inflammation and even cancer (Michalik et al., 2004). In a clinical setting, PPAR- γ targeting is used in the treatment of insulin-insensitive, Type II diabetes. Thiazolidiones, a class of synthetic PPAR ligands specific for PPAR- γ , increase insulin sensitivity in adipocytes and cause a reduction in serum glucose levels in diabetic patients (Lebovitz and Banerji, 2001).

Recent preclinical studies have shown that targeting of PPAR- γ using thiazolidione derivatives – Rosiglitazone, Ciglitazone and Troglitazone – can induce decreases in tumour growth and apoptosis in numerous cancer subtypes (Hamaguchi et al., 2010, Mody et al., 2007). PPAR- γ has been shown to be widely expressed in cancer and its regulation of cellular differentiation and metabolism is one mechanism proposed for its anti-cancer activity (Rosen and Spiegelman, 2001, Michalik et al., 2004). Combinational studies involving thiazolidione compounds and platinum-based drugs, including cisplatin, have reported promising, synergistic anti-cancer activities (Girnun et al., 2007, Reddy et al., 2008). The mechanism of synergism between PPAR- γ ligation and Cisplatin has been proposed to be via PPAR- γ -mediated downregulation of metallothioneins, proteins involved in resistance to platinum-based therapy (Girnun et al., 2007). The efficacy of PPAR- γ targeting specifically in MPM has also been recently proposed. A study by *Hamaguchi et al*

has reported on the anti-cancer activity of thiazolidione compounds in *in vitro* and *in vivo* MPM models (Hamaguchi et al., 2010). Troglitazone was shown to induce G0/G1 arrest and apoptosis as a single-agent therapy and cause an additive inhibitory effect on MPM tumour growth when in combination with Cisplatin.

The serendipitous discovery of MDG 44 containing the same scaffold as an experimental PPAR- γ modulator, together with the above reports on the anti-cancer activity of PPAR- γ , qualified this target for further investigation with MDG 44. Specific cellular activities attributed to PPAR- γ activation in cancer matched those cellular effects reported for MDG 44 treatment in MPM, making PPAR- γ a rational target for study. Of particular interest was the synergistic / additive anti-cancer activity observed in combinational studies involving Cisplatin and PPAR- γ agonists. In this Chapter, experiments were performed to test whether MDG 44 was a ligand for PPAR- γ and if its apoptotic activity in MPM was mediated through PPAR- γ activation. The role of MDG 44 and PPAR- γ activation in the formation of large cytoplasmic vesicles in MPM cells was also investigated. A theory was proposed that the large cytoplasmic vesicles formed in response to MDG 44 incubation were in fact peroxisomes.

Peroxisomes are cellular organelles ubiquitous in eukaryotes, involved in fatty acid metabolism (beta oxidation) and detoxification of molecules through the peroxidation reaction (hydrogen peroxide oxidation of toxic intermediates) (Schrader and Fahimi, 2008). Peroxisome number, division, proliferation and turnover is a highly regulated process, sensitive to peroxisomal metabolic requirements of the cell – increases in fatty acid uptake causes an increase in peroxisomal activity (Yan et al., 2005). In mammalian cells, peroxisome proliferation, the process of rapid increase in peroxisome number (Yan et al., 2005), can be regulated by PPAR nuclear receptors. PPAR- γ ligation and activation can initiate the transcription of *Pex* genes, a family of proteins associated with peroxisome regulation. Upregulation of *Pex11 α/β* by PPAR activation is sufficient to drive the proliferation of peroxisomes in mammalian cells (Schrader et al., 1998, Shimizu et al., 2004). While investigating PPAR- γ as the molecular target of MDG 44, the role of PPAR- γ in the formation of cytoplasmic vesicles in MPM was studied and experiments were performed to determine if these vesicles were peroxisomes.

The final target for investigation in this Chapter was that of the Ubiquitin-Proteasome System (UPS) and its mechanistic interaction with autophagy. The UPS system

is associated with the selective degradation of short-lived proteins which participate in transient cellular processes of cell cycle transition, cell growth and apoptosis (Wu et al., 2010a). The UPS is also a regulator of protein quality, contributing to the clearance of misfolded proteins, which could otherwise cause toxicity and cell death (Kostova et al., 2007). The UPS exist as a two tiered network: The chymotrypsin-like activity of the 20S catalytic subunit within the 26S proteasome facilitates degradation of targeted proteins; the ubiquitin enzyme network of activating (E1), conjugating (E2) and ligation (RING-E3 / HECT-E3 domain) enzymes, “tag” selected proteins, through Lys48 poly-ubiquitin conjugation, for proteasomal degradation by the 26S proteasome (Eldridge and O'Brien, 2010). The ubiquitin network of enzymes, containing hundreds of target-specific E3-ligases, provides regulation and specificity to the proteasomal degradation process. Ubiquitination activities have recently been shown to induce diverse effects in cells, besides simply ubiquitin-tagging proteins for proteasomal degradation. The formation of alternately conjugated ubiquitin chains can have a diverse effect on protein activities and fates. The altered topology of Lys68 linked poly-ubiquitin chains, compared to Lys48 ubiquitin proteasomal degradation tags, can form binding sites for protein adapters and regulate signal transduction events, as outlined previously with cIAP-1/2 (RING-E3 ligase) regulation of Rip1 signalling (Bertrand et al., 2008).

Autophagy is an alternate protein degradation pathway in which long-lived proteins, protein aggregates and defective organelles can be sequestered into double membrane enclosed autophagosomes and ultimately subjected to lysosomal degradation (Mizushima et al., 2008). The autophagic pathway is characterised by this morphological change in which increased formation of double-membraned cytoplasmic vesicles occurs (Bursch, 2001). As reported above, MDG 44 treatment was seen to induce the formation of large cytoplasmic vesicles, suspected to be autophagosomes, in MPM cells. In this Chapter, the autophagy hypothesis was tested. The effect of MDG 44 treatment on the accumulation of autophagosomes and on complete autophagic flux within MPM cells was examined. This was assaying by the detection of markers for autophagosome formation (LC3-II) and for lysosomal degradation (p62). The contribution of autophagy to MDG 44 induced PCD was also assessed.

The UPS system and in particular proteasome inhibition was proposed as a direct molecular target for MDG 44. As outlined above, experimentation in Chapter 4 revealed that MDG 44 induced increased expression of the proteasome-regulated protein, c-IAP-1.

cIAP-1 is a regulator of signal transduction and apoptosis and, as such, its cellular expression levels are highly regulated by the UPS. cIAP-1 itself contains a RING E3-ligase domain, which under pro-apoptotic conditions (SMAC release) induces auto-ubiquitination and rapid proteasomal degradation (Yang and Du, 2004). The accumulation in cIAP-1 expression in response to MDG 44 incubation suggested proteasome inhibition as an interesting putative molecular target.

Also of interest for MDG 44 target investigation, was the reported mechanistic link between proteasome inhibition and induction of autophagy. A number of mechanisms have been reported as to how direct proteasome inhibition can induce increased autophagic flux. Both the UPS and autophagy are two sides of a complimentary protein degradation program. In general, it has been observed that inhibition of the proteasome results in a compensatory increase in autophagic activity in order to remove misfolded and ubiquitinated proteins that accumulate due to diminished proteasomal activity. Poly-ubiquitinated proteins that accumulate due to proteasome inhibition can form protein aggregates or aggresomes, which can be localised to the autophagosome by p62, for eventual lysosomal degradation (Bjorkoy et al., 2005). Proteasome inhibition has also been shown to induce ER stress and the unfolded protein response, also resulting in autophagy activation to remove accumulated misfolded protein (Ding et al., 2007). This complementary activity exists in attempts to maintain cellular homeostasis. Inhibition of the proteasome has also been shown to prevent normal proteasomal regulation of particular autophagy proteins expression. The accumulation of these proteins facilitates increased autophagic flux. These proteins include a raptor interacting protein that negatively regulates mTor activity (Wu et al., 2010b) and LC3, a constitutive protein in autophagosome formation (Wu et al., 2008).

Proteasome inhibitors are an expanding class of chemotherapeutic agents, under investigation in a number of malignancies (Wu et al., 2010a). Proteasome inhibitors have been reported to possess diverse mechanisms of anti-cancer activity. Inhibition of the proteasome disrupts the regulated expression of cell cycle regulators (p53 and p21) (Blagosklonny et al., 1996, Maki et al., 1996) and pro- and anti-apoptotic protein families (Bcl-2) (Lomonosova et al., 2009). This loss of control of expression can result in increased capacity for apoptotic induction. Accumulation of poly-ubiquitinated and misfolded proteins can induce ER stress and PCD (Ding et al., 2007). However, as previously described, incubation with proteasome inhibitors can also induce autophagy and elicit a

cytoprotective response in certain cancers (Zhu et al., 2010). Recently, the proteasome inhibitor, Bortezomib was approved for clinical use in the treatment of multiple myeloma and mantle cell lymphoma (Orlowski et al., 2002, Bedford et al., 2011). These compounds have classically shown best activity in haematological malignancies characterised by a dependence on proteasomal activity (Bianchi et al., 2009). However, numerous recent studies have reported on the anti-cancer activity of various proteasome inhibitors in solid tumours, including Malignant Pleural Mesothelioma. A genetic array study of malignant mesothelioma subtypes identified altered UPS gene expression being associated with poor-prognosis in mesothelioma (biphasic subtype) (Borczuk et al., 2007). The UPS represented a viable therapeutic target and treatment with the proteasome inhibitor, Bortezomib, was shown to synergistically increase the efficacy of platinum based therapy (oxaliplatin) *in vitro*. Other studies have reported on the apoptotic activity of proteasome inhibitors MG132 and Bortezomib in *in vitro* and *in vivo* models in MPM (Sartore-Bianchi et al., 2007, Yuan et al., 2008). Of particular interest was a study by Gordon *et al*, in which the *in vitro* efficacy of Bortezomib in combination studies with both Cisplatin and Pemetrexed was analysed in a panel of MPM cell lines, which included the cell lines, CRL 5820 (NCI-H28) and CRL 5915 (NCI-H2052) (Gordon et al., 2008). Bortezomib treatment induced cell cycle arrest, apoptosis and synergised with cisplatin and pemetrexed in a sequence dependent manner. Treatment with Bortezomib was also able to selectively increase expression of cIAP-1 in CRL 5915 and CRL 5820 cell lines, with no effect on cIAP-2 or XIAP expression.

The effects of proteasome inhibition on cIAP-1 expression, synergism with Cisplatin and induction of autophagy matched cellular effects observed or proposed with MDG 44. In this study, the UPS pathway and particularly the proteasome was analysed as being a potential molecular target of MDG 44.

Results

6.1 A compound patent search for 2-Indolinone / MDG 44 scaffold compounds did not identify any molecular targets for investigation with MDG 44

In an attempt to identify avenues for investigating the molecular target of MDG 44, a patent search for small molecules inhibitors which were similar in chemical structure to

MDG 44 was performed (Patent search performed by Georgio Carta, Molecular Design Group, Trinity College Dublin). It was hoped that identifying compounds of a similar basic scaffold and with a known molecular target would help propose rational targets for investigating the apoptotic mechanism of action of MDG 44. Three filed patents were identified containing compounds with the 2-indolinone / MDG basic scaffold (Fig 6.2.A).

The first patent (Patent I.D WO/2009/133294) protected a class of Spleen tyrosine kinase (Syk) inhibitors which act as immunomodulators, inhibiting Mast cell degranulation. This class of compounds include the lead compound C-13 and its active analogue, Compound α , which contains the 2-indolinone MDG scaffold, as well as 2,4-dimethoxyphenyl and Naphthylmethyl substitutions at positions R1 and R2 on the MDG basic scaffold (Fig 6.2.B – i – ii). Syk is a cytoplasmic tyrosine kinase predominantly involved in signal transduction as an adapter molecule in multi-protein receptor complexes (Riccaboni et al., 2010). In immune cells, in particular B-cells and Mast cells, Syk is a key modulator of immunoreceptor signalling and, as such, is an attractive target for the design of therapies for allergy and auto-immune disorders. Syk associates with the IgE receptor complex, Fc ϵ R1, in Mast cells, transducing signals for Mast cell degranulation – histamine and cytokine release, promoting inflammation. In an allergic response, inhibiting Syk signal transduction downstream of allergen bound IgE receptor ligation prevents Mast cell degranulation and an inappropriate immune response. C-13 and Compound α have been shown to act as anti-allergy compounds, barring Mast cell degranulation by inhibiting the protein-protein interaction between the N-terminal SH2 domain of Syk and the Fc ϵ R1 receptor complex (Fig 6.2.B.iii) (Villoutreix et al., 2011) These compounds did not bind to the tyrosine kinase active site of Syk.

The second patent (Patent I.D WO/2008/083326) investigated described a class of peptidic and small molecule inhibitors of Ewings Sarcoma family tumours (ESFT), specifically targeting the protein-protein interaction between the ESFT fusion protein EWS-FLI1 and RNA helicase A (RHA). ESFT is a rare disorder of bone and soft tissue characterised by the chromosomal translocation t(11:22), involving the Ewings Sarcoma breakpoint region 1 gene (EWSR1; chromosome 22) and Friend Leukaemia integration 1 gene (FLI1; chromosome 11), forming the fusion protein EWS-FLI1 (Erkizan et al., 2010). This oncogenic transcription factor, or a similar variant, are present in 95% of ESFT and its association with RHA is thought to be essential for cell transformation and sustaining tumour growth (Toretsky et al., 2006). Compounds NSC635437 and YK-4-279, described

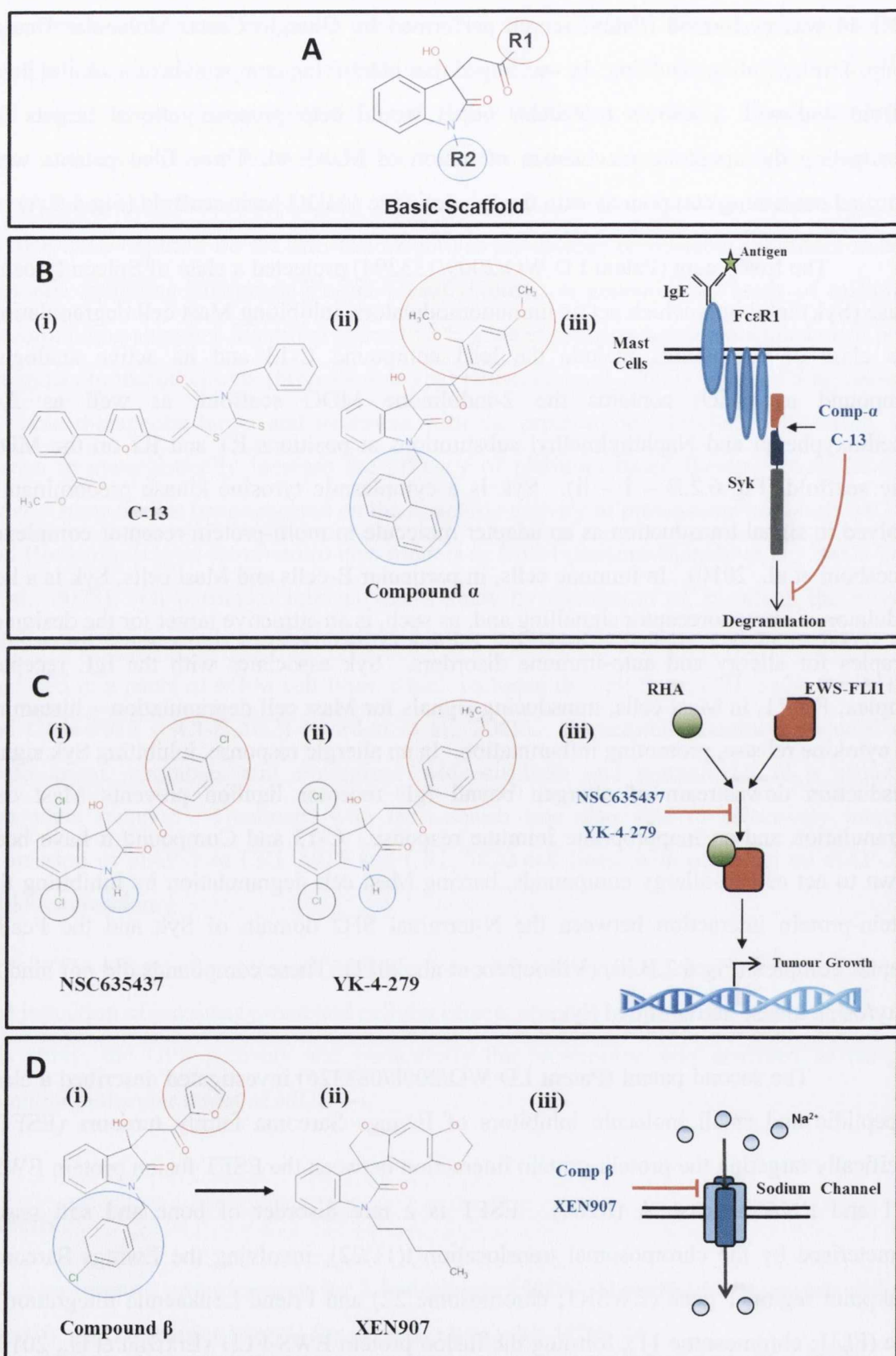


Figure 6.2: Chemical structures and mechanisms of action for MDG 44 like molecules identified via a compound patent search.

in the filed patent, contain the 2-indolinone MDG scaffold, with particular substitutions indicated in Figure 6.2.C. Published results on NSC635437 and its optimised variant YK-4-279 have been shown to inhibit tumour growth in Ewings Sarcoma via inhibiting the interaction of EWS-FLI1 and RHA (Fig 6.2.C.iii) (Erkizan et al., 2009). YK-4-279 has also shown activity in prostate cancers expressing ESFT fusion protein oncogenes (Rahim et al., 2011).

The final patent (Patent I.D WO/2008/046083) described a class of 2-indolinone sodium channel blockers with potential as therapeutic agents in the treatment of hypercholesterolemia, benign prostatic hyperplasia, pruritis and cancer. A published report detailed the activity of compound- β and its optimised analogue, XEN907 (Fig 6.2.D.i-ii), as inhibitors of the sodium channel subtype, $Na_v1.7$ (Chowdhury et al., 2011). Compound- β possesses the closest similarity to MDG 44, containing the basic 2-indolinone scaffold, as well as furan and chlorophenylmethyl substitutions at positions R1 and R2 respectively. In the filed patent and the above reference, these compounds were shown to have activity as direct inhibitors of sodium channel opening (Fig 6.2.D.iii). In vitro and in vivo assays had shown efficacy for these compounds in reducing serum cholesterol levels (Hypercholesterolemia) and potential in the treatment of pain or neurological disorders. While efficacy for these compounds in the treatment of certain hormone sensitive cancers is proposed, no data for such activity is reported.

None of the targets identified using the compound patent screen were taken forward and investigated as a putative mechanism for the apoptotic activity of MDG 44 observed in MPM.

6.2 MDG 44 did not show affinity for PPAR- γ in a TR-FRET PPAR- γ competitive binding assay

The affinity of MDG 44 for its proposed molecular target of PPAR- γ was assayed using a TR-FRET PPAR- γ competitive binding assay, as outlined in Section 2.13. The assay employed the principle of time-resolved fluorescence resonance energy transfer (TR-FRET) to determine the ability of test compounds to outcompete a fluorescent PPAR- γ ligand (fluoromone) for the LBD binding pocket. If a compound has affinity for PPAR- γ , displacement of the fluoromone disrupts the proximal fluorescence energy transfer from an

LBD bound terbium “donor” molecule to the fluoromone “accepter”. Loss of this FRET signal, expressed as the ratio of FRET emission (520nm) to terbium fluorescent emission (495nm), is indicative of competitive binding in this assay. The TR-FRET PPAR- γ competitive binding assay used was a commercially available assay kit (LantaScreenTM; Invitrogen) and the experimentation on MDG 44 was performed by Daniel Nevin, a collaborator in Molecular Design Group, Trinity College Dublin.

The TR-FRET assay was performed with incubations of increasing concentrations of MDG 44 (10nM – 50 μ M), vehicle and the known PPAR- γ agonist, GW1929 (Brown et al., 1999). Results revealed that the positive control, GW1929, showed strong affinity for the PPAR- γ binding pocket, with complete inhibition of FRET signal (FRET emission ratio) in response to 1 μ M incubation (Fig 6.3). However, incubations with MDG 44, up to the highest concentration assayed of 50 μ M, did not induce any significant decrease in the FRET emission ratio, compared to vehicle control. These results suggested MDG 44 did not have affinity for the PPAR- γ binding pocket.

6.3 Known PPAR- γ ligands possessed anti-proliferative activity in MPM cells

To validate the proof-in-concept that PPAR- γ ligation could induce cell death in MPM, cell viability experiments were performed testing the known PPAR- γ ligands, Rosiglitazone, Ciglitazone and GW1929. Both Rosiglitazone and Ciglitazone are members of the thiazolidinedione class of PPAR- γ agonist, which have previously reported to induce cell death as a monotherapy and in combination with platinum-based drugs in numerous cancer cell lines, including MPM (Girnun et al., 2007, Hamaguchi et al., 2010, Mody et al., 2007). GW1929 is another PPAR- γ agonist of alternate structure, with high affinity for the PPAR- γ binding pocket (Brown et al., 1999). Cell viability experiments were performed by Alamar Blue assay in CRL 5915 cells. Cells were incubated with increasing concentrations of Rosiglitazone, Ciglitazone and GW1929 for 48h prior to cell viability analysis. Results revealed that both Ciglitazone and GW1929 displayed anti-proliferative activity in CRL 5915 cells, with IC₅₀ values for cell viability of 13.7 μ M ($\pm$ 1.1) and 44.6 μ M ($\pm$ 1.8), respectively (Fig 6.4.A). However, Rosiglitazone did not induce any significant decrease in cell viability up to the highest concentration assayed (100 μ M). These results confirmed the proof-in-concept that PPAR- γ could induce cell death in MPM cell lines.

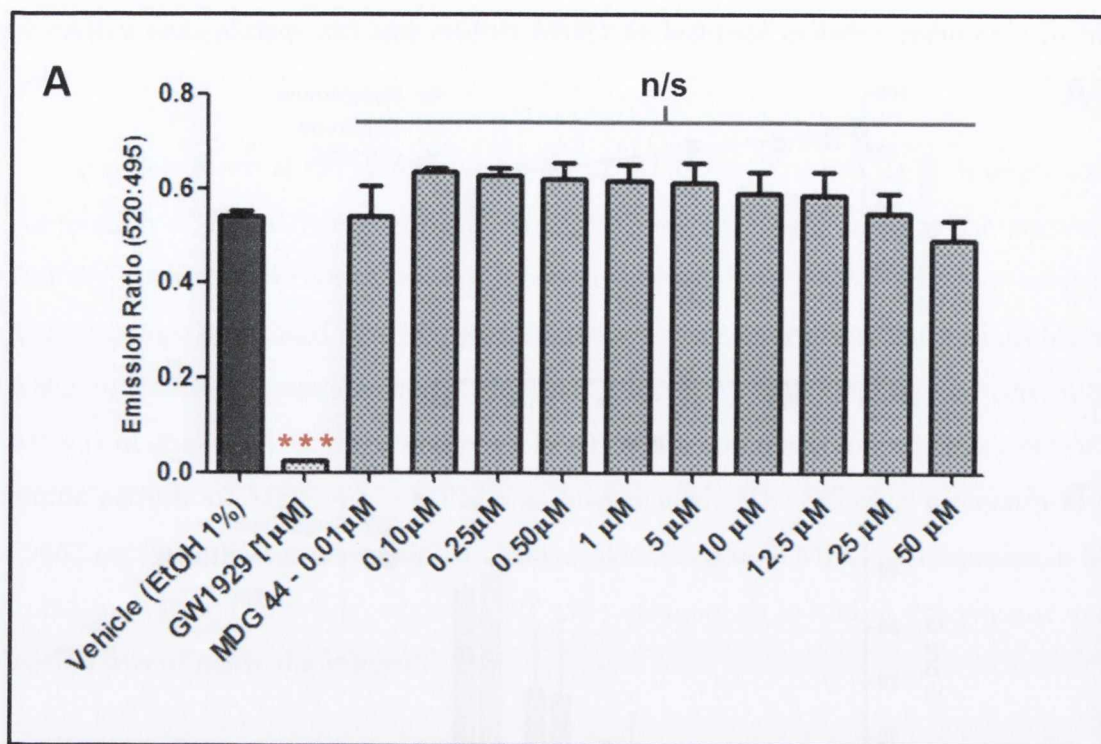


Figure 6.3: MDG 44 did not act as a PPAR- γ ligand

MDG 44 was tested in a PPAR- γ TR-FRET competitive binding assay to determine if it is a ligand for the PPAR- γ binding pocket (A). TR-FRET reagents were incubated with Vehicle (EtOH 1%), GW1929 [1 μ M], a known PPAR- γ agonist, and increasing concentrations of MDG 44. The competitive binding FRET assay and data manipulation was performed as outlined in Section 2.13. *** $P < 0.001$; n/s = No significant difference (Determined using Graphpad Prism 4 statistical software). [Experiment performed by Daniel Nevin, Molecular Design Group, School of Biochemistry and Immunology, Trinity College Dublin].

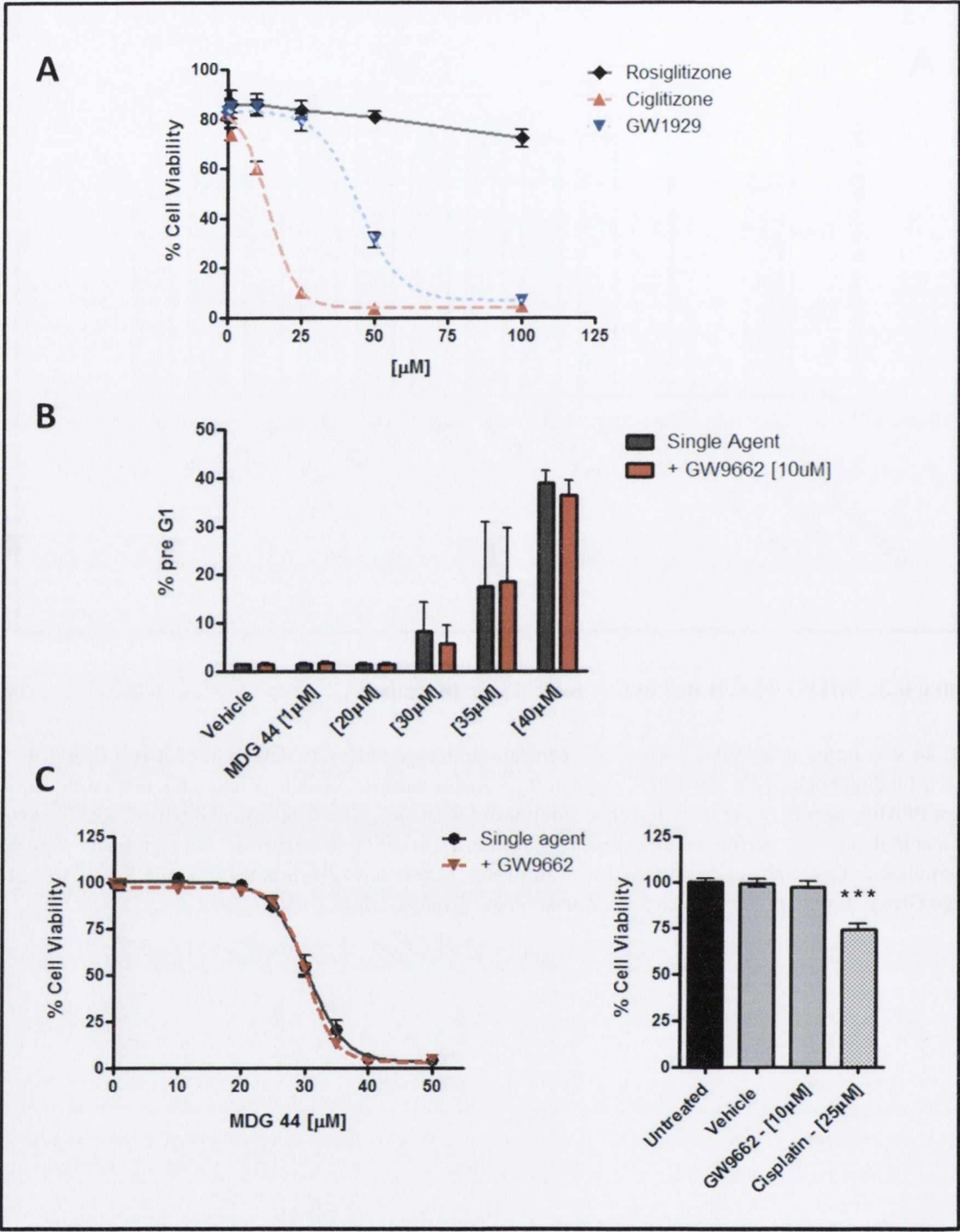


Figure 6.4: PPAR- γ antagonist GW9662 did not inhibit MDG 44 induced apoptosis

CRL 5915 cells treated with increasing concentrations of PPAR- γ agonists Rosiglitazone, Ciglitazone and GW1929 for 48h and induction of cell death was analysed by Alamar Blue cell viability assay (A). CRL 5915 cells treated with MDG 44, with or without PPAR- γ antagonist GW1929 [10 μM] for 18h and induction of apoptosis determined by PI staining and cell-cycle Flow Cytometry analysis (B). CRL 5915 cells treated with increasing concentrations of MDG 44, with or without PPAR- γ antagonist GW1929 [10 μM] for 24h and induction of cell death was analysed by Alamar Blue cell viability assay (C).

6.4 PPAR- γ antagonism did not inhibit MDG 44 induced cellular responses in MPM cells

In order to test if the cellular activities of MDG 44 were mediated through binding and activation of PPAR- γ , experiments with MDG 44 were performed in the presence of GW9662 (2-chloro-5-nitrobenzanilide), a potent, irreversible PPAR- γ selective antagonist. Previous studies have used this PPAR- γ inhibitory assay to confirm the anti-proliferative activities of PPAR- γ ligands as being mediated directly by their binding and activation of PPAR- γ (Liu et al., 2010). The ability of PPAR- γ antagonism to inhibit the cytotoxic and apoptotic activity of MDG 44 in MPM was investigated. The effect of co-treatment with GW9662 on the cellular morphological changes associated with MDG 44 treatment in MPM was also analysed. The ability, or not, of PPAR- γ antagonism to inhibit cytoplasmic vesicle formation was of particular interest.

6.4.1 PPAR- γ antagonist, GW9662, did not inhibit MDG 44 induced cell death in CRL 5915 cells

Both apoptosis and cell viability assays were performed to investigate the effect of PPAR- γ antagonism on MDG 44 induced cell death in MPM. CRL 5915 cells were pre-treated for 2h with vehicle or GW9662, prior to treatment with MDG 44. GW9662 was assayed at a concentration of 10 μ M. Initial optimisation experiments indicated no apoptotic or cytotoxic effect with treatment at this concentration in CRL 5915 cells and was above the concentration used for PPAR- γ inhibitory assays performed in previous reports [1 μ M] (Liu et al., 2010). Apoptotic analysis revealed that PPAR- γ antagonism had no effect on apoptotic induction by MDG 44 (Fig 6.4.B). 40 μ M MDG 44 induced 38.9% ($\pm$ 2.7) apoptosis (pre G1 population), compared with 36.5% ($\pm$ 3.0) with pre-treatment with GW9662.

Similar results were observed with cell viability analysis. Cells were treated with increasing concentrations of MDG 44 alone or with pre-treatment with GW9662 (Fig 6.4.C). IC₅₀ values for cell viability in response to single or dual-agent treatments were 30.4 μ M ($\pm$ 0.4) and 30.2 μ M ($\pm$ 0.2), respectively. These results indicated that PPAR- γ antagonism had no effect on MDG 44 induced cell death in CRL 5915 cells.

6.4.2 PPAR- γ antagonist, GW9662, did not inhibit MDG 44 induced formation of cytoplasmic vesicles in CRL 5915 cells

The effect of PPAR- γ antagonism on MDG 44 induced morphological changes in MPM was also assayed in CRL 5915 cells. Cells were pre-treated with vehicle or GW9662 for 2h prior to treatment with indicated concentrations of MDG 44 (Fig 6.5.a – b). After 18h incubation, morphological analysis was performed by cytopinning cells onto slides and RapiDiff staining of the nucleus and cytoplasm. Cells treated with vehicle or GW9662 alone were observed to be healthy, viable cells containing an intact nucleus, plasma membrane and no cytoplasmic vesicles were detected (Fig 6.5.a). In incubations with 30 μ M and 35 μ M MDG 44, formation of large cytoplasmic vesicles was observed (Fig 6.5.b). In cells pre-treated with GW9662, no difference in the type or amount of vesicles present in the cytoplasm was observed. Results indicate that PPAR- γ antagonism had no effect on MDG 44 induced formation of cytoplasmic vesicles in MPM cells.

6.5 Examination of cytoplasmic vesicles as being Peroxisomes: Catalase Spectrophotometric enzyme assay

In conjunction with experimentation investigating PPAR- γ as being the molecular target of MDG 44, experiments were performed to determine the nature of the cytoplasmic vesicles induced by MDG 44 treatment in MPM. A theory was proposed that MDG 44 ligation and activation of PPAR- γ could induce the transcription of *Pex* genes, a family of protein regulators of Peroxisome division and proliferation (Yan et al., 2005). An examination into whether the cytoplasmic vesicles induced by MDG 44 treatment were Peroxisomes was performed. Catalase, a peroxisomal enzyme involved in the oxidation of toxic intermediates (Peroxidation reaction) and decomposition of excess hydrogen peroxide to H₂O₂ and O₂ (Catalase reaction), was used as a marker for the peroxisome (Mozaffar et al., 1986). A spectrophotometric enzyme experiment, assaying the decomposition of hydrogen peroxide by Catalase, was used as a method to quantify Catalase activity and in turn the level of peroxisomal activity in cells. The decomposition reaction catalysed by Catalase was illustrated in Figure 6.6.A and the experimental method for this assay was described in full, in Section 2.13.

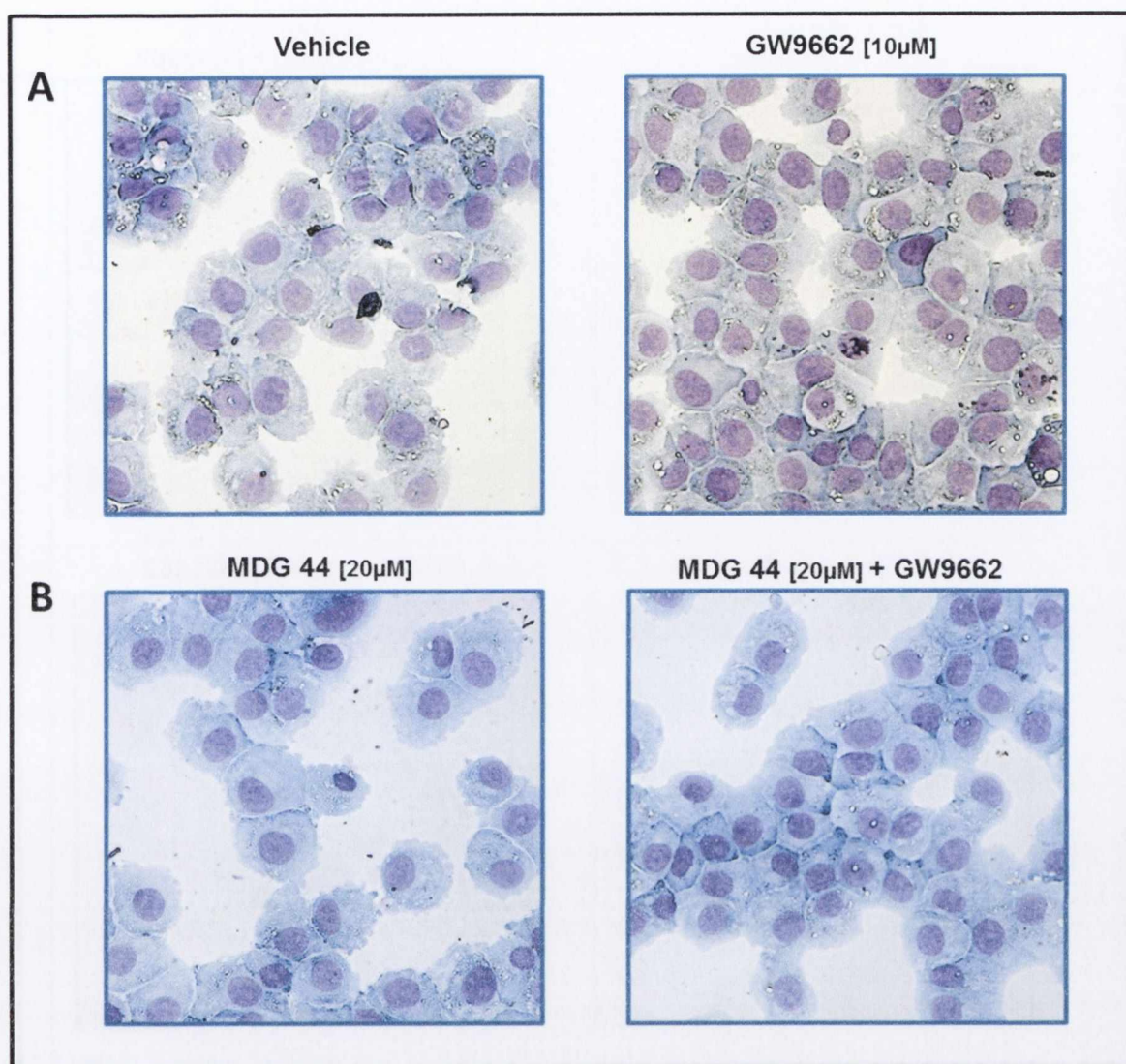


Figure 6.5.a: GW9662 did not inhibit MDG 44 induced formation of cytoplasmic vesicles in CRL 5915 cells

CRL 5915 cells were treated with controls – Vehicle and GW9662 [10µM] (A) – or MDG 44 [20µM] +/- GW9662 (B) for 18h. CRL 5915 cells were seeded at a density of 0.12×10^6 cells / well (3ml). Cytospin slides were prepared and stained as outlined in Section 2.11. Slides were viewed and pictures taken using a Zeiss Axiovert microscope, under 20X magnification. Changes in cellular morphology in response to treatment with test compounds were noted. Results are representative of two independent experiments.

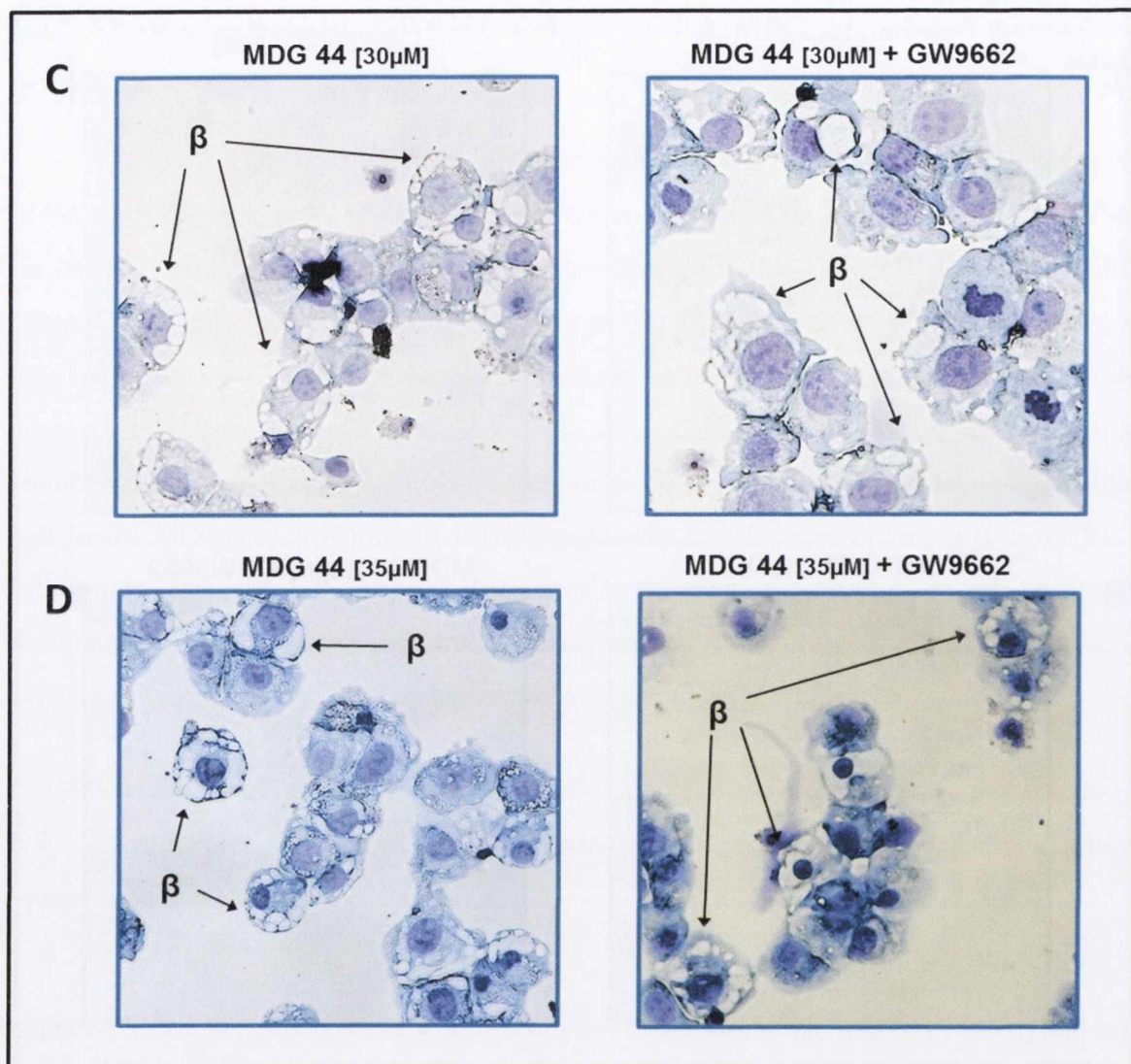


Figure 6.5.b: GW9662 did not inhibit MDG 44 induced formation of cytoplasmic vesicles in CRL 5915 cells

CRL 5915 cells were treated with MDG 44 [30μM] +/- GW9662 [10μM] (C) and MDG 44 [35μM] +/- GW9662 [10μM] (D) for 18h. CRL 5915 cells were seeded at a density of 0.12×10^6 cells / well (3ml). Cytospin slides were prepared and stained as outlined in Section 2.11. Slides were viewed and pictures taken using a Zeiss Axiovert microscope, under 20X magnification. Changes in cellular morphology in response to treatment with test compounds were noted: Formation of large cytoplasmic vesicles (β). Results are representative of two independent experiments.

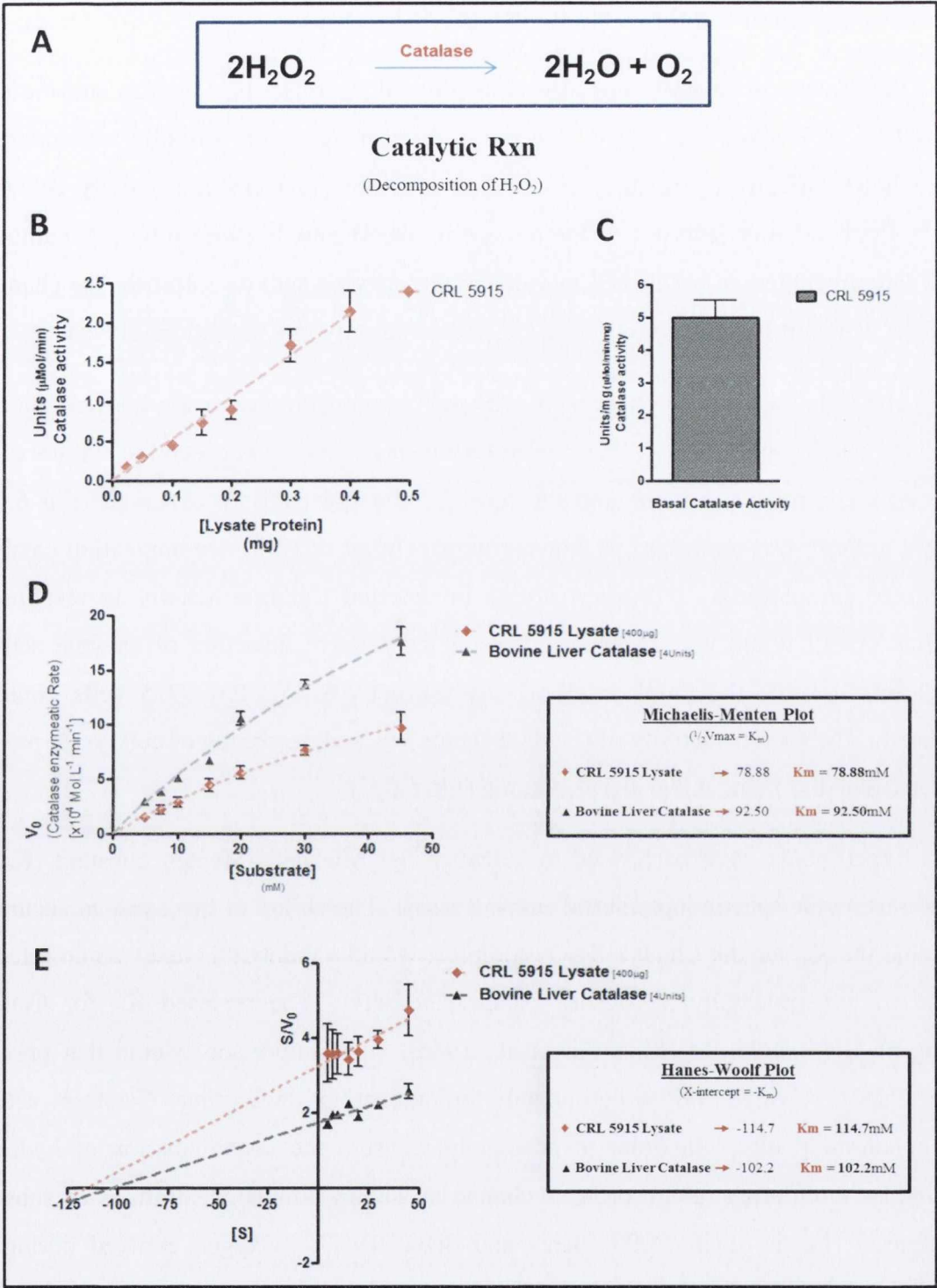


Figure 6.6: Catalase assay – Control experiments and K_m calculations

The Catalase enzyme is responsible for catalysing the decomposition of hydrogen peroxide ($2\text{H}_2\text{O}_2$) to water ($2\text{H}_2\text{O}$) and oxygen (O_2) (A). Increasing concentrations of CRL 5915 whole cell lysate protein was assayed for Catalase activity (B). The basal level of Catalase specific activity (Units/mg protein) in untreated CRL 5915 cells was determined to be $4.999 (\pm 0.5185)$ Units/mg (C). Experiments were performed to attempt to calculate the K_m for Catalase activity of CRL 5915 whole cell lysate protein [400 μg] (◆) and a positive control of Bovine Liver Catalase [4 units/addition] (▲). The predicted K_m of Catalase for each sample was determined using Michaelis-Menten (D) and Hanes-Woolf (E) plots. Catalase activity was determined by spectrophotometric enzyme assay, as outlined in Section 2.13. Each data point represents the mean $\pm$ SEM of at least three independent experiments.

6.5.1 Investigation of Catalase activity in CRL 5915 cells

Initial experimentation looked to validate and optimise the Catalase enzyme assay using CRL 5915 whole cell lysate. Decomposition of hydrogen peroxide was monitored spectrophotometrically by reading absorbance at 240nm and enzyme activity calculated using a chart recorder read-out of the change in absorbance per unit time. A number of initial experiments were performed to validate the enzyme activity / absorbance change at 240nm as being attributable specifically to Catalase activity in CRL 5915 cell lysate.

The Catalase enzyme experiment was performed using increasing concentrations of CRL 5915 whole cell lysate. A linear relationship was observed between the amount of cell lysate assayed (mg of protein) and the level of Catalase activity observed (Fig 6.6.B). Catalase activity was expressed as enzyme units – μmol of H_2O_2 decomposition catalysed per minute ($\mu\text{mol}/\text{min}$). A linear increase in detected Catalase activity in response to increases in cell lysate was an expected result for positive detection of enzyme activity. Using these results, the basal level of Catalase activity in CRL 5915 cells could be calculated. The specific activity of Catalase (Units of Catalase per mg of cell lysate protein) was determined as being $5.0 (\pm 0.5)$ Units/mg (Fig 6.6.C)

Experiments were performed to calculate the Michaelis-Menten constant (K_m) of Catalase using the spectrophotometric enzyme assay. The ability of this assay to accurately determine the K_m for the Catalase: H_2O_2 complex, would validate this assay as an effective method of experimentally measuring Catalase activity. The reported K_m for Catalase binding of H_2O_2 is 25mM (Mozaffar et al., 1986). It is important to note that previous studies have reported on experimental limitations to measuring Catalase activity spectrophotometrically. In order to accurately measure the decomposition of hydrogen peroxide by monitoring the absorbance change at 240nm, low concentrations of substrate are required (Lewis et al., 2009, Beers and Sizer, 1952). Oxygen evolved during the Catalase reaction possesses limited solubility in solution and excess formation of oxygen bubbles in the reaction mixture will cause errors when monitoring absorbance change.

K_m calculations were performed using CRL 5915 cell lysate ($400\mu\text{g}$ / addition) and Bovine liver Catalase (4 units / addition) (Sigma). Both samples of Catalase enzyme were assayed at seven different concentrations of hydrogen peroxide substrate between 2.5mM – 45mM. Initial velocities were calculated and K_m values predicted using Michaelis-Menten (Fig 6.6.D) and Hanes-Woolf (Fig 6.6.E) plots. Linear and non-linear regression curve fit

analysis was performed using Graphpad Prism statistical software. Predicted K_m values for CRL 5915 lysate and Bovine liver Catalase using the Michaelis-Menten plot ($K_m = \frac{1}{2} V_{max}$) were 78.9mM and 92.5mM, respectively. Using the Hanes-Woolf plot (X-intercept = $-K_m$), predicted K_m values for CRL 5915 lysate and Bovine liver Catalase were 114.7mM and 102.2mM, respectively. Very similar values for the K_m of Catalase were predicted for both CRL 5915 lysate and isolated Bovine liver Catalase, indicating that the activity observed with CRL 5915 lysate in this assay was due to Catalase enzyme activity. While experimental limitations in the measurement of Catalase activity spectrophotometrically have been noted, this assay was able to predict K_m values for CRL 5915 lysate and Bovine liver Catalase in range of reported K_m values for the Catalase:H₂O₂ complex (25mM).

A final assay-validation experiment involving the Catalase inhibitor sodium azide was performed. Sodium azide is a noted inhibitor of enzymes containing metal ion cofactors, including Superoxide dismutase, Peroxidase and Catalase. The Catalase enzyme consists of a tetramer of four polypeptide chains, each containing a porphyrin heme group essential for catalytic activity (Percy, 1984). Sodium azide can complex with ferrous ions of the heme groups of Catalase and inhibit activity. Previous studies have employed sodium azide [1mM] as an inhibitor of Catalase in *in vitro* and *in vivo* experiments investigating Catalase enzymatic activity (Gregory et al., 1977, Sanchis-Segura et al., 1999).

CRL 5915 cell lysate was assayed for Catalase activity with or without the presence of sodium azide, in the *in vitro* enzyme assay described above. Bovine liver Catalase was used as an assay control. The addition of 5 Units of enzyme was made after testing CRL 5915 activity in inhibitory assays. The ability of sodium azide to also inhibit Bovine liver Catalase activity confirmed inhibition of the Catalase enzyme in each assay. The initial velocity / enzymatic rate of Catalase activity was calculated – defined as mol of H₂O₂ decomposition catalysed per minute, per litre of reaction mixture ($\times 10^{-4} \text{ mol min}^{-1} \text{ l}^{-1}$). The catalase enzymatic rate detected in CRL 5915 lysate samples (300µg protein) was $4.3 (\pm 0.3) \times 10^{-4} \text{ mol min}^{-1} \text{ l}^{-1}$, however in the presence of sodium azide, the Catalase inhibitor was able to completely abolish Catalase activity (Fig 6.7.D). Representative results (recorder trace read-outs) from sodium azide inhibitory assays were illustrated in Figure 6.7.A – C. These results included assays investigating the Catalase activity of CRL 5915 cell lysate alone (Fig 6.7.B); the Catalase activity of CRL 5915 cell lysate, with pre-incubation with sodium azide (Fig 6.7.C); and the Catalase activity of CRL 5915 cell lysate before and after the addition of sodium azide (Fig 6.7.A). These results all indicated that

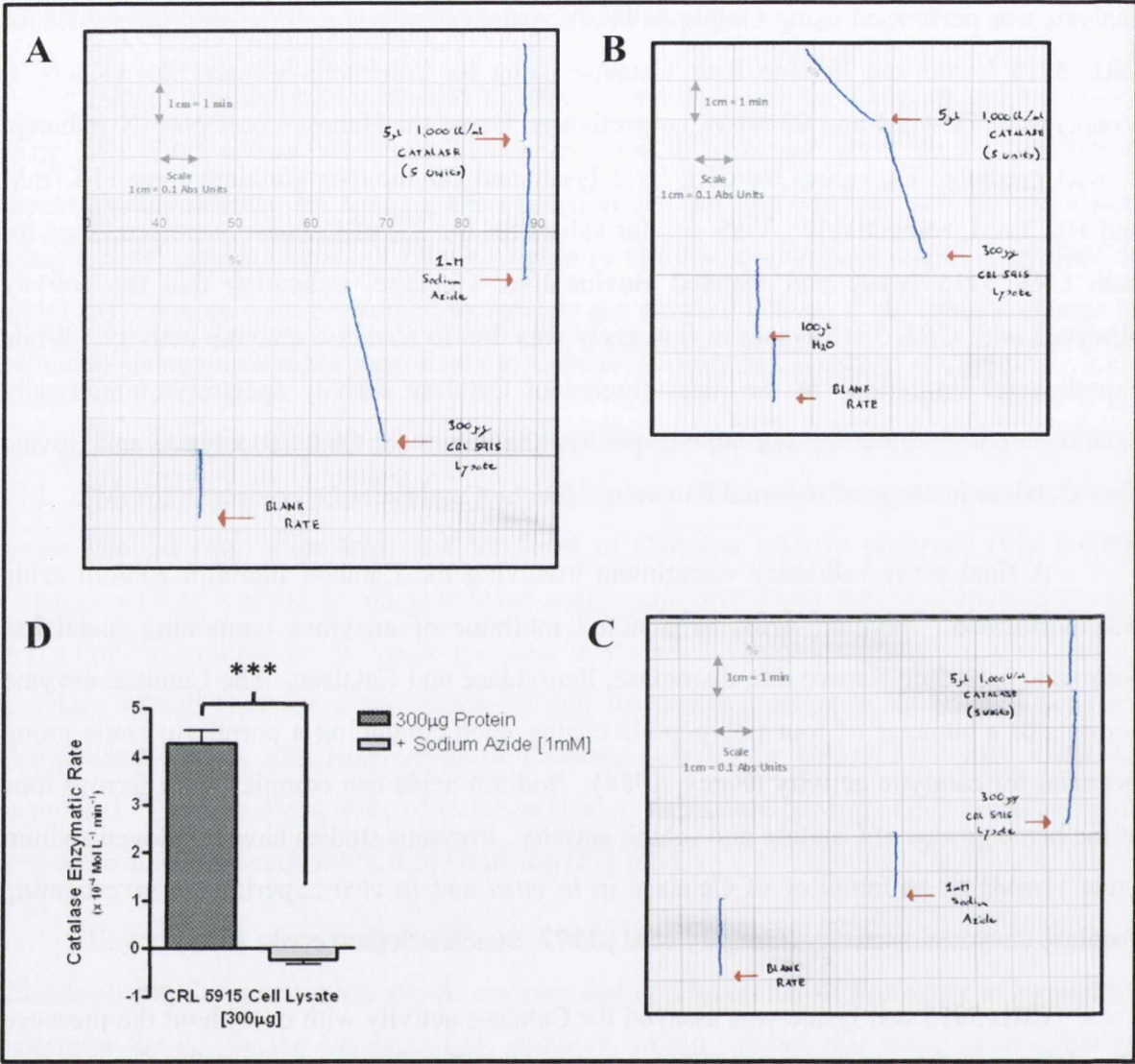


Figure 6.7: Catalase assay – Sodium azide inhibited Catalase activity in a spectrophotometric enzyme analysis of CRL 5915 cell lysate

Spectrophotometric enzyme assay analysis of Catalase activity was performed using CRL 5915 whole cell lysate protein [300 μg] in the presence or absence of the Catalase enzyme inhibitor, sodium azide [1 mM] (A, B & C). The addition of sodium azide prior to CRL 5915 whole cell lysate protein inhibited Catalase activity (decomposition of hydrogen peroxide) (D). Catalase enzymatic rate is represented as the Mol/l decomposition of H₂O₂ per min (x 10⁴ mol l⁻¹ min⁻¹). Catalase activity was determined by spectrophotometric enzyme assay, as outlined in Section 2.13. Results are representative the mean ± SEM of at four independent experiments. ***P<0.001 (Determined using Graphpad Prism 4 statistical software).

sodium azide was able to inhibit the enzyme activity observed in CRL 5915 cells. All the control experiments performed indicated that this *in vitro* enzyme assay was a valid method of assaying and quantifying Catalase activity from CRL 5915 whole cell lysates.

6.5.2 MDG 44 treatment did not induce increased Catalase activity in CRL 5915 cells

The accumulation of peroxisomes in response to MDG 44 treatment in MPM cells was investigated using the described Catalase spectrophotometric enzyme assay. Catalase enzyme activity was used as a marker for peroxisomal activity. An increase in Catalase activity in response to treatment was considered indicative of an accumulation or increase in number of peroxisomes in cells. CRL 5915 cells were treated with vehicle, MDG 44 or left untreated. After 4h incubation, whole cell lysates were prepared and Catalase activity was assayed as outlined previously (Section 2.13). The specific activity of Catalase (Units of Catalase per mg of cell lysate added – Units/mg) for each sample was calculated and graphed in Figure 6.8. Results revealed that no significant increase in Catalase activity was observed in CRL 5915 cells in response to incubation with MDG 44. The lack of increase in Catalase activity, a marker for peroxisomes, strongly indicated that the large cytoplasmic vesicles induced by MDG 44 treatment in MPM cells, were not peroxisomes.

6.6 Examination of the accumulation of autophagosomes in MPM cells in response to MDG 44 and inhibitors of autophagy

The final avenue of target investigation examined in this study was that of the complementary cellular degradation programs of Macroautophagy and the ubiquitin-proteasome system. Initial experimentation proposed to investigate whether the cytoplasmic vesicles induced by MDG 44 incubation in MPM cells were autophagosomes, as originally proposed. Experiments were also performed to determine if induction of autophagy was involved in the execution of the cell death program activated by MDG 44. Experimentation in Chapter 5 had confirmed that a caspase-dependant, apoptotic program of cell death was induced by MDG 44 in CRL 5915 cells, however, concurrent activation and interplay between the these two PCD pathways had previously been proposed

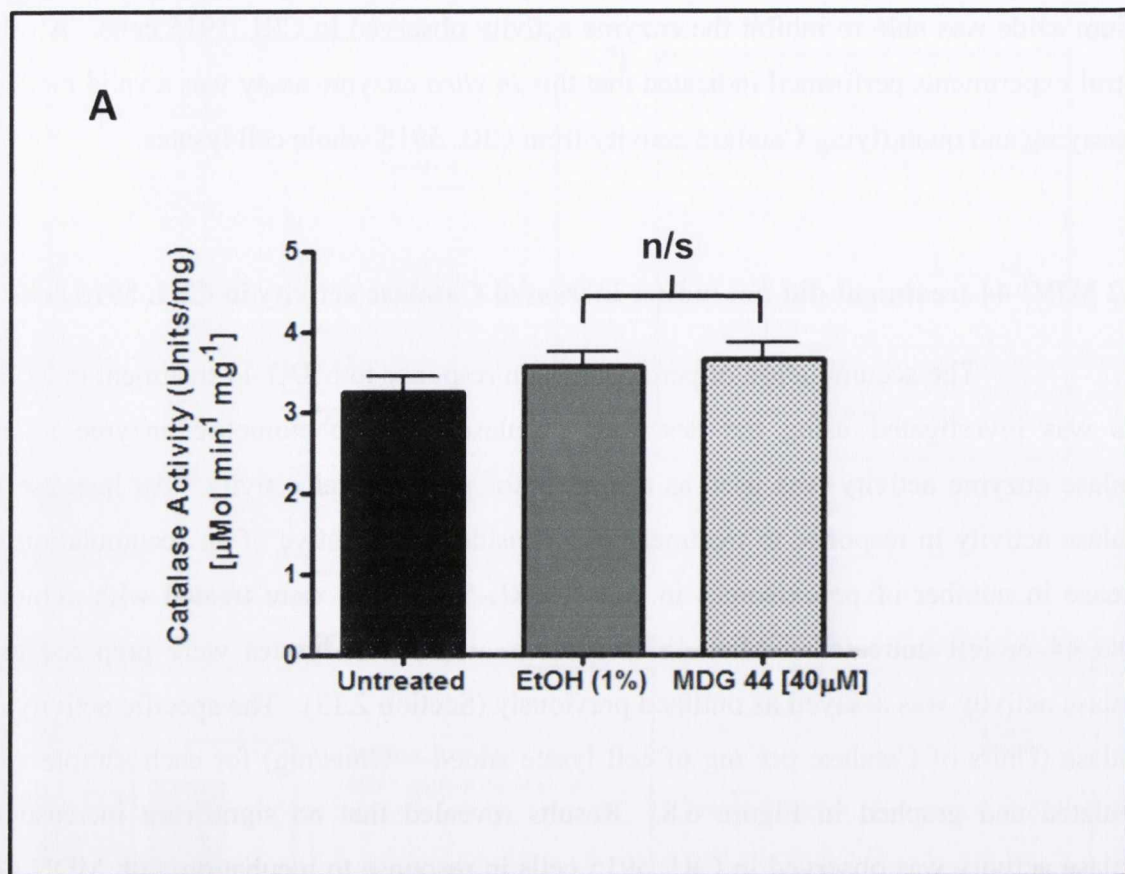


Figure 6.8: MDG 44 treatment did not induce an increase in Catalase activity in CRL 5915 cells

CRL 5915 cells were left untreated or treated with vehicle (EtOH 1%) or MDG 44 [40μM] for 4h and then analysed for Catalase activity (A). CRL 5915 cells were seeded at a density of 0.325×10^6 cells / T25 (5ml). Whole cell lysates were prepared and Catalase activity was determined by spectrophotometric enzyme assay, as outlined in Section 2.10. The specific activity of Catalase in the cell lysate of each treatment sample was compared – the amount (μMol) of H₂O₂ decomposed per minute, per mg of cell lysate protein (Units/mg or μMol min⁻¹ mg⁻¹). Results represent the mean ± SEM of four independent experiments. n/s = No significant difference (Determined using Graphpad Prism 4 statistical software).

(Gonzalez-Polo et al., 2005). Subsequent to this analysis, the activity of MDG 44 as a potential proteasome inhibitor was investigated.

Initial autophagy experimentation employed a known inducer of autophagosome accumulation in cells. Chloroquine, a clinical agent in the treatment of malaria and autoimmune disorders, is also a reported inhibitor of autophagy (Shacka et al., 2006). Within the cell, this weak base can concentrate in acidic vesicles, including lysosomes, disrupting the pH of the organelle. Chloroquine treatment results in inhibition of lysosome enzyme function and lysosomal acidification. Interference with the lysosome in this way prevents its fusion with the autophagosome and the formation of autolysosomes (Boya et al., 2005). This disruption to “autophagic flux” – The uptake of cellular material (cytoplasmic protein, lipids, organelles, etc.) into autophagosomes and the ultimate degradation and recycling of these components within the autolysosome – prevents the final step of lysosomal fusion and degradation of autophagosomes, resulting in their accumulation within the cytoplasm of Chloroquine treated cells.

Western Blot detection of the autophagosomal marker, microtubule-associated protein Light Chain 3 (LC3), was used as a method for quantifying accumulation of autophagosomes within cells. This protein, originally identified as a subunit of the microtubule association proteins 1A/1B (Mann and Hammarback, 1994), is a mammalian homolog of the autophagy-related protein Atg8 and is a central component in autophagosome formation (Yang and Klionsky, 2010). LC3 exists in cells in two forms; the cytosolic protein LC3-I and its lipidated form LC3-II. Autophagy-related proteins Atg7 and Atg3 (E1- and E2-like ubiquitin enzymes) conjugate the phospholipid, phosphatidylethanolamine (PE) to LC3-I to form LC3-II (Kabeya et al., 2000). During initiation of phagophore formation, lipidated LC3-II localises to the PAS (phagophore-assembly-site) and participates in elongation of the isolation membrane into a mature autophagosome (Yang and Klionsky, 2010). LC3-II is a constitutive part of the autophagosome and is widely used as a marker for the detection of autophagy and quantification of autophagosomal formation (Tanida et al., 2005).

Initial control experiments were performed, using Chloroquine, to examine the effect of inducing accumulation of autophagosomes in MPM cells. Quantification of autophagosome formation / accumulation using the LC3-II marker was examined by

Western Blot. The effect of Chloroquine treatment and autophagosome accumulation on cellular morphology in MPM cells was also analysed.

6.6.1 Chloroquine induced accumulation of autophagosomes in MPM cells; detected by LC3 Western Blot analysis

CRL 5915 cells were used to investigate the effect of Chloroquine treatment on autophagy in MPM. The LC3 Western Blot assay was verified as a method of detecting increases in autophagosome number in this cell line. The increased detected expression of the lipidated form of the LC3 protein (LC3-II) is indicative of an increase in autophagosomal number within the cell. Previous studies had reported on the cytotoxic activity of Chloroquine in certain cancer models (Boya et al., 2003) and so, initial control experiments were performed to test the tolerance of CRL 5915 cells to Chloroquine treatment. CRL 5915 cells were incubated with Chloroquine (10 μ M – 25 μ M) for 18h prior to fixing, PI staining and apoptotic analysis (Fig 6.9.A). Results revealed that Chloroquine was tolerated in CRL 5915 cells, with no significant induction of apoptosis detected up to the highest concentration assayed (25 μ M – 1.4% ($\pm$ 0.2) pre G1 apoptosis).

For autophagosome detection, Cells were incubated with either vehicle or Chloroquine [25 μ M] for 1h, 2h, 4h and 6h time points. At each experimental endpoint, whole cell lysates were prepared and LC3 examination performed by Western Blot. Immunoblotting analysis using Anti-LC3 primary antibody (Sigma – L7543) detected both cytosolic LC3-I and lipidated LC3-II forms of LC3. The apparent molecular mass detected for LC3-I and LC3-II was ~16kDa and ~14kDa, respectively. Due to the lipidation of LC3-II, the extreme hydrophobicity of the PE conjugate makes LC3-II migrate further when subjected to SDS-PAGE and, as such, has an apparent smaller molecular mass to that of its unlipidated form, LC3-I (Mizushima and Yoshimori, 2007). Immunoblotting analysis revealed that Chloroquine treatment induced highly significant increases in LC3-II expression compared to vehicle controls at each time point assayed (Fig 6.9.B). A significant increase in LC3-II expression was detected after 1h incubation with Chloroquine and the level of LC3-II expression increased with time, up to 6h incubation. This result confirmed that Chloroquine treatment induced a rapid increase in autophagosome number in MPM cells, indicative of accumulation of autophagosomes in response to inhibition of lysosomal fusion.

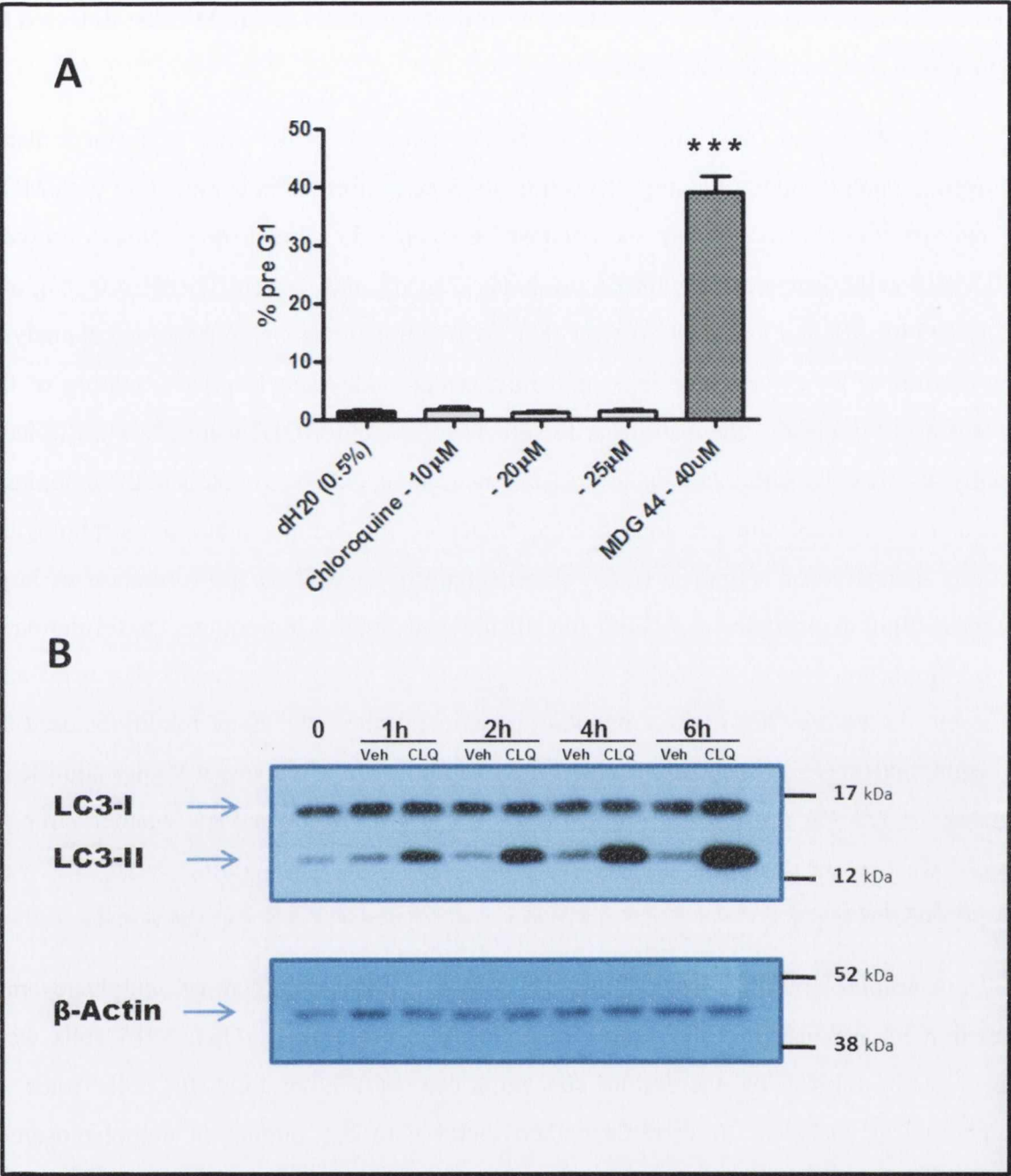


Figure 6.9: Chloroquine treatment induced an increase in formation of the autophagosomal marker LC3-II in CRL 5915 cells

(A) Chloroquine treatment does not induce apoptosis in CRL 5915 cells. CRL 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml). Cells were treated with indicated concentrations of vehicle (dH₂O 0.5%), Chloroquine or MDG 44 for 18h prior to fixing, PI staining and cell-cycle Flow Cytometry analysis, as outlined in Section 2.7. Results are representative of the mean $\pm$ SEM of four independent experiments. ***P<0.001 (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

(B) CRL 5915 cells were seeded at a density of 0.3125×10^6 cells / T25 (5ml). Cells were left untreated or treated with dH₂O ("Veh") or Chloroquine [25µM] ("CLQ") for 1h, 2h, 4h and 6h time points. At each experimental endpoint whole cell lysates were prepared as described in Section 2.5.1. Protein (20µg) was resolved on a 12.5% SDS-PAGE gel and expression of LC3 was examined as outlined in Section 2.15.2. Blots were stripped and re-probed with anti-β-actin (Primary Ab – 1:5,000) as a protein loading control. Results are representative of two independent experiments.

6.6.2 Chloroquine induced accumulation of autophagosomes in MPM cells; detected by Cytospin analysis and RapiDiff staining

Morphological analysis of Chloroquine treated cells was performed using Cytospining and RapiDiff staining. Experiments were performed in conjunction with MDG 44 treatment in order to compare and contrast the changes in cellular morphology observed. CRL 5915 cells were treated with Chloroquine [25 μ M], and its vehicle (dH₂O 0.5%), and MDG 44 [40 μ M], and its vehicle (EtOH 1%). After 4h incubations, morphological analysis was performed by cytospining cells onto microscope slides and RapiDiff staining of the nucleus and cytoplasm. Incubations with vehicle (dH₂O and EtOH) were observed to have healthy, viable cells with intact nuclei, plasma membrane and no structures in the cytoplasm (Fig 6.10). In line with previous results, MDG 44 incubations induced morphological features indicative of apoptosis (DNA fragmentation), as well as the formation of large vesicles within the cytoplasm of cells. In cells incubated with Chloroquine, the formation of large cytoplasmic vesicles, similar to those present in MDG 44 treated cells, were also observed. In the previous LC3 immunoblot experiment, the same assay conditions used for Cytospin analysis – 4h incubation with 25 μ M Chloroquine – induced a highly significant increase in LC3-II expression (Fig 6.9.B). These two results taken together strongly suggested that the cytoplasmic vesicles observed with Chloroquine treatment were autophagosomes.

A second experiment examining the dose dependant induction of autophagosomal formation in response to Chloroquine treatment was performed. CRL 5915 cells were treated with vehicle or increasing concentrations of Chloroquine for 24h prior to morphological analysis. A dose dependant increase in the amount of autophagosomes accumulating in cells was observed. Incubations with vehicle and 10 μ M Chloroquine did not induce formation of autophagosomes in cells (Fig 6.11). No change in cellular morphology was observed; cells were viable with intact nuclei and plasma membrane. Increasing the concentration of Chloroquine treatment to 20 μ M and 25 μ M resulted in the detection of autophagosomes within cells. A successive increase in the amount of autophagosomes detected in cells was observed with increased concentrations of Chloroquine.

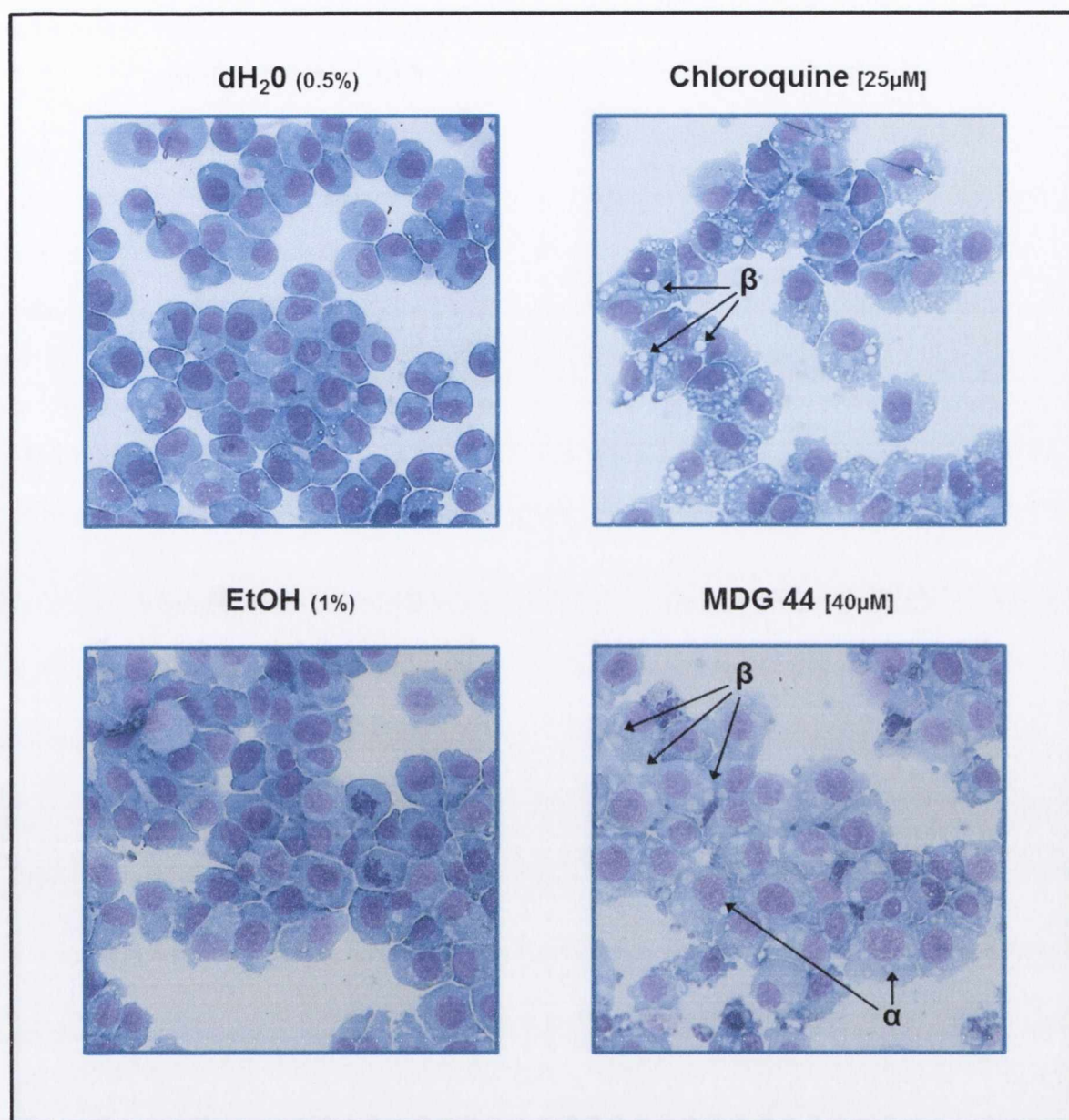


Figure 6.10: Chloroquine treatment induced the formation of large cytoplasmic vesicles in CRL 5915 cells

CRL 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml) and treated with Vehicle (dH₂O or EtOH) Chloroquine [25μM] or MDG 44 [40μM] for 4h. Cytospin slides were prepared and stained as outlined in Section 2.11. Slides were viewed and pictures taken using a Zeiss Axiovert microscope, under 20X magnification. Changes in cellular morphology in response to treatment with test compounds were noted: DNA fragmentation (α); Formation of large cytoplasmic vesicles (β). Results are representative of three independent experiments.

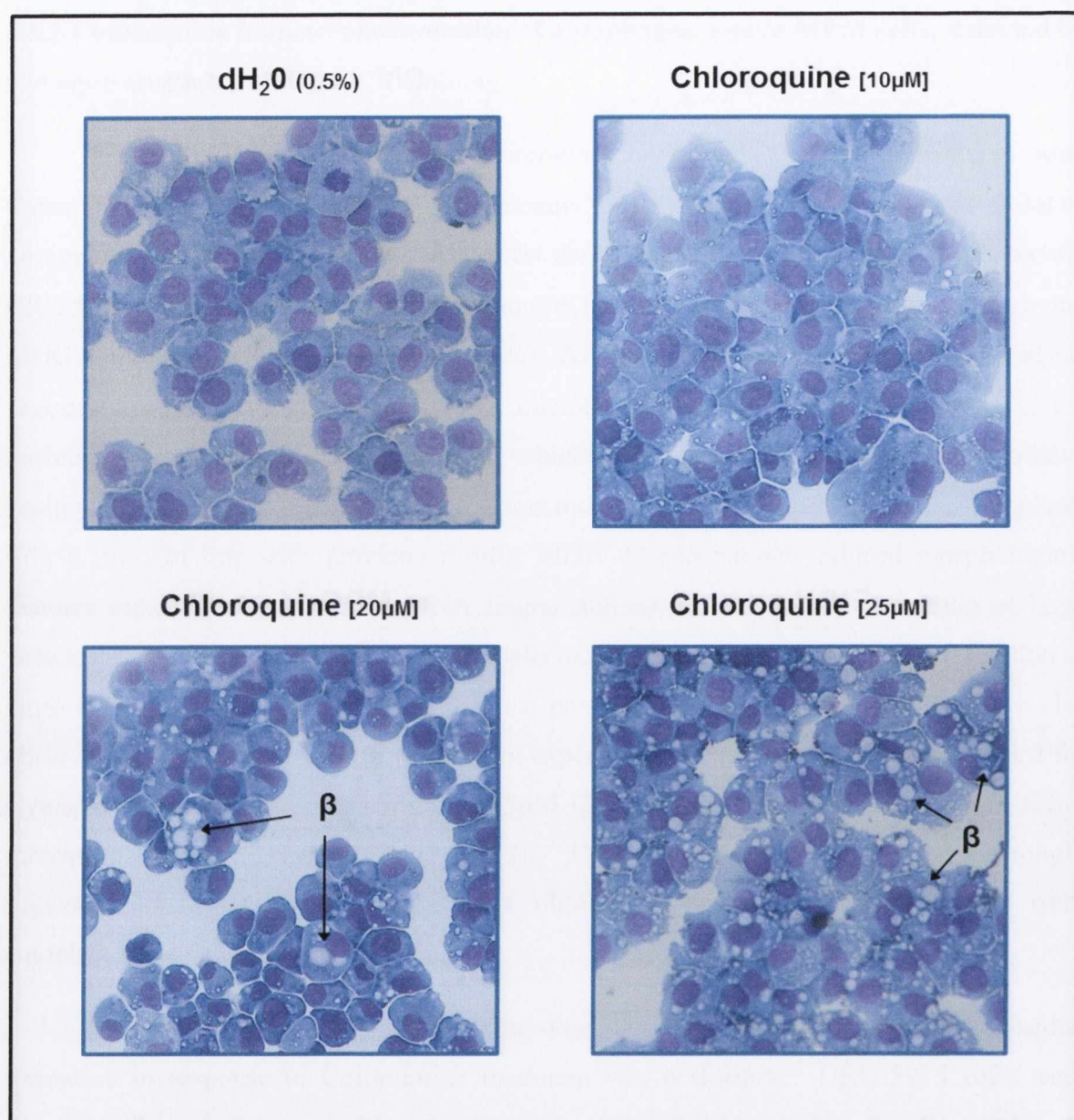


Figure 6.11: Chloroquine treatment induced the formation of large cytoplasmic vesicles in CRL 5915 cells

CRL 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml) and treated with Vehicle (dH₂O) or indicated concentrations of Chloroquine for 24h. Cytospin slides were prepared and stained as outlined in Section 2.11. Slides were viewed and pictures taken using a Zeiss Axiovert microscope, under 20X magnification. Changes in cellular morphology in response to treatment with test compounds were noted: Formation of large cytoplasmic vesicles (β). Results are representative of three independent experiments.

6.7 Examination of the effect of MDG 44 treatment on Autophagic Flux in MPM cell

Investigations were made into the effect of MDG 44 treatment on Autophagy in MPM cells. Previous results had indicated that MDG 44 treatment induced the formation of large cytoplasmic vesicles in MPM, which were suspected to be autophagosomes. Inducing the accumulation of autophagosomes suggested that MDG 44 treatment may somehow affect autophagosomal processes within MPM cells, be that inducing increased autophagy or inhibition at some point along the pathway. Numerous autophagy modulators are noted to affect autophagy at different stages of the process, from inhibiting lysosomal degradation of components, lysosomal fusion or initial activation of the pathway. It was proposed that induction of autophagy by MDG 44 may be a consequence of engagement with its molecular target.

Autophagic flux experiments were performed to analyse the effect of MDG 44 incubation on the entire process of autophagy within the cell. Autophagic flux is a term which describes the uptake and eventual lysosomal turnover of cellular components through autophagy. Experimentally, autophagic flux can be assayed by monitoring the protein expression levels of markers for autophagosome formation and autolysosome protein degradation. Monitoring the complete flux of components from autophagosome formation to lysosomal degradation is a better marker for autophagy. An increase or decrease in autophagy in the cell can be quantified and induction of defective autophagic processes be identified. In autophagic flux experiments, the lipidated form of LC3 (LC3-II) was used as the protein marker for autophagosome formation and p62 (Sequestosome 1) was used as the marker for lysosomal turnover. P62 is commonly used as a marker for autophagic flux (Bjorkoy et al., 2005, Mizushima and Yoshimori, 2007). This protein can associate directly with LC3 on the inner leaflet of the autophagosome membrane and can be taken up into autophagosomes as part of poly-ubiquitinated protein aggregates, where it is eventually degraded in the autolysosome (Bjorkoy et al., 2005). Increased expression of p62 is also a measure of defective autophagic flux in which components taken up into the autophagosome are not eventually degraded.

Autophagic flux experiments were performed with and without the presence of lysosomal cathepsin inhibitors, E64d and Pepstatin A. These compounds inhibit the lysosomal enzymes, Calpain, Cathepsin B, Cathepsin H and Cathepsin L and prevent the degradation of cellular components within the autolysosome (Umezawa et al., 1970, Tamai

et al., 1987). Cathepsin inhibitors are commonly used in autophagic flux experiments due to the difficulty in the detection of an increase in the autophagosome marker, LC3-II, with increased autophagic flux (Tanida et al., 2005). Once the autophagosome fuses with the lysosome, LC3-II is degraded by lysosomal enzymes (Cathepsins), as well as its delivered cargo. The use of cathepsin inhibitors prevents this lysosomal degradation of LC3-II, allowing this marker for autophagosome formation be more easily detected.

6.7.1 Nutrient starvation induced an increase in Autophagic flux in MPM cells

Nutrient starvation of cells is a classic inducer of increased autophagic flux. During nutrient starvation conditions, cells catabolise its own cellular components in an attempt to maintain cellular energy homeostasis (Deter et al., 1967). Nutrient starvation of CRL 5915 cells was used as a positive control to test this method of measuring autophagic flux in this cell line. Cells were incubated in nutrient rich media or nutrient starvation media (Hank's balance salt solution - HBSS) with or without the addition of cathepsin inhibitors, E64d and Pepstatin A, for 2h and 4h time points. The assay procedure was performed as outlined in full, in Section 2.15.

Nutrient starvation of cells, at both time points, induced increases in LC3-II expression, both with and without the addition of cathepsin inhibitors (Fig 6.12.A). In treatments without cathepsin inhibitors (lane 1 and 3), a distinct increase in LC3-II expression was still observed with nutrient starvation. This indicated the assay was sensitive enough for the detection of increases in autophagosome number, independent of the use of cathepsin inhibitors. Treatment with cathepsin inhibitors can induce an increase in LC3-II expression on their own. Inhibition of normal lysosomal turnover of LC3-II results in its accumulation and increased expression (Tanida et al., 2005). An increase in LC3-II expression with cathepsin inhibitor treatment alone was observed at both time points (lane 1 and 2). The validity of the assay and use of cathepsin inhibitors was still observed however. In nutrient rich and nutrient starvation incubations also containing cathepsin inhibitors (lane 2 and lane 4), nutrient starvation induced a further increase in LC3-II expression, observed at both time points assayed. These results confirmed that nutrient starvation induced an increase in the number of autophagosomes within cells.

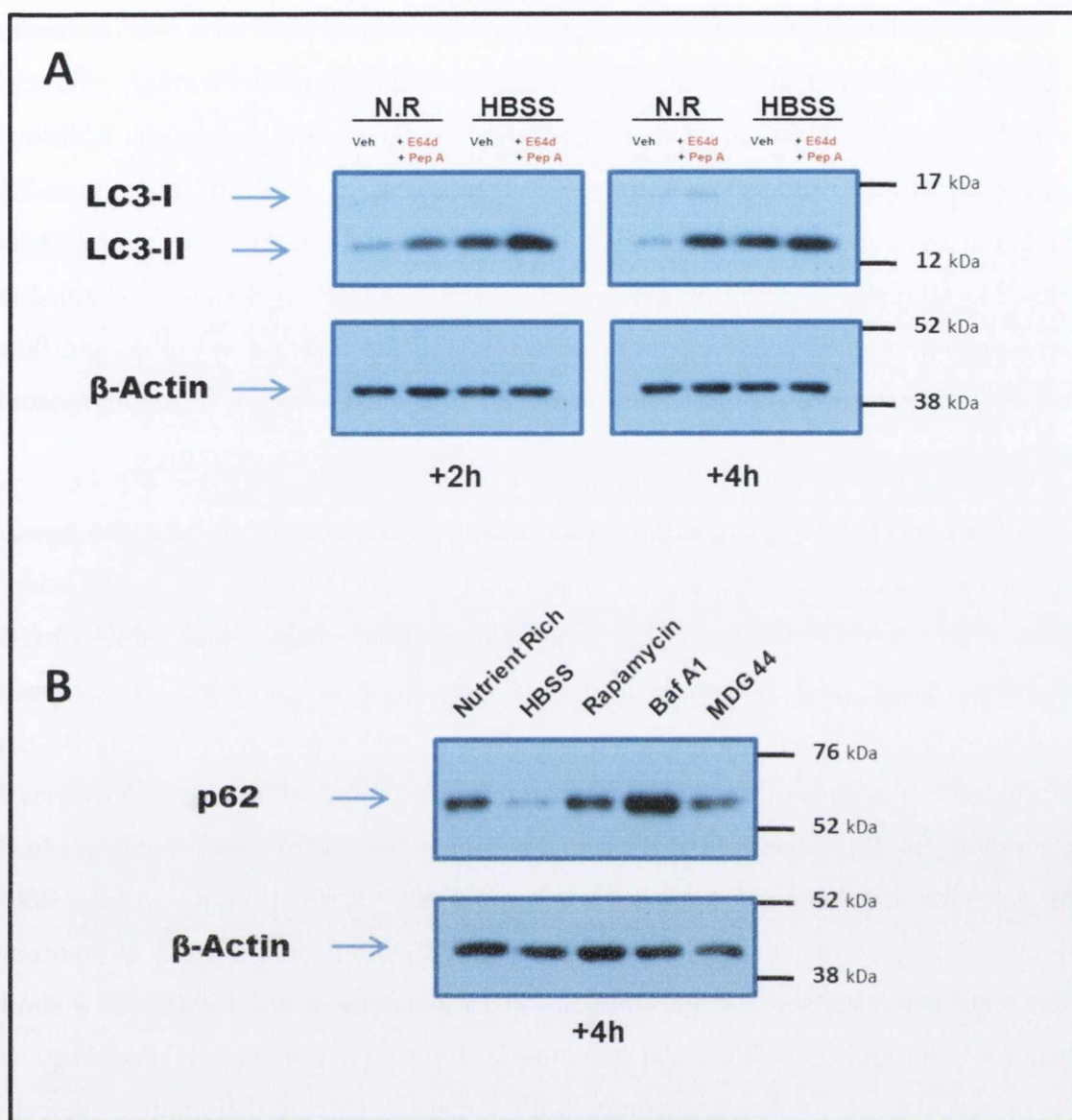


Figure 6.12: Nutrient starvation induced an increase in “autophagic flux” in CRL 5915 cells

(A) CRL 5915 cells were incubated in nutrient rich media (N.R) or nutrient starvation media (HBSS – Hanks Balanced Salt Solution), with or without the addition of cathepsin inhibitors E64d (10μg/ml) and Pepstatin A (10μg/ml), for 2h and 4h. At each experimental endpoint whole cell lysates were prepared as described in Section 2.5.1. Protein (20μg) was resolved on a 12.5% SDS-PAGE gel and expression of LC3 was examined as outlined in Section 2.15.2. Blots were stripped and re-probed with anti-β-actin (Primary Ab – 1:5,000) as a protein loading control. Results are representative of two independent experiments.

(B) CRL 5915 cells were incubated in nutrient rich media, HBSS or nutrient rich media supplemented with Rapamycin [1μM], Bafilomycin A1 [10nM] or MDG 44 [30μM] for 4h. Whole cell lysates were prepared as described in Section 2.5.1. Protein (20μg) was resolved on a 12.5% SDS-PAGE gel and expression of p62 was examined as outlined in Section 2.15.2. Blots were stripped and re-probed with anti-β-actin (Primary Ab – 1:5,000) as a protein loading control. Results are representative of three independent experiments.

Confirmation of increased autophagic flux with nutrient starvation was obtained by Western Blot analysis of protein expression of the lysosomal turnover marker, p62. Cells incubated with nutrient rich and nutrient starvation media for 4h, without the addition of cathepsin inhibitors, were assayed for p62 degradation, as outlined in Section 2.15. Incubation with nutrient starvation media induced significant degradation of the p62 protein, compared to nutrient rich samples (Fig 6.12.B – Lane 1 and 2). This result confirmed that nutrient starvation of CRL 5915 cells induced a significant increase in autophagic flux – increased autophagosome formation and increased lysosomal turnover of autophagosomal cargo.

The effects of other modulators of autophagy on p62 expression were also assessed in this assay. Cells were treated with Rapamycin and Bafilomycin A1 for 4h prior to analysis of p62 expression. Rapamycin is an inducer of autophagy which inhibits mTOR (mammalian target of rapamycin), a negative regulator of initiation of autophagy. Incubation with Rapamycin had no significant effect on p62 expression (Fig 6.12.B – lane 3). An inducer of autophagy would be expected to prompt degradation of p62; however, the concentration of Rapamycin [1 μ M] and incubation time (4h) were believed to be insufficient for induction of autophagy (Tung et al., 2010, Bjorkoy et al., 2005). Bafilomycin A1 is a vacuolar-ATPase inhibitor which inhibits acidification of lysosomes and prevents autophagosome / lysosome fusion (Yamamoto et al., 1998). In a similar manner to Chloroquine, Bafilomycin A1 inhibits lysosomal turnover in autophagy and causes accumulation of autophagosomes within cells. This initiation of a defective autophagic phenotype was detected by the accumulation of p62 (Fig 6.12.B – lane 4).

These control results confirmed the capacity of this autophagic flux assay to detect induction of autophagy (nutrient starvation) and detect the accumulation of autophagosomes during inhibition of autophagy (Bafilomycin A1).

6.7.2 MDG 44 induced increased expression of the autophagosome marker, LC3-II in CRL 5915 cells

Autophagic flux experiments were performed to investigate the effect of MDG 44 treatment on the induction of autophagy and accumulation of autophagosomes in CRL 5915 cells. Two sets of experiments analysing the effect of MDG 44 on autophagic flux were

performed. Cells were incubated with vehicle or MDG 44 [40 μ M] for a short time course of 2h, 4h and 6h incubations (Fig 6.13.A). In a second experimental set up, cells were incubated with vehicle or MDG 44 [30 μ M] for a longer time course of 4h, 12h, 16h and 20h incubations (Fig 6.13.B). In both sets of experiments, cells were also incubated with autophagy inhibitors, Chloroquine and Bafilomycin A1. Autophagic flux analysis was performed as outlined in Section 2.15. Immunoblot analysis of p62, LC3-I, LC3-II and β -actin, illustrated in Figure 6.13.A and Figure 6.13.B, were all from a single representative experiment of each time course.

In the short time course experiments, the assay conditions used – MDG 44 concentration and incubation times – were set to match that of the original Cytospin experiment in which formation of cytoplasmic vesicles were first observed (Chapter 5; Fig 5.17). A significant increase in expression of LC3-II in response to MDG 44 [40 μ M] treatment was detected after both 4h and 6h incubations (Fig 6.13.A). Concurrent decreases in LC3-I expression was observed, indicative of processing of LC3-I to the lipidated LC3-II form, present in autophagosomes. However, significant degradation of p62 was not observed with MDG 44 incubation. No significant or consistent degradation of p62 could be detected in any of the experimental replicates.

A second experimental set up, employing longer incubation times and lower MDG 44 treatment concentrations, was used. 40 μ M incubations with MDG 44 induced apoptotic cell death in CRL 5915 cells. The test concentration of MDG 44 was lowered and incubation time increased in an attempt to better observe the effect of MDG 44 incubation on autophagic flux. Treatment with MDG 44 again induced significant increases in LC3-II expression, this time with 30 μ M incubation of MDG 44 for 12h, 16h and 20h time points (Fig 6.13.B). Indications of LC3-I processing was also observed in this time course. However, while LC3-I processing and significant increases in LC3-II expression were detected, again no concurrent degradation of p62 was observed with MDG 44 incubation. Similar responses were observed with incubations with the autophagy inhibitors, Chloroquine and Bafilomycin A1. Both compounds induced significant increases in LC3-II expression, indicative of autophagosome formation and accumulation, however no degradation of p62 was observed.

Autophagic flux analysis indicated that MDG 44 induced increased expression of LC3-II, indicative of increased autophagosome formation within MPM cells. While

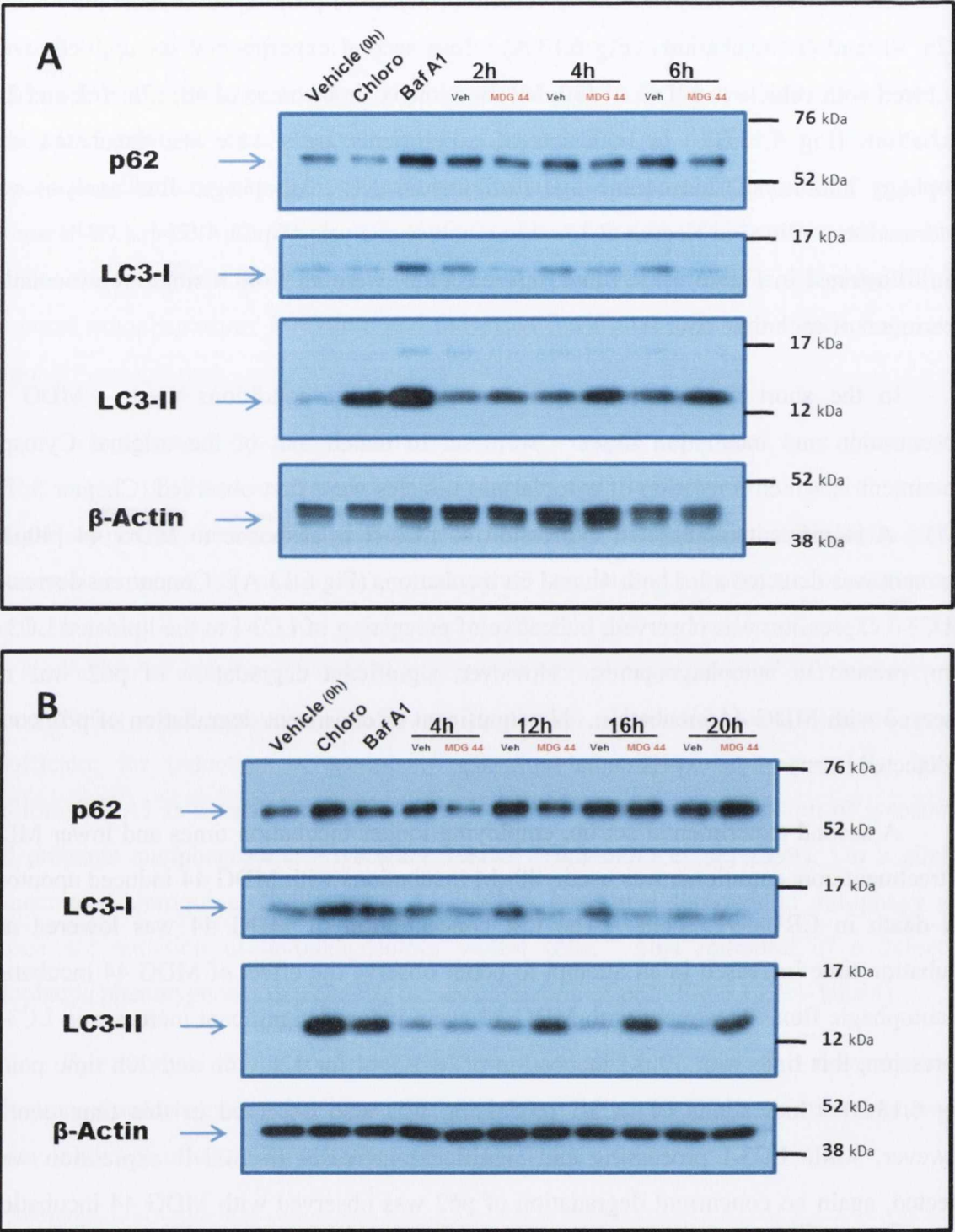


Figure 6.13: MDG 44 treatment induced an increase in the processing and accumulation of the autophagosomal marker LC3-II

CRL 5915 cells were treated with vehicle (EtOH 1%) or MDG 44 at a concentration of 40μM for short time points (0, 2h, 4h & 6h) (A) or at a concentration of 30μM for longer time points (0, 4h, 12h, 16h & 20h) (B) to examine the effect of MDG 44 on p62 and LC3 processing. Concurrently, cells were treated with positive controls Chloroquine [25μM] and Bafilomycin A1 [10nM]. At each experimental endpoint whole cell lysates were prepared as described in Section 2.5.1. Protein (20μg) was resolved on a 12.5% SDS-PAGE gel and expression of LC3 and p62 was examined as outlined in Section 2.15.2. Blots were stripped and re-probed with anti-β-actin (Primary Ab – 1:5,000) as a protein loading control. Results are representative of three (A) and two (B) independent experiments.

increased formation of autophagosomes was detected (LC3-II), increased lysosomal turnover of p62 was not observed. The accumulation of autophagosomes without the lysosomal degradation of cargo may indicate MDG 44 induces a defective form of autophagic flux in MPM cells.

6.8 MDG 44 induced the formation of LC3-GFP positive structure in LC3-GFP stably transfected macrophages

Confocal microscopy analysis was used to confirm the result that MDG 44 treatment could induce the formation and accumulation of autophagosomes and visualise the presence of these structures within cells. An LC3-GFP, stably transfected macrophage cell line was used for this experimentation. The macrophage cell line was transfected with an expression plasmid containing the LC3-GFP tagged gene and a promoter region inducing ubiquitous overexpression of the fluorescently-labelled LC3 protein. In this cell line, the formation and accumulation of autophagosomes can be visualised by way of the fluorescently tagged LC3 protein. In normal, healthy cell with basal levels of autophagic flux, most LC3 protein will be present in its cytosolic form (LC3-I) and distributed uniformly throughout the cytoplasm. During confocal microscopy analysis of LC3-GFP cells, this phenomenon was represented as uniform GFP fluorescence throughout the cytoplasm. Formation of autophagosomes requires the recruitment of large amounts of lipidated LC3 protein (LC3-II) to the outer and inner leaflet of the maturing autophagosome (Yang and Klionsky, 2010). In LC3-GFP cells, this coalescence of large amounts of LC3 protein to relatively small structures within the cytoplasm was visualised as the formation of GFP “Bright” structures with the cytoplasm. LC3-GFP transfection and overexpression has been previously used as a method of detecting LC3 positive autophagosomal structures in cells (Wilson et al., 2011, Yang et al., 2008).

Initial experimental analysis was performed using an Andor Revolution XD spinning disk confocal microscope with single fluorescent channel detection of GFP fluorescence within live cells. LC3-GFP cells were incubated with vehicle and MDG 44, as well as the autophagy inhibitors Chloroquine and Bafilomycin A1, for 4h prior to live cell imaging. Chloroquine and Bafilomycin A1 were used as positive controls in this assay as compounds known to induce accumulation of autophagosomes within cells. In Vehicle treated cells, uniform GFP fluorescence was observed throughout the cell (Fig 6.14). Incubations with

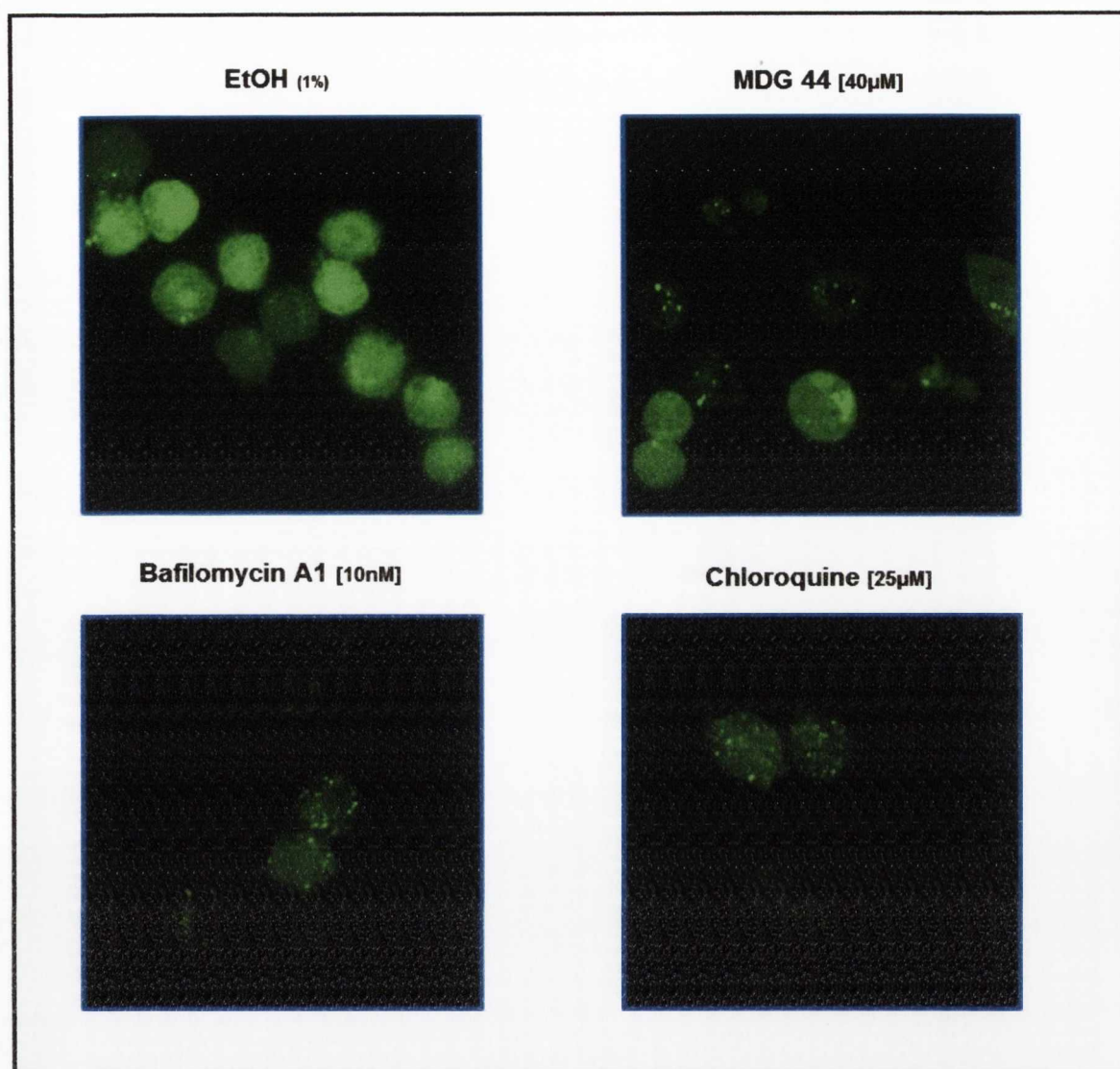


Figure 6.14: MDG 44 treatment induced the formation of LC3 positive cytoplasmic structures in LC3-GFP stably transfected Macrophages

LC3-GFP stably transfected Macrophages were seeded at density of 0.5×10^6 cells on glass bottom, 2ml volume confocal wells. Cells were treated with vehicle (EtOH 1%) or indicated concentrations of MDG 44, Bafilomycin A1 and Chloroquine for 4h. Live cell imaging of LC3-GFP Macrophages was performed using an Andor Revolution XD Spinning Disk Confocal Microscope.

Bafilomycin A1 and Chloroquine induced the formation of LC3 positive, GFP “Bright” structures within cells, indicative of autophagosomes. Similar LC3-GFP structures were observed in MDG 44 treated cells.

In subsequent confocal microscopy analysis, LC3-GFP cells were counter stained with the fluorescent nuclear dye, DAPI, and live cell fluorescent imaging was performed using an Olympus FV1000 Point-scanning confocal microscope. Images of the GFP channel alone, GFP combined with DAPI and the merge of GFP, DAPI and Phase contrast images were taken. Cells were treated with vehicle or MDG 44 for 4h prior to live cell imaging. Positive control experiments, included incubation with nutrient rich media, nutrient starvation media (HBSS), Chloroquine and Bafilomycin A1. These incubations were also performed for 4h, in conjunction with MDG 44 experimentation. Cells incubated with nutrient rich media showed no autophagosome formation, with uniform distribution of LC3-GFP protein throughout the cytoplasm (Fig 6.15.a). Cells incubated in nutrient starvation media (HBSS) induced an increase in LC3 positive, GFP “Bright” autophagosomal structures within the cytoplasm. Incubation with the autophagy inhibitors Chloroquine and Bafilomycin A1 both induced significant accumulation of autophagosomes. Bafilomycin A1 was particularly potent with a very large amount of LC3 positive, GFP “Bright” structures detected within the cytoplasm of treated cells (Fig 6.15.a). Similar results were observed for MDG 44 incubations in LC3-GFP cells (Fig 6.15.b – 6.15.c). Treatment with vehicle had no inductive effect on the formation of autophagosomes. However, incubation with MDG 44 at 35 μ M and 40 μ M induced significant accumulation of LC3 positive, GFP “Bright” structures within the cytoplasm of cells.

6.9 PI3-Kinase inhibitor, 3-Methyl Adenine, did not inhibit MDG 44 induced cell death in CRL 5915 cells

Experiments were performed to examine the contribution of the activation of autophagy on the induction of cell death by MDG 44. An inhibitory assay was designed to test if preventing the induction of autophagy would affect the level of MDG 44 induced PCD in MPM. The phosphatidylinositol-3-kinase (PI3K) inhibitor, 3-methyl adenine (3-MA), was used to inhibit autophagy upstream of the initiation of phagophore assembly and autophagosome maturation. This technique has been widely used in previous reports to

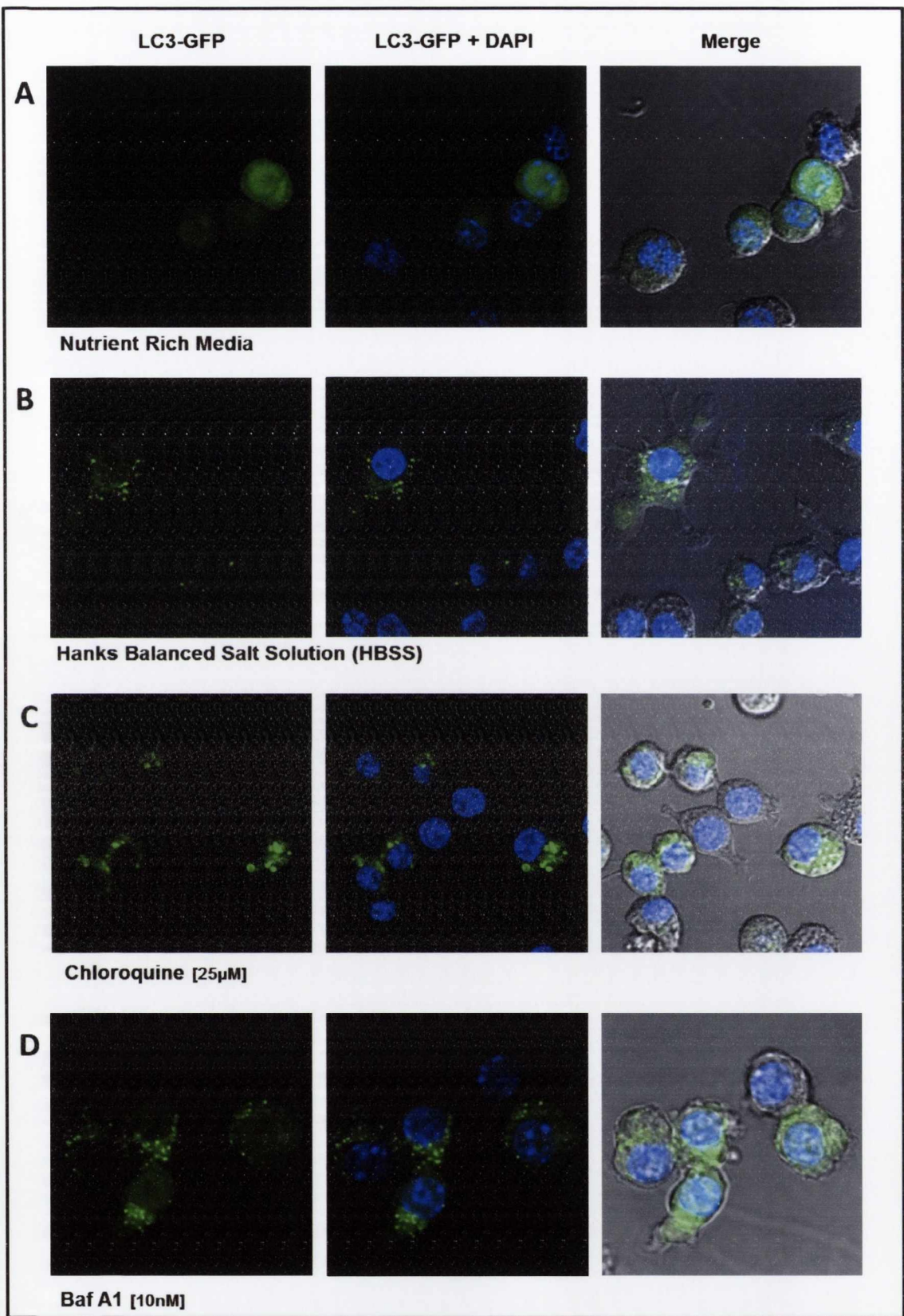


Figure 6.15.a: Nutrient starvation, Chloroquine and Bafilomycin A1 induced the formation of LC3 positive cytoplasmic structures in LC3-GFP stably transfected Macrophages

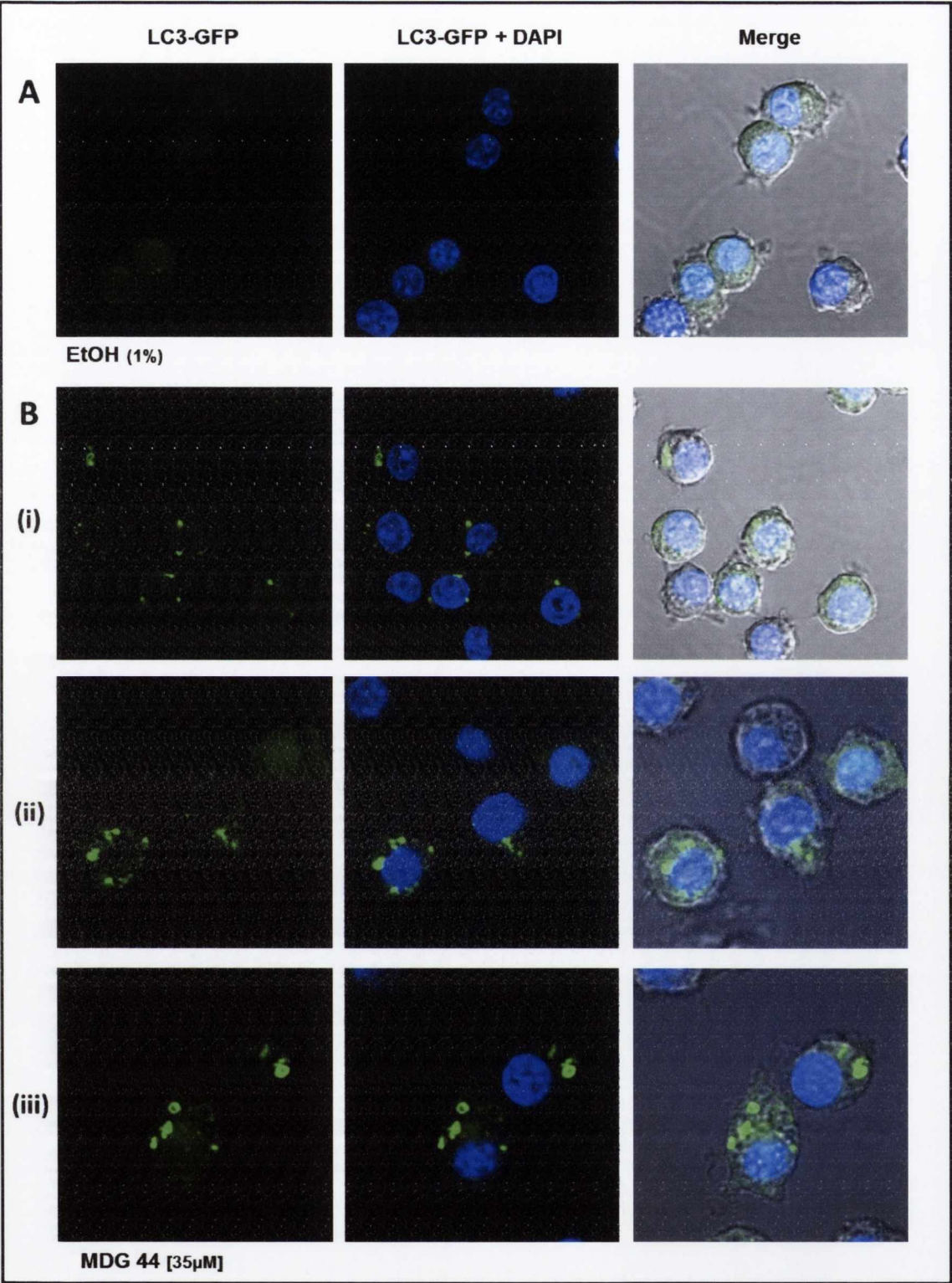


Figure 6.15.b: MDG 44 [35μM] treatment induced the formation of LC3 positive cytoplasmic structures in LC3-GFP stably transfected Macrophages

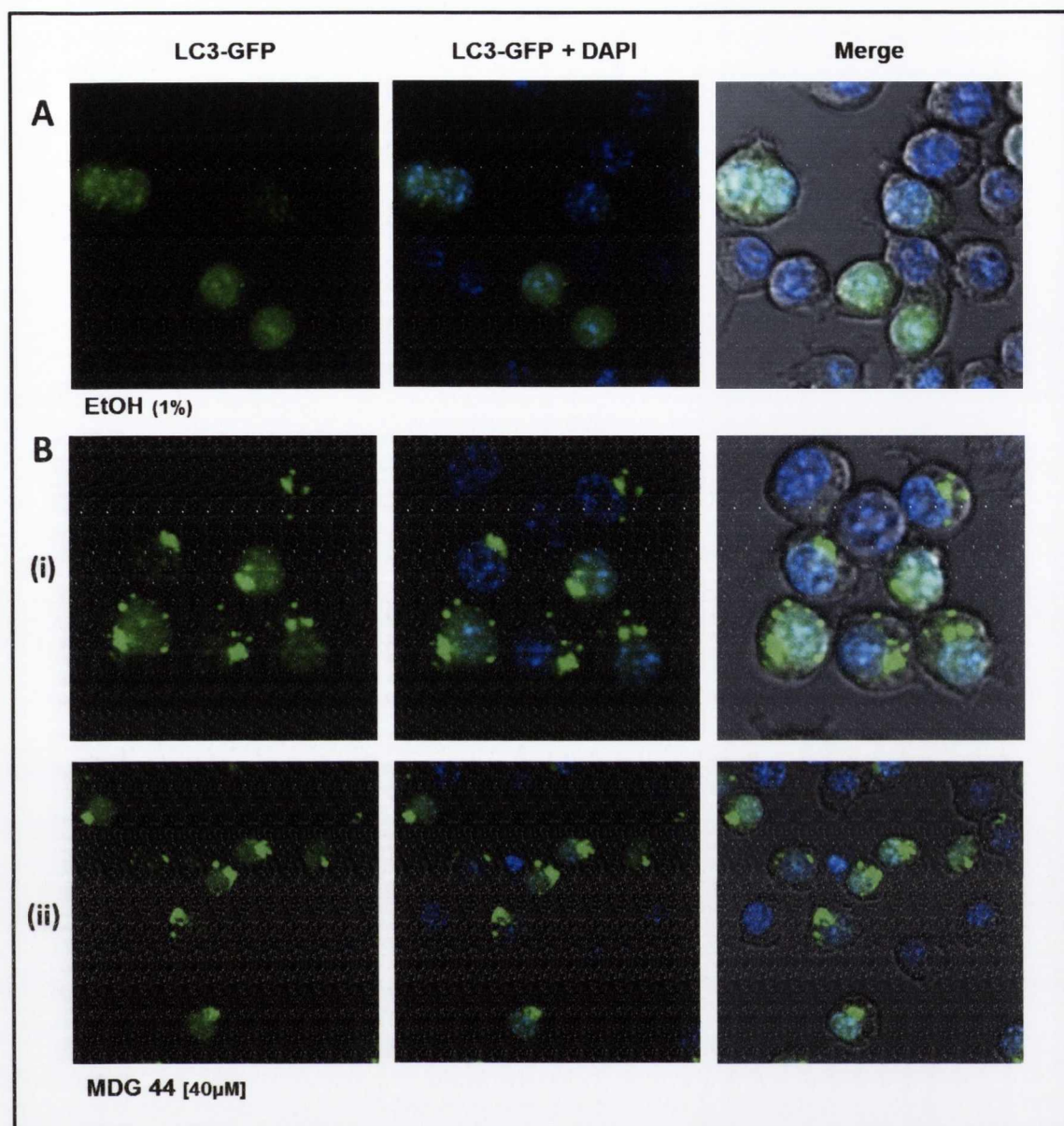


Figure 6.15.c: MDG 44 [40μM] treatment induced the formation of LC3 positive cytoplasmic structures in LC3-GFP stably transfected Macrophages

Figure Legend –Fig. 6.15.a – c

(**Fig 5.x.a**) Cells were incubated in nutrient rich media (**A**), nutrient starvation media (Hanks Balanced Salt solution – HBSS) (**B**) or nutrient rich media supplemented with Chloroquine [25μM] (**C**) or Bafilomycin A1 [10nM] (**D**) for 4h. (**Fig 5.x.b**) Cells were incubated with Vehicle (EtOH 1%) (**A**) or MDG 44 [35μM] (**B i – iii**) for 4h. (**Fig 5.x.c**) Cells were incubated with Vehicle (EtOH 1%) (**A**) or MDG 44 [40μM] (**B i – iii**) for 4h. LC3-GFP stably transfected Macrophages were seeded at density of 0.13×10^6 cells on glass bottom, 2ml volume confocal wells. Live cell imaging of LC3-GFP Macrophages was performed using an Olympus FV1000 Point-Scanning Confocal Microscope. The nuclear DNA of cells was stained with DAPI (Blue). The images shown are of LC3-GFP alone (Green – Left panel), LC3-GFP + DAPI (Green + Blue respectively – Centre panel) and LC3-GFP + DAPI + phase contrast image (Merge – Right panel). Results are representative images of three independent experiments.

inhibit autophagy induction *in vitro* (Seglen and Gordon, 1982, Takatsuka et al., 2004). CRL 5915 cells were pre-treated with vehicle or 3-MA for 1h prior to treatment with MDG 44 for 6h and 24h incubations. PI cell cycle analysis revealed that 3-MA had no inhibitory effect on the induction of cell death by MDG 44 at either time point (Fig 6.16.A). Cell viability assays were also performed in which CRL 5915 cells were pre-treated with 3-MA prior to incubation with increasing concentrations of MDG 44 for 24h. Cell viability analysis was performed by Alamar Blue assay. Again, 3-MA had no inhibitory effect on induction of cell death by MDG 44 (Fig 6.16.B). IC₅₀ values for cell viability of treatments with MDG 44 alone and MDG 44, plus 3-MA were 33.7 μ M ($\pm$ 0.9) and 35.1 μ M ($\pm$ 0.7), respectively.

6.10 Examination of MDG 44 as a proteasome inhibitor

The final target of interest in this study was that of the ubiquitin-proteasome system (UPS) and investigations were performed to assess the activity of MDG 44 as a direct inhibitor of the 26S proteasome. Maintaining competent ubiquitination and proteosomal activities within the cell is an essential process for cellular homeostasis. The UPS participates in the non-lysosomal degradation of short lived proteins involved in highly regulated cellular processes, including regulation of signal transduction and cell cycle transition (Schwartz and Ciechanover, 1999). Inhibition of the proteasome causes a number of distinct responses within the cells including inhibition of signal transduction pathways, accumulation of poly-ubiquitinated proteins and activation of cell stress pathways, culminating in activation of PCD (Wu et al., 2010a). In this study, to assess the activity of MDG 44 as a proteasome inhibitor, classical assays used to detect specific indirect consequences of proteasome inhibition were performed. These assays included examining the accumulation of poly-ubiquitinated protein in response to MDG 44 incubation and assessing the effect of MDG 44 on the NF κ B:I κ B interface.

NF κ B is a transcription factor with pleiotropic functions and activated in numerous signalling events within the cell, including death receptor ligation and Toll-Like Receptor (TLR) immune signalling (Grimm and Baeuerle, 1993). Constitutive NF κ B activation is prevented by its association with its regulatory accessory protein, I κ B. NF κ B activation is initiated by mitogen activated protein kinase (MAPK) phosphorylation of I κ B, targeting the protein for ubiquitin tagging and subsequent proteosomal degradation. NF κ B activation and

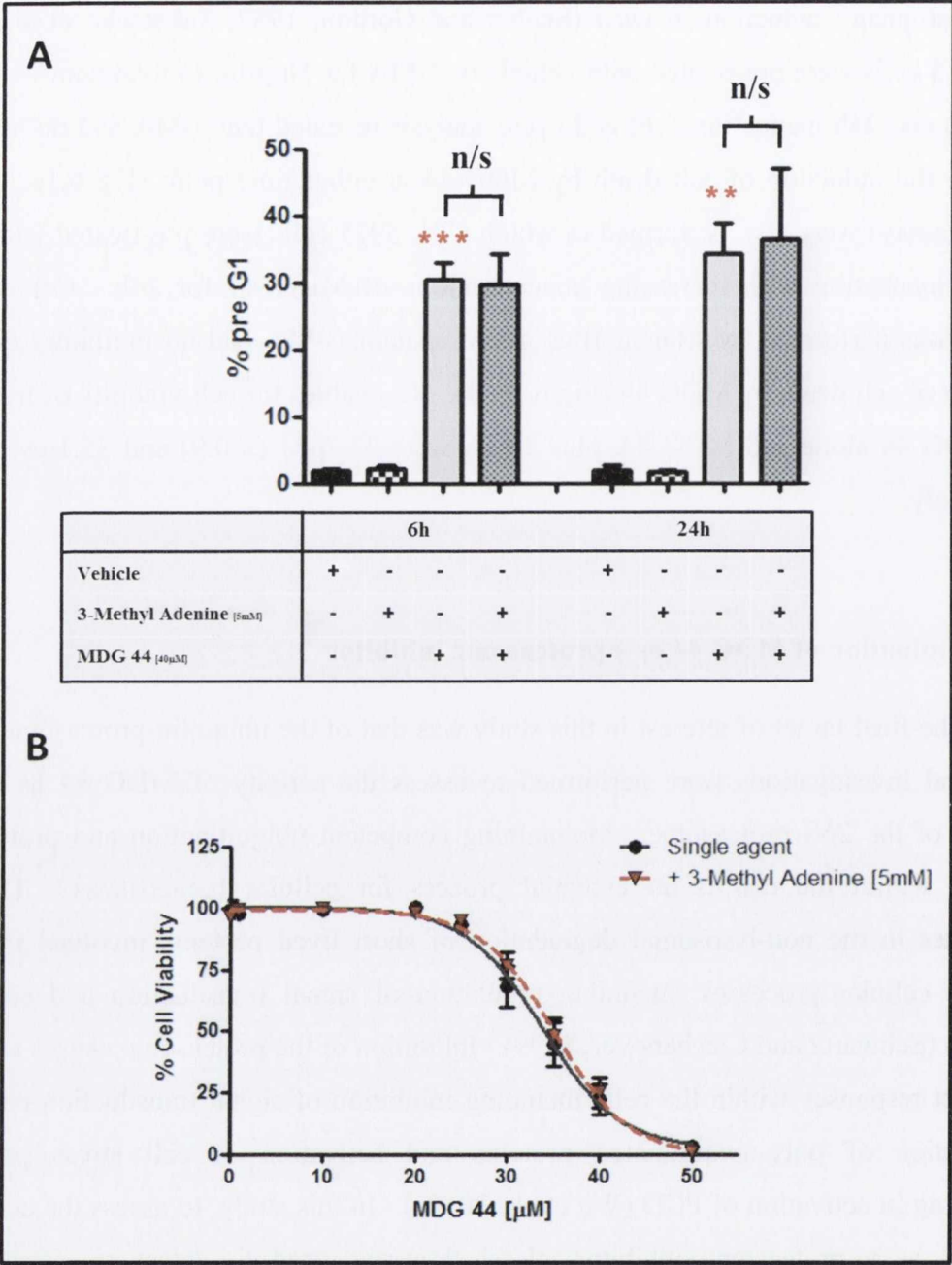


Figure 6.16 PI3K / Autophagy inhibitor, 3-Methyl Adenine, did not inhibit MDG 44 induced cell death in CRL 5915 cells

(A) CRL 5915 cells were treated with MDG 44 [40μM] +/- 3-Methyl Adenine [5mM] for 6h or 24h and percentage induction of apoptosis was determined. CR 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml). Cells were pre-treated for 1h with 3-MA prior to treatment with MDG 44 for indicated time points. PI staining and cell-cycle flow cytometry analysis is outlined in Section 2.7. . Values are representative of mean $\pm$ SEM of four independent experiments. **P<0.01 ***P<0.001; n/s (no significant difference P>0.05) (Determined using unpaired Student's t-test, part of Graphpad Prism 4 statistical software).

(B) CRL 5915 cells treated in a dose dependant manner with MDG 44 +/- 3-Methyl Adenine [5mM] for 24h. CRL 5915 cells were seeded at a density of 0.5×10^4 cells / well (200μl) on a 96 well plate. Cells were pre-treated for 1h with 3-MA prior to treatment with MDG 44 for indicated time points. Alamar blue cell viability analysis was performed as outlined in Section 2.6. Values are representative of mean $\pm$ SEM of four independent experiments. Non-linear regression curve fit analysis was performed (Graphpad Prism 4 Statistical software). EC50 values generated for MDG 44 treatment alone or in combination with 3-MA were 33.73μM ($\pm$ 0.9μM) and 35.10μM ($\pm$ 0.7μM), respectively.

downstream signal transduction is dependent on this ubiquitin-proteasome regulated process (Henkel et al., 1993). In this study, the ability of MDG 44 to inhibit both NF κ B activation and degradation of I κ B was assessed. Numerous previous studies have reported on the ability of proteasome inhibitors to elicit such activities in cells (Kim, 2002, Traenckner et al., 1994).

6.10.1 MDG 44 inhibited NF κ B activation in the Hek-Blue-hTLR4 / NF κ B reporter assay

A Hek-Blue-hTLR4 NF κ B reporter assay was used to investigate inhibition of NF κ B by MDG 44. Hek-Blue-hTLR4 cells are a commercially available cell line (Invitrogen) transfected with expression plasmids containing hTLR4, its co-receptor genes MD2/CD14 and a secreted embryonic alkaline phosphatase (SEAP) reporter gene flanked by five NF κ B response elements in its promoter region. TLR4 ligation and subsequent activation of NF κ B was quantified by measuring the expression and secretion of the NF κ B reporter SEAP. Quanti-Blue, a medium containing alkaline phosphate substrate and which changes colour in the presence of alkaline phosphatase (SEAP), was used to quantify NF κ B activation spectrophotometrically. The experimental procedure and data analysis used was outlined in Section 2.16.

Hek-Blue cells were pre-treated with increasing concentrations of MDG 44 for 1h, prior to incubation with the TLR4 native ligand, Lipopolysaccharide (LPS), to induce NF κ B activation. LPS incubations were performed for 6h and 24h time points prior to Quanti-Blue analysis. As a positive control, test wells were also pre-treated with the known proteasome inhibitor, MG132 (Ding et al., 2007), prior to incubation with LPS for both time periods. As well as assaying NF κ B activation via Quanti-Blue, Alamar-Blue cell viability analysis was performed on treated Hek-Blue cells at the same time. It was necessary to control against any inhibition of NF κ B activation being attributable to the cytotoxic activity of the test compounds and controls assayed. After 6h incubations, MG132 induced significant reductions in NF κ B activation with 5 μ M treatment and complete inhibition of NF κ B activation was observed with 7.5 μ M incubation (Fig 6.17.a.C). Alamar-Blue cell viability analysis of MG132 incubations revealed only slight reductions (10% – 15%) in the cell viability of Hek-Blue cells, indicating specific targeting and inhibition of NF κ B activation by the proteasome inhibitor.

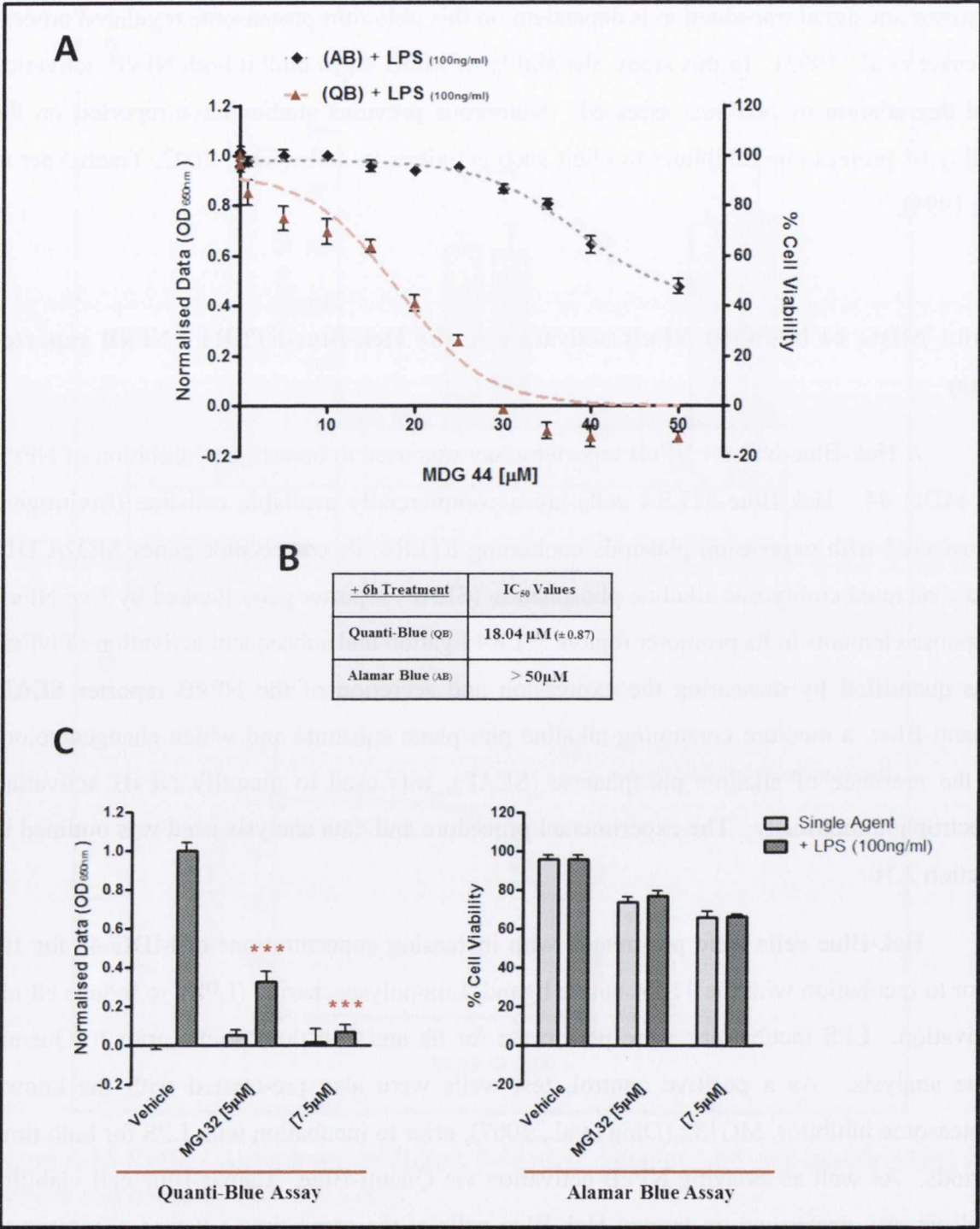


Figure 6.17.a: MDG 44 inhibited induction of NF κ B activation in a Hek-Blue-hTLR-4 / NF κ B reporter assay

Hek-Blue-hTLR4 cells treated with indicated concentrations of MDG 44 (**A**) or the proteasome inhibitor MG132 (**C**) for 6h. NF κ B activation was monitored by Quanti-BlueTM detection of secreted embryonic alkaline phosphatase (SEAP) reporter gene protein expression levels in the supernatant of treated cells (full experimental method and data analysis described in Section 2.17). Simultaneously, the effect of test compound treatment on the cell viability of Hek-Blue-hTLR4 cells was determined by Alamar Blue cell viability assay, as outlined in Section 2.6. IC₅₀ values for MDG 44 inhibition of NF κ B activation and cell viability are shown (**B**). Results are representative of the mean $\pm$ SEM of four independent triplicate experiments. Graphpad Prism 4 statistical software was used to perform non-linear regression curve fitting and statistical analysis (unpaired Student's t-test; ***P<0.001).

MDG 44 treatment induced significant, dose-dependent inhibition of NF κ B activation after 6h LPS incubation (Fig 6.17.a). Alamar-Blue cell viability results indicated that inhibition of NF κ B was completely independent of the cytotoxic activity of MDG 44. The IC₅₀ value for NF κ B inhibition was 18.0 μ M ($\pm$ 0.9) compared with the IC₅₀ value for cell viability of MDG 44 being outside the assay range ($>50\mu$ M) (Fig 6.17.a). Similar results were observed after 24 LPS incubation assays (Fig 6.17.b). These results confirmed that MDG 44 induced inhibition of NF κ B, in a dose-dependent manner.

6.10.2 MDG 44 inhibited TNF- α induced I κ B degradation in a dose dependant manner in CRL 5915 cells

As outlined above, NF κ B activation requires both the phosphorylation of I κ B and the ubiquitination / proteasomal degradation of its regulatory protein. Proteasome inhibitors prevent the activation of NF κ B primarily due to the inhibited degradation of I κ B. In a complementary experiment to the Hek-Blue assay investigating inhibition of NF κ B activation, an experiment was designed to examine the effect of MDG 44 on the proteasomal degradation of I κ B. CRL 5915 cells were treated with TNF- α to induce NF κ B activation / I κ B degradation; Expression of the TNFR in CRL 5915 cells was reported previously (Chapter 4; Fig 4.10). In an initial control experiment, CRL 5915 cells were incubated with vehicle or the proteasome inhibitor, MG132, for 1h prior to treatment with TNF- α . Incubations with TNF- α were performed for 0, 5 min, 15 min and 30 min, and whole cell lysates were prepared at each experimental end point. Induction of I κ B degradation was detected by Western Bot analysis. Results illustrated in Figure 6.18.A indicated that incubations with TNF- α alone induced rapid degradation of I κ B. Degradation of the I κ B band was first detected after 15 min TNF- α incubation and complete degradation of I κ B was observed after 30 min. In contrast, pre-treatment with MG132 completely inhibited the degradation of I κ B, at all incubation periods with TNF- α .

From the control assay, a TNF- α incubation period of 30 min was chosen for I κ B degradation experimentation with MDG 44. Complete degradation of I κ B was detected after 30 min incubation with TNF- α , however, inhibition of the proteasome could still prevent the degradation of I κ B at this time point. In this experiment, CRL 5915 cells were pre-treated with increasing concentrations of MDG 44 for 1h, prior to incubation with TNF- α . Pre-treatment with MG132, followed by incubation with TNF- α was also performed as a

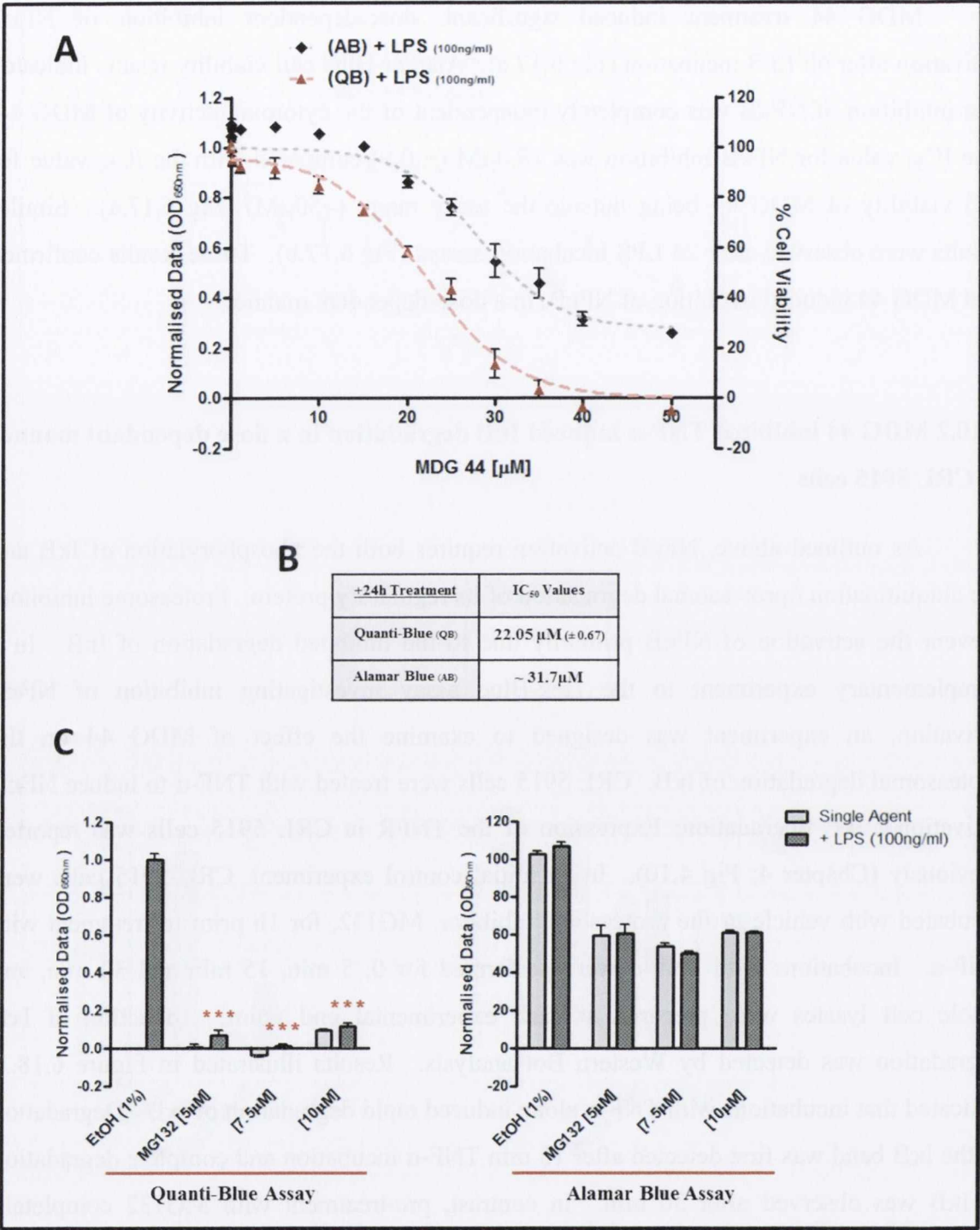


Figure 6.17.b: MDG 44 inhibited induction of NF κ B activation in a Hek-Blue-hTLR-4 / NF κ B reporter assay

Hek-Blue-hTLR4 cells treated with indicated concentrations of MDG 44 (A) or the proteasome inhibitor MG132 (C) for 24h. NF κ B activation was monitored by Quanti-Blue™ detection of secreted embryonic alkaline phosphatase (SEAP) reporter gene protein expression levels in the supernatant of treated cells (full experimental method and data analysis described in Section 2.17). Simultaneously, the effect of test compound treatment on the cell viability of Hek-Blue-hTLR4 cells was determined by Alamar Blue cell viability assay, as outlined in Section 2.6. IC₅₀ values for MDG 44 inhibition of NF κ B activation and cell viability are shown (B). Results are representative of the mean $\pm$ SEM of four independent triplicate experiments. Graphpad Prism 4 statistical software was used to perform non-linear regression curve fitting and statistical analysis (unpaired Student's t-test; ***P<0.001).

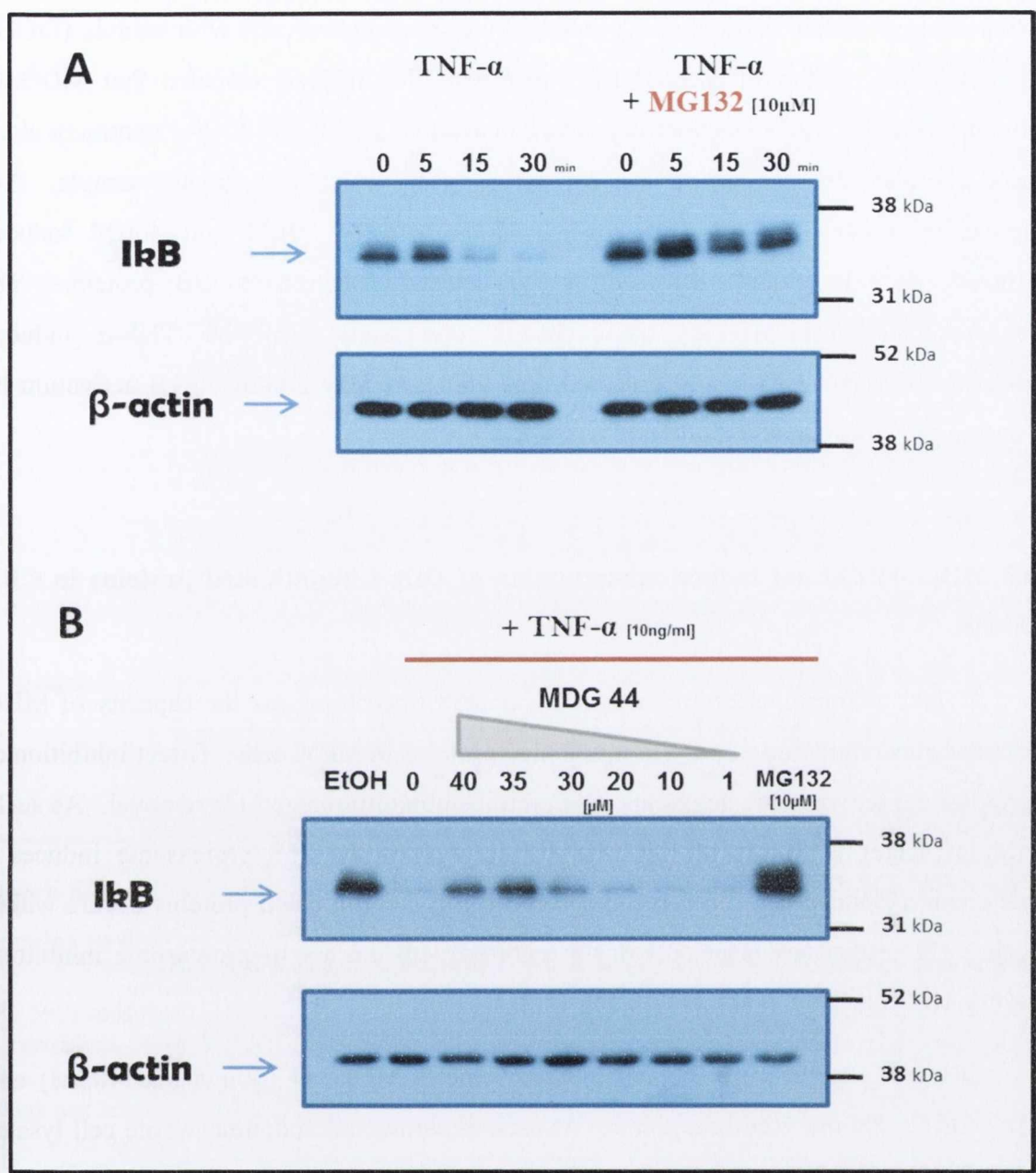


Figure 6.18: MDG 44 inhibited TNF- α induced I κ B degradation in a dose dependant manner in CRL 5915 cells

CRL 5915 cells treated with TNF- α [10ng/ml] with or without pre-treatment with the proteasome inhibitor MG132 [10 μ M] for 0, 5, 15 and 30 min (A). CRL 5915 cells treated with indicated concentrations of MDG 44 or MG132 prior to treatment with vehicle or TNF- α for 30 min (B). CRL 5915 cells were seeded at a density of 0.12×10^6 cells / well (2.5ml). Cells were pre-treated with MDG 44 or MG132 for 1h prior to addition of TNF- α for indicated time points. At the experimental end points, whole cell lysates were prepared as described in Section 2.5.1. Protein (20 μ g) was resolved on 10% SDS-PAGE gels and transferred to PVDF membrane, as outlined in Section 2.18. Blots were blocked in 5% Marvel TBS-Tween and probed with anti-I κ B (Primary Ab – 1:2,500). Blots were stripped and re-probed with anti- β actin (Primary – 1:5,000) as a loading control. Results are representative of three independent experiments.

positive control. Other assay controls included pre-treatment of cells with vehicle (EtOH), with or without incubation with TNF- α . Western Blot analysis revealed that MDG 44 inhibited I κ B degradation in a dose dependant manner (Fig 6.18.B). TNF- α treatment alone induced complete degradation of I κ B, compared to the vehicle only treated sample. Pre-treatment with MDG 44 at four concentrations between 20 μ M and 40 μ M induced significant, dose-dependent increases in the detectable levels of I κ B protein. The proteasome inhibitor, MG132, as expected, completely inhibited TNF- α induced degradation of I κ B. This result indicated that MDG 44 may inhibit NF κ B activation by preventing proteasomal degradation of I κ B.

6.10.3 MDG 44 did not induce accumulation of Poly-Ubiquitinated proteins in CRL 5915 cells

A final proteasome inhibition assay was performed to assess the capacity of MDG 44 to induce accumulation of poly-ubiquitinated proteins in MPM cells. Direct inhibition of the proteasome prevents the degradation of proteins ubiquitin-tagged for removal. As such, the use of direct inhibitors of the catalytic activity of the 26S proteasome induces a characteristic response in which accumulation of poly-ubiquitinated proteins occurs within the cell. This effect has been previously reported with the use of proteasome inhibitors, including MG132 (Ding et al., 2007, Fang et al., 2011).

CRL 5915 cells were incubated with vehicle, MDG 44 (30 μ M and 40 μ M) and MG132 for 4h, 8h and 20h time points. At each experimental endpoint, whole cell lysates were prepared and Immunoblot analysis for ubiquitinated proteins performed. Immunoblotting using anti-ubiquitin detected a “smear” of mostly high molecular weight, polyubiquitinated proteins in each lane (Fig 6.19). Native ubiquitin protein (8.5 kDa), unconjugated to protein targets was not detected in this assay. Basal levels of poly-ubiquitinated proteins were detected in vehicle treated samples. In comparison to these control levels, incubation with the proteasome inhibitor MG132 induces a significant increase in the amount of poly-ubiquitinated proteins detected. This response is observed at all three time points assayed. However, incubations with both 30 μ M and 40 μ M MDG 44, at all three time points, induced no change in the amount of poly-ubiquitinated proteins within the cell, when compared to vehicle samples. The lack of accumulation of poly-ubiquitinated proteins in treated cells suggest that MDG 44 does not directly inhibit the

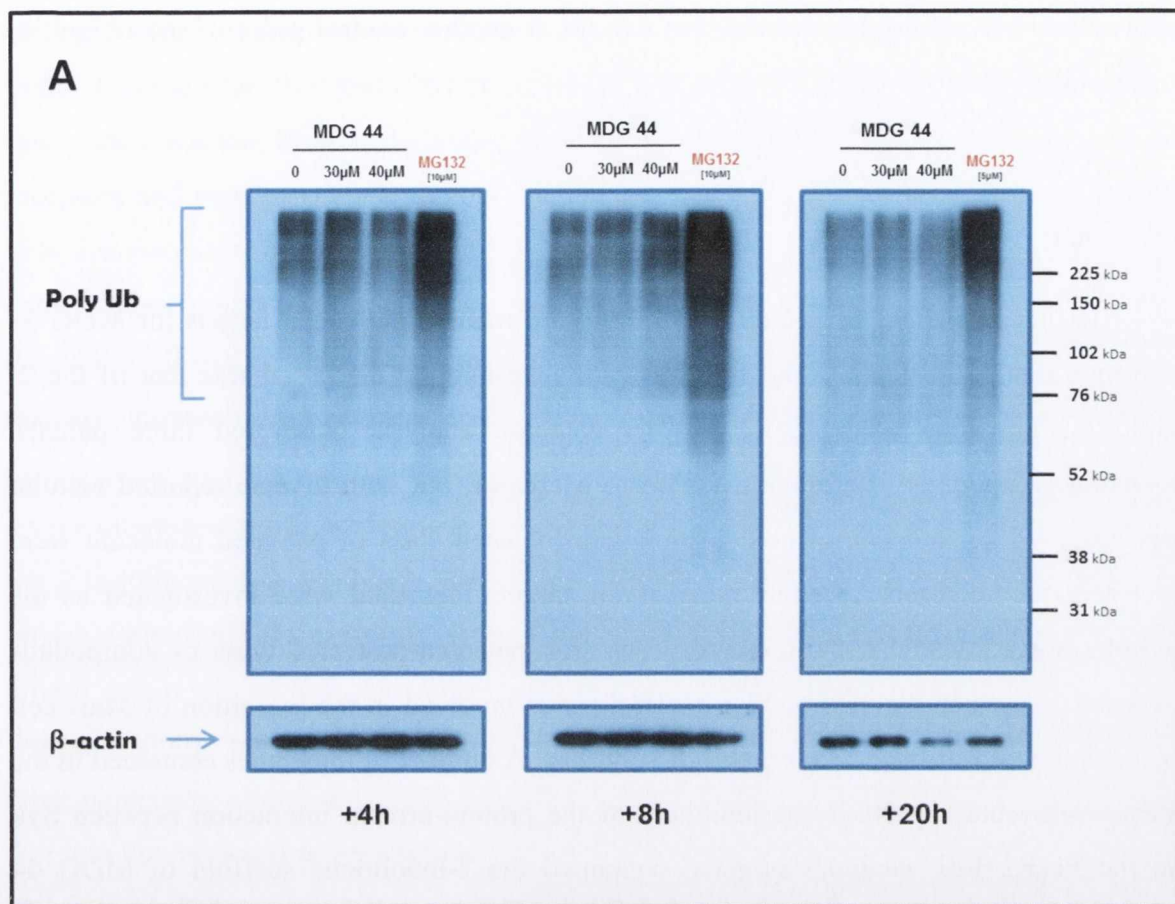


Figure 6.19: MDG 44 treatment did not induce accumulation of Poly-Ubiquitinated proteins in CRL 5915 cells

CRL 5915 cells were seeded at a density of 0.13×10^6 cells / well (2.25ml). Cells were treated with indicated concentrations of Vehicle (EtOH 1%), MDG 44 or proteasome inhibitor MG132 for 4h, 8h and 20h time points. At each experimental endpoint whole cell lysates were prepared as described in Section 2.5.1. Protein (30 μg) was resolved on an 8% SDS-PAGE gel and transferred to PVDF membrane, as outlined in Section 2.5.4. Blots were blocked in 5% Marvel TBS-Tween and probed with anti-Ubiquitin (Primary Ab – 1:1,000). Blots were stripped and re-probed with anti-β actin (Primary – 1:5,000) as a loading control, as outlined in Section 2.5.6. Results are representative of three independent experiments.

catalytic activity of the proteasome, but my act at another control point of the ubiquitin-proteasome system.

Discussion

In this chapter, a large and diverse array of putative molecular targets for MDG 44 were investigated in depth. The first mode of investigation discussed was that of the 2-indolinone scaffold patent search. This database analysis uncovered three patents, protecting compounds of similar structure to MDG 44, but with diverse reported cellular activities. While interesting molecular targets for each class of patented molecule were described, for numerous reasons, none of the targets identified were investigated as the possible target for MDG 44 in MPM. The first patented-protected class of compounds discussed, described a series of Syk kinase ligands involved in the inhibition of Mast cell degranulation in response to allergen/IgE stimulus. A number of molecules contained in the patent, with reported activity as inhibitors of the protein-protein interaction between Syk and the FcεR1 IgE receptor complex, contained the 2-indolinone scaffold of MDG 44 (Compound α) (Villoutreix et al., 2011). Syk kinase is ubiquitously expressed in hematopoietic cells and is most noted for its activity in immunoregulation and immunoreceptor signalling (Riccaboni et al., 2010). Its activity as an immunomodulator makes Syk a target of immense clinical significance in the treatment of autoimmune and allergic disorders like Rheumatoid arthritis, Systemic lupus erythematosus and asthma. Syk kinase targeting in cancer has been reported, specifically in the treatment of lymphoma and leukaemia. The anti-cancer agents Staurosporine and Imatinib mesylate (Gleevec) are both tyrosine kinase inhibitors (TKI) with affinity for Syk (Riccaboni et al., 2010). Gleevec is an anti-cancer agent in clinical use for the treatment of chronic myelogenous leukaemia (CML), inhibiting tumour growth via inhibition of the oncogenic, tyrosine kinase fusion-protein, BCR/abl (O'Dwyer and Druker, 2000).

It was decided, after consideration of the cellular activates of Syk kinase and the reported mechanism of action of the 2-indolinone inhibitors, that Syk kinase did not represent a rational target for investigation with MDG 44. Syk expression and activity is predominantly contained to immune cells. While Syk is a reported target with anti-cancer activity, this was again limited to lymphoma and leukaemia, not solid tumours. Recently however, the use of TKIs in the treatment of MPM has been revealed as a promising new

strategy, with a number of compounds under investigation in Phase I and Phase II clinical trials (Jakobsen and Sorensen, 2011). The TKI Gleevec has reported affinity for the proto-oncogene c-Kit and Platelet-derived growth factor receptor (PDGFR), two tyrosine kinase receptors and poor prognostic factors in MPM. Specifically, PDGFR has been associated with disease progression, causing thrombocytopenia, and its inhibition in overexpressed MPM cells induced suppression of tumour growth (Edwards et al., 2001, Vogelzang et al., 2005). Four Phase-II trials employing Gleevec as a single agent therapy have reported very limited success; however combination trials with Pemetrexed / Cisplatin and another with Gemcitabine are proposed (Jakobsen and Sorensen, 2011). While tyrosine kinases represent a very interesting novel strategy for treatment in MPM, the specific mechanism of action of the 2-indolinone Syk inhibitors did not suggest MDG 44 may work by way of tyrosine kinase inhibition. The patented compounds did not target the ATP binding pocket of the kinase active site of Syk, like Gleevec or other tyrosine kinase inhibitors (O'Dwyer and Druker, 2000), but rather an alternate binding site specific for its interaction with immunoreceptor complexes. The specificity of the compounds activity, coupled with the fact that the proposed target (Syk-Immunoreceptor interaction) did not translate to potential activity in MPM, was the reason why the Syk kinase was not chosen for investigation with MDG 44.

The second target identified through the patent search was that of the Ewings Sarcoma Family Tumours (ESFT) fusion protein, EWS-FLI1. 2-indolinone compounds were shown to inhibit the interaction between the oncogenic transcription factor, EWS-FLI1, and its accessory protein, RHA, disrupting its transcriptional regulation of cell transformation and tumour growth (Erkizan et al., 2009). While this target was specific to cancer, the EWS-FLI1 fusion protein arises through a chromosomal translocation specific to ESFT and (Erkizan et al., 2010), as such, this target did not translate to activity in MPM. The specificity of the target meant it was not applicable for target investigation with MDG 44.

The final putative target identified using the patent search was that of sodium channel proteins. The patent identified a class of compounds including 2-indolinone structures with activity as sodium channel blockers. Reported activity for these compounds in the patent and in a published report by *Chowdhury et al* was related to disorders such as hypercholesterolemia, benign prostatic hyperplasia and the treatment of pain (Chowdhury et al., 2011). In the patent, the efficacy for these compounds in cancer treatment was

proposed, however no data to qualify this activity was reported. As well as the other targets identified through the patent search, sodium channel proteins were not investigated as a possible target for MDG 44.

The first molecular target chosen for investigation with MDG 44 in this Chapter was the nuclear receptor, PPAR- γ . Again, structural analysis of compounds was used to propose this target for investigation. Similarly with the patent search, a compound was identified with similar structure to MDG 44 and a known molecular target. An experimental PPAR- γ modulator was identified, MDG 588, which contained the identical 2-indolinone / MDG basic scaffold to that of MDG 44 and similar hydrophobic substitutions at position R1 on the scaffold. This structural analysis, as well as published data on the anti-cancer activity of PPAR- γ , supported the theory of PPAR- γ being a rational molecular target for MDG 44. Numerous papers, including studies in MPM (Hamaguchi et al., 2010), had reported on the anti-cancer activity of various PPAR- γ agonists as single-agents (Mody et al., 2007) and in combination with platinum-based therapy (Cisplatin) (Girnun et al., 2007, Reddy et al., 2008). A small number of contradictory reports do exist, reporting that PPAR- γ ligands, including Thiazolidinedione derivatives, mediated cell death in cancer through alternate, yet unknown mechanisms (Al-Alem et al., 2011, Zaytseva et al., 2011, Seargent et al., 2004). Experiments testing known PPAR- γ ligands on CRL 5915 cells, confirmed that these classes of compounds could induce cell death in MPM.

The capacity of MDG 44 to bind PPAR- γ and mediate its apoptotic and cellular effects through PPAR- γ activation, were assayed in three ways – “On Target” competitive binding assay; PPAR- γ inhibitory assays; and peroxisomal activity assays. The initial test for affinity of MDG 44 for the PPAR- γ binding pocket, by way of TR-FRET competitive binding assay, produced negative results for PPAR- γ being the molecular target of MDG 44. Incubation with MDG 44 had no positive effect on the displacement the fluoromone from the LBD, indicating it did not have affinity for PPAR- γ . This “Off Target” effect for MDG 44 was mirrored in PPAR- γ inhibitory assays. Pre-incubation of CRL 5915 cells with the PPAR- γ antagonist GW9662, had no inhibitory effect on MDG 44 induced cell death, apoptosis and the formation of large cytoplasmic vesicles. This result strongly indicated that MDG 44 mediated effects in MPM were not being elicited through PPAR- γ activation. Catalase activity assays, performed to determine if the cytoplasmic vesicles induced by MDG 44 treatment in MPM were peroxisomes, further confirmed the absence of PPAR- γ and peroxisomal activity in MDG 44 mediated effects in MPM. The assay was optimised

and validated as a true measure of Catalase activity in CRL 5915 whole cell lysate. MDG 44 treatments were performed at concentrations and time points at which optimal cytoplasmic vesicle formation was observed - 40 μ M MDG 44 for 4h (Chapter 5; Fig 5.17.a – 5.17.b). Incubation with MDG 44 induced no increase in Catalase activity in CRL 5915 cells. No increase in Catalase activity, a common marker for Peroxisomes and peroxisomal activity (Mozaffar et al., 1986), refuted the suggestion that these cytoplasmic vesicles were peroxisomes. This result correlated with that of the PPAR- γ “On target” and inhibitory assays and confirmed that PPAR- γ was not the molecular target for MDG 44.

However, investigations into autophagy and the ubiquitin-proteasome system produced some promising results. Examination into the induced formation of autophagosomes revealed that MDG 44 caused both the increased protein expression of the autophagosome marker LC3-II and induced formation of LC3 positive structures within the cytoplasm. Analysis using Chloroquine, a known inhibitor of autophagy and inducer of autophagosome accumulation in cells (Shacka et al., 2006), indicated that this compound also induced significant increases in LC3-II expression, as expected. Using CytoSpin and RapiDiff staining analysis, the accumulation of autophagosomes within the cell could be detected as large cytoplasmic vesicles within the cells. These structures were similar to the cytoplasmic vesicles induced by MDG 44 incubation. These results strongly suggested that MDG 44 did in fact induce an increase in autophagosome number within MPM cells.

Analysis of complete autophagic flux however, revealed that while increased LC3-II expression was observed, no consistent degradation of the lysosomal turnover marker, p62, could be detected. Induction of autophagy and increased autophagic flux in CRL 5915 cells was confirmed using nutrient starvation as a positive control. Incubating cells in nutrient deprived conditions induced the increase in LC3-II and concurrent p62 degradation, indicating an increase in autophagic flux. Observations with MDG 44 may suggest induction of a type of defective autophagic flux. Increased LC3-II and p62 expression was observed in treatments with the autophagic flux inhibitors Chloroquine and Bafilomycin A1. Both compounds inhibit fusion of the lysosome and autophagosome, causing accumulation of autophagosomes (LC3-II) and protein cargo (p62) (Yamamoto et al., 1998). While no degradation of p62 was detected, no significant increase in response to MDG 44 treatment was observed either. Further analysis is required to differentially confirm the increase in autophagosome number by means other than LC3 analysis and investigate the effect of MDG 44 on increased autophagic flux, or its inhibition.

An apoptotic program of cell death induced by MDG 44 had been confirmed in Chapter 4; however, cellular crosstalk between apoptosis and autophagy programs of cell PCD can occur within cells and the induced formation of autophagosomes suggested autophagy may be a part of the cell death program (Gonzalez-Polo et al., 2005). Analysis of the contribution of autophagy to the induction of PCD by MDG 44 was performed using 3-methyl adenine (3-MA). Inhibition of PI3K, a signal transducer essential in activating of the initial steps of autophagy and phagophore formation (Seglen and Gordon, 1982), had no inhibitory effect on MDG 44 induced cell death. The lack of contribution of autophagy to cell death, or as a cytoprotective event (no increased activity with 3-MA was observed), suggested induction of autophagy was a consequence of MDG 44 engaging with its molecular target. This result was in line with the theory that MDG 44 may work through targeting the proteasome.

Experimentation on MDG 44 targeting the proteasome provided some interesting results. MDG 44 induced dose-dependent inhibition of both NF κ B activation and I κ B degradation. This result indicated that MDG 44 could inhibit NF κ B activation via preventing proteasomal degradation of its regulatory subunit, I κ B. This activity matched that of MG132, a direct inhibitor of the proteasome. However, poly-ubiquitination analysis by Western Blot revealed MDG 44 did not induce accumulation of poly-ubiquitinated proteins. This effect is a direct consequence of specific inhibition of the 26S proteasome and indicative of activity of proteasome inhibitors. The positive control for proteasome inhibition, MG132, did induce significant accumulation of poly-ubiquitinated proteins.

This final result questioned the activity of MDG 44 as a direct proteasome inhibitor, but its molecular target may still reside within the UPS pathway. Inhibiting the ubiquitination of I κ B, as opposed to the proteasome itself, would also result in inhibited proteasomal degradation of I κ B and inhibition of NF κ B activation. This result suggested that MDG 44 could target members of the E1, E2, E3-ubiquitin enzyme pathway and this target will be under investigation in future experimentation.

In conclusion, experimentation in this Chapter ruled out a number of putative mechanisms of action for MDG 44 and identified components of the UPS pathway as potential targets for this compound. A patent database search identified a number of diverse molecular targets for 2-indolinone scaffold containing compounds, but none represented a rational target for MDG 44 in MPM. Biochemical analysis ruled out PPAR- γ as potential

target for MDG 44. No target affinity, dependence on PPAR- γ activation for apoptotic induction or increase in peroxisome proliferation was observed. Autophagy analysis confirmed MDG 44 induced an increase in formation of autophagosomes, but is suspected to induce a defective autophagic phenotype. Experimentation into the mechanistic link between proteasome inhibition and induction of autophagy produced promising results. However, while the originally proposed target of the 26S proteasome was ruled out, the ubiquitination enzyme pathway was identified a promising target for future study.

Chapter 7

Design and biochemical evaluation of second generation MDG 44 analogues

Introduction

In conjunction with the mechanistic analysis of MDG 44-mediated cell death in Chapter 5 and 6, attempts were made to develop more potent analogues of MDG 44. Previous experimentation in Chapter 5 indicated that MDG 44 had an IC_{50} value for decreasing cell viability of $31.3\mu\text{M}$ (± 0.6) (48h incubation) and could synergistically enhance Cisplatin induced apoptosis at sub-lethal concentrations of MDG 44 as low as $15\mu\text{M}$. While MDG 44 displayed reasonable mid-micomolar activity in *in-vitro* assays, improvement in drug potency would be desired to aid the future potential of this class of compound as a chemotherapeutic in MPM or other malignancies. An important secondary objective in the design and testing of new MDG analogues was the identification of molecules with improved aqueous solubility compared to that of MDG leads. MDG 42 and MDG 44 displayed sparing solubility in aqueous solution. Both compounds stock solutions were prepared in 100% ethanol and were immiscible and precipitated in 1% ethanol-aqueous solutions at concentrations above 60 - $70\mu\text{M}$. The design of analogues with chemical properties for greater aqueous solubility would improve the classification of this class of compound as being truly “Drug-like” and aid the potential for oral availability of a final drug.

A number of different approaches can be taken in the design of analogues and creation of compound libraries for drug screening. A common approach involves the chemical process of “Scaffold Decoration” – the addition of various types of functional groups and structural moieties at particular points on a basic molecular scaffold permissible for substitution (Dallinger and Kappe, 2005). This process is restricted only by limitations in chemical synthesis and can lead to the generation of large compound-libraries of analogues applicable for high and low through-put screening analysis. Compound generation and screening in this manner can help identify new lead analogues with increased activity or potency. Structure-Activity Relationship (SAR) analysis matches the read-out data from a screening assay (“On target” activity or cellular potency etc.) with the type and position of structural moieties substituted onto screened analogues. This type of computational analysis can help identify substitutions that limit or substitutions that are essential for mediating activity and can greatly inform the generation of successive series of analogues (Tong et al., 2003). However, large amounts of data and large compound-libraries are required for a truly quantitative form of Structure-Activity Relationship analysis (QSAR) to be performed (Kubinyi, 1990).

This technique is commonly used in pharmacology and medicinal chemistry drug discovery studies. Examples of this include a study by *Cloonan et al.*, investigating the potential of Serotonin re-uptake transporter (SERT) ligands as pro-apoptotic agents in the treatment of cancer (Cloonan et al., 2009). A medium scale library (250) of structurally diverse analogues of 4-Methylthioamphetamine (4-MTA), a known SERT ligand with diverse activity (Huang et al., 1992, Hurtado-Guzman et al., 2003), were synthesised and screened for SERT dependant anti-proliferative activity in B-cell lymphoma, leukaemia and breast cancer cell lines. Results showed that the anti-proliferative activity of analogues was not dependant on SERT binding, but the screen did identify a number of analogues with more potent anti-cancer activity than 4-MTA. While the molecular target for these compounds was not known, the 4-MTA analogues showed potential for development as chemotherapeutic agents.

As opposed to being an entirely random procedure, a selective and predictive method of substitution and analogue generation is most commonly used where applicable. In a related study to ours, also on the development of novel SMAC-mimetic compounds, molecular analogues of Embelin were designed and tested for their affinity to the XIAP-BIR3 binding pocket (Glover et al., 2003). A selective approach to analogue generation was performed, with substitutions only made at the eleven carbon hydrophobic tail of Embelin. Through the initial computational design process in the identification of Embelin as a SMAC mimetic, it was predicted that the hydrophobic tail of Embelin was essential for its affinity for the hydrophobic binding pocket of XIAP-BIR3 (Nikolovska-Coleska et al., 2004). Analogues were synthesised with substitutions of carbon tails of alternate length and tails containing phenyl groups. This selective approach to analogue design identified structures with significantly increased potency compared to that of the lead compound Embelin.

The full use of computational design techniques can help to streamline the labour intensive and sometimes random method of analogue synthesis by employing a rational approach to the design and selection of analogues for screening. In a study by *Hardcastle et al.* (Hardcastle et al., 2006), a structural based design approach was used in the design of more potent analogues of MDM2-p53 inhibitors. Analogues were based on previously identified lead compounds containing an isodolinone scaffold (Hardcastle et al., 2005), similar to that of the 2-indolinone / MDG basic scaffold. Using compound docking software (easyDockTM), isodolinone lead compounds were virtually docked into the MDM2-

p53 binding pocket, obtained from a published co-crystal structure of these two proteins in interaction (Kussie et al., 1996). A predicted binding mode for these lead compounds was obtained and the position of the isodolinone scaffold was preserved during virtual screening of structures onto this binding pose. Analogues containing the isodolinone scaffold and with substitutions computationally predicted to increase interaction with the protein binding pocket (minimal criteria: one additional H-bond interaction) were chosen for biochemical analysis. This structure-based computational design methodology represented a rational and quantitative approach for the identification of more potent analogues of a particular class of molecule, and in this and subsequent studies was successful in identifying more potent isodolinone based inhibitors of the MDM2-p53 interaction (Hardcastle et al., 2006, Watson et al., 2011).

Numerous other computational design techniques can aid in the identification of more potent analogues. Pharmacophore generation from known ligands of a target of interest, in particular targets with co-crystal structures of ligands or lead compounds in-situ in their biological binding pose are strong bases for the design of analogues. Pharmacophores are computationally generated feature maps of structural moieties, functional groups and space restrictions of ligands and ligand / binding pocket interactions deemed essential for compound affinity (Khedkar et al., 2007). Virtual screening of compound databases onto this ligand feature map aids the identification of compounds or analogues of lead compounds which satisfy the pharmacophore and have predicted affinity for a target. This approach is widely used, including studies designing analogues of SMAC mimetic compounds (AVPI pharmacophore) (Sun et al., 2004, Li et al., 2004, Crisostomo et al., 2009) and was the basis for the design of the original MDG 44 class of compounds, as discussed previously (Chapter 4).

As outlined above, computational techniques, be they structural-based design, ligand-based pharmacophore design or a combination of processes (Knox et al., 2009), significantly aid drug design and second generation drug development. However, for the identification of active MDG analogues, such techniques were not applicable. As the cellular target of MDG 44 had not been fully elucidated, structural information on the binding pocket or known ligands or inhibitors of the target were not available to facilitate a computational approach to the design of MDG 44 analogues. As such, the approach taken for the design of MDG 44 analogues involved considering all known actives in the initial series of compounds generated and identifying structural commonalities between

these compounds. Using this information, types of substitutions and points on the basic 2-indolinone scaffold applicable for substitution were chosen. Analogues were searched for and obtained from commercial compound databases (Specs; ChemDiv).

The molecular structures of MDG 44, as well as the second active compound, MDG 42, were considered for basic structure-activity relationship (SAR) analysis in order to identify essential moieties and help propose positions or group substitutions that could produce more potent analogues (Fig 7.1.A – 7.1.B). Both compounds were based on the initial 2-indolinone / MDG scaffold (Fig 7.1.C) and both MDG 42 and 44 also possessed a *tetra*-butylbenzene group at the R1 position. Structures differed at the R2 position – MDG 44 possessed a 1, 2, 4-trimethylbenzene group, while an ethylbenzene group was present in MDG 42 at the same position.

Points on the basic scaffold chosen for alteration were illustrated in Figure 7.1.C. Decoration of benzene rings and addition of alternate ring structures were considered for positions R1 and R2. Of particular interest was the importance of the *tetra*-butylbenzene group at position R1, present in lead compounds MDG 44 and MDG 42. The importance of this moiety for compound activity was investigated. Analogues containing this moiety, other bulky carbon substitutions at the *para*-position of the phenyl group or alternate phenyl group decorations at R1 were chosen for testing. Similar carbon ring substitutions were considered at point R2 when choosing analogues. Other classes of substitutions chosen for positions R1 and R2 included methoxyphenyl, dimethoxyphenyl, ethoxyphenyl, benzodioxol, bromophenyl, fluorophenyl, chlorophenyl, pyrrol and naphthyl containing structures. R1 and R2 were primary points for alteration when considering analogues, with decoration at position (c), the central 2-indolinone scaffold benzene ring, chosen in a small number of analogues. The MDG primary scaffold was not altered in any other way. Of the initial screen of MDG 44 analogues, all compounds were obtained from the SPECS® compound database.

In this Chapter, analogues of MDG 44 were screened in MPM cell lines in an attempt to identify more potent inducers of apoptosis in this cancer model. IC₅₀ and EC₅₀ values for inhibition of cell viability and induction of apoptosis were investigated for lead analogues identified in the initial screen. The synergistic capacity of lead analogues to enhance Cisplatin-induced apoptosis was also determined. The objective of this study was to identify a lead compound with increased single-agent and synergistic potency and which

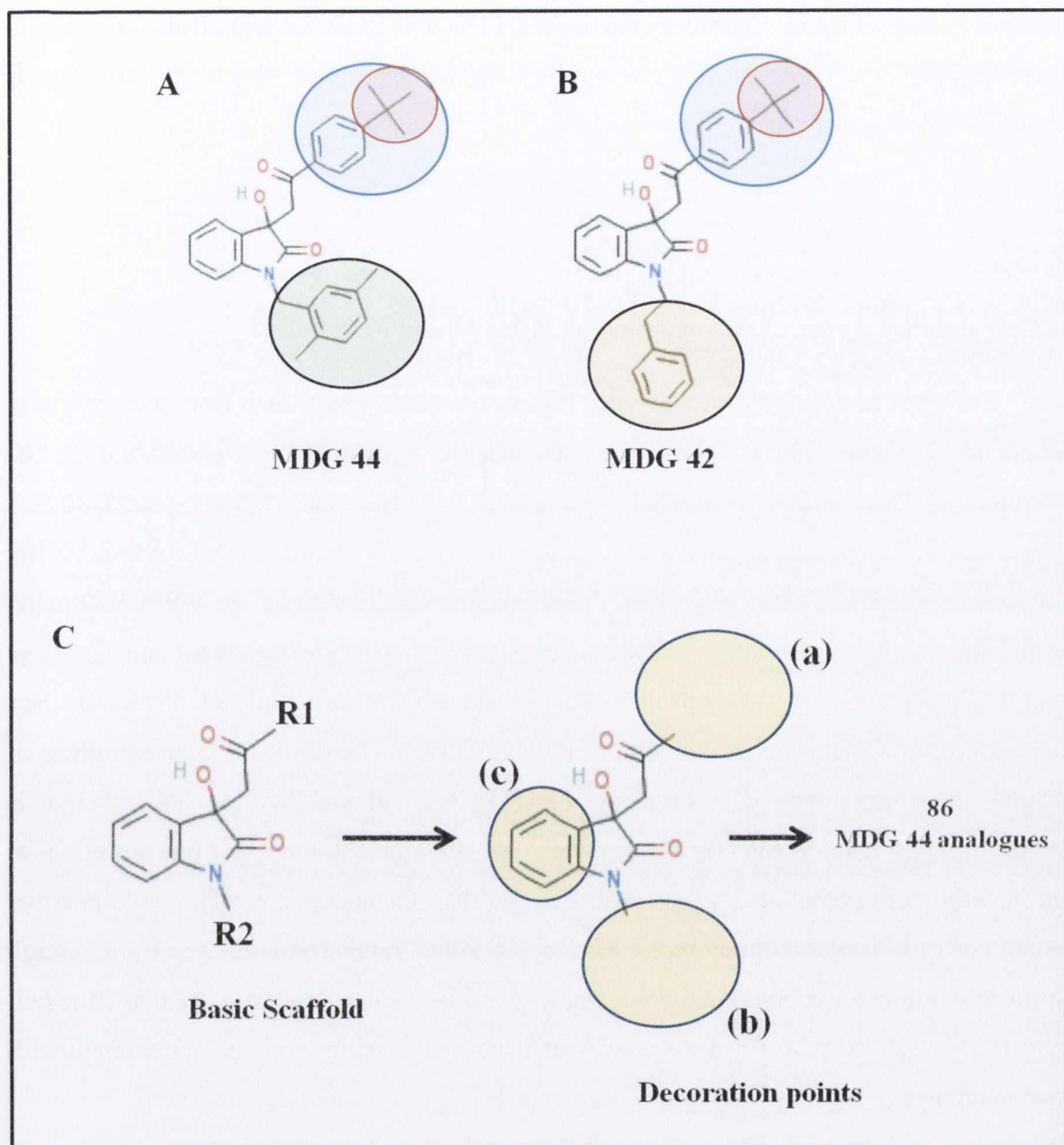


Figure 7.1: Design of 86 MDG 44 analogues.

Molecular structures of MDG 44 (**A**) and MDG 42 (**B**), active compounds in MPM cell lines. One substitution was identical in MDG 44 and MDG 42 (○), and of particular interest was a butyl substitution at the para-position (○). Substitutions at the R2 position differ between MDG 44 (○) and MDG 42 (○). (**C**) Basic MDG compound scaffold and decoration points – (a) & (b) – which were chosen for MDG 44 analogues.

displayed greater aqueous solubility than that of MDG 44. Basic SAR analysis linking anti-cancer activity and types of substitutions present on analogues was hoped to identify structural moieties essential for activity.

Results

7.1 Cell viability screen of 86 analogues of MDG 44 and MDG 42

From the design profile established, 86 compounds were chosen from a commercial compound database (Specs) which met the specific structural criteria laid out in the Introduction. These analogues of MDG 44 and MDG 42, designated CDX 1 – CDX 86 (see Appendix I for compound structures and full chemical name), were screened for their ability to induce cell death in CRL 5915 cells. This cell line was chosen as the MPM test model for this assay as it had previously shown to be sensitive to MDG 44 treatment and extensive synergism and mechanistic analysis of MDG 44 had been performed in CRL 5915 cells (See Chapter 5 & 6). Cells were treated with analogues CDX 1 – 86 for 48h at concentrations of 32.5 μ M, the approximate IC₅₀ value of MDG 44 for cell viability in CRL 5915 cells (Chapter 5, Fig 5.4.A), and 10 μ M, a lower, cut off concentration for the detection of significantly more potent analogues. It was hoped that the analogue screen would uncover compounds of at least low micromolar efficacy in MPM, representing a significant enough improvement in cellular potency to be taken forward for further analysis. Alamar Blue cell viability analysis was performed to quantify cell death induced in response to treatment with these analogues.

Cell viability results for each of the MDG 44 analogues (CDX 1 – 86) screened were depicted in Figure 7.2 – 7.4. Twenty eight different analogues induced a reduction in cell viability of 50% or greater with 32.5 μ M drug incubation, indicating equal or greater potency compared to the reference molecule MDG 44 (IC₅₀ – 32.5 μ M). For more precise analysis, analogues were ranked in order based on their percentage cell viability after 10 μ M incubations (Fig 7.4.B). Seven compounds which showed the greatest potency in CRL 5915 cells – CDX 45, 25, 24, 66, 13, 78 & 68 – were indicated in the screen and taken forward for further analysis (Fig 7.4.B).

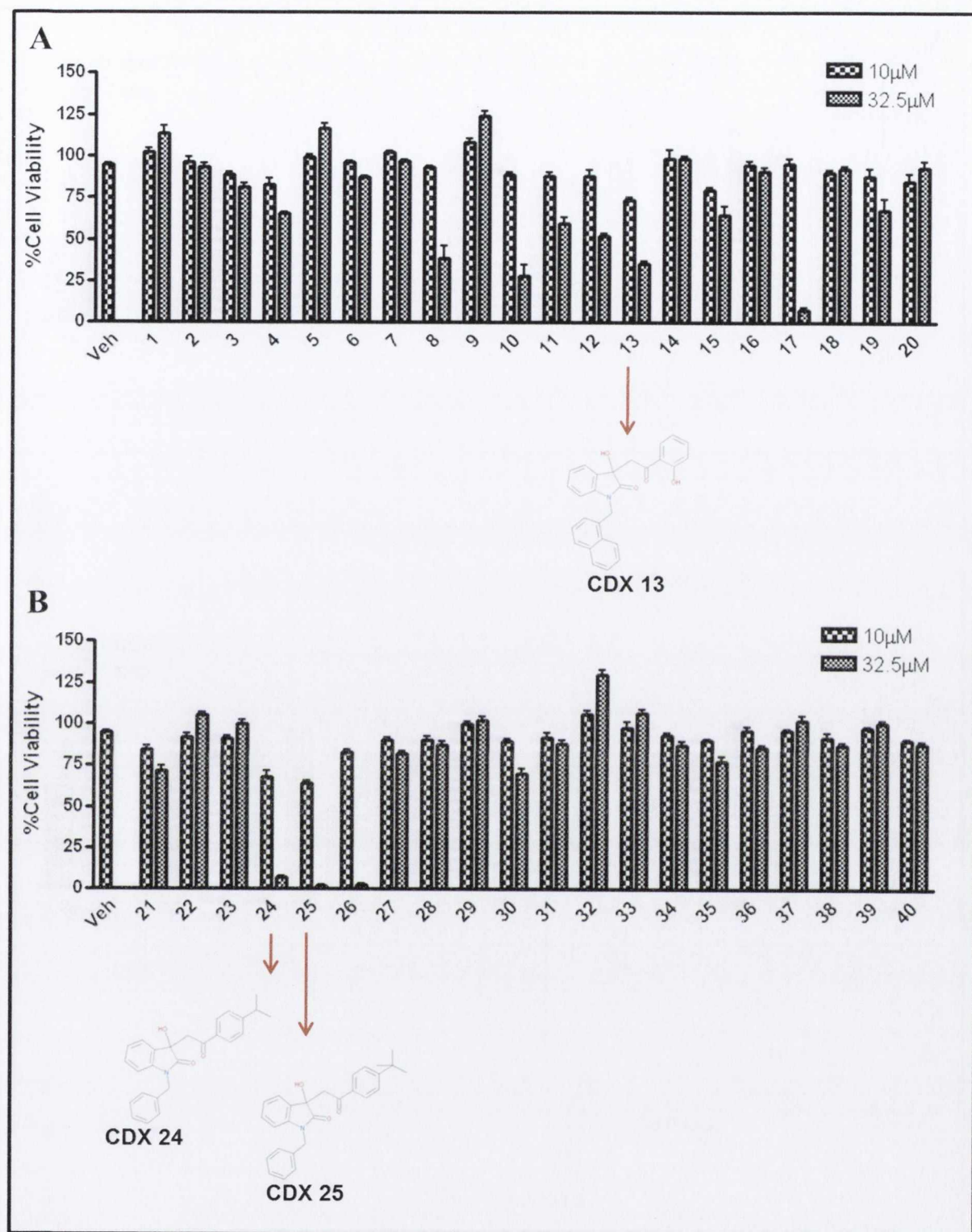


Figure 7.2: MDG 44 analogue screen: Cell viability results.

Cell viability results of MDG 44 analogues CDX 1 – 20 (**A**) and CDX 21 – 40 (**B**), tested at 10µM and 32.5µM in CRL 5915 cells. Molecular structures of analogues of interest are included. CRL 5915 cells were seeded at a density of 0.5×10^4 cells / well (200µl) of a 96 well plate and treated with indicated analogues for 48h. Cell viability was determined by Alamar Blue assay, as outlined in Section 2.6.

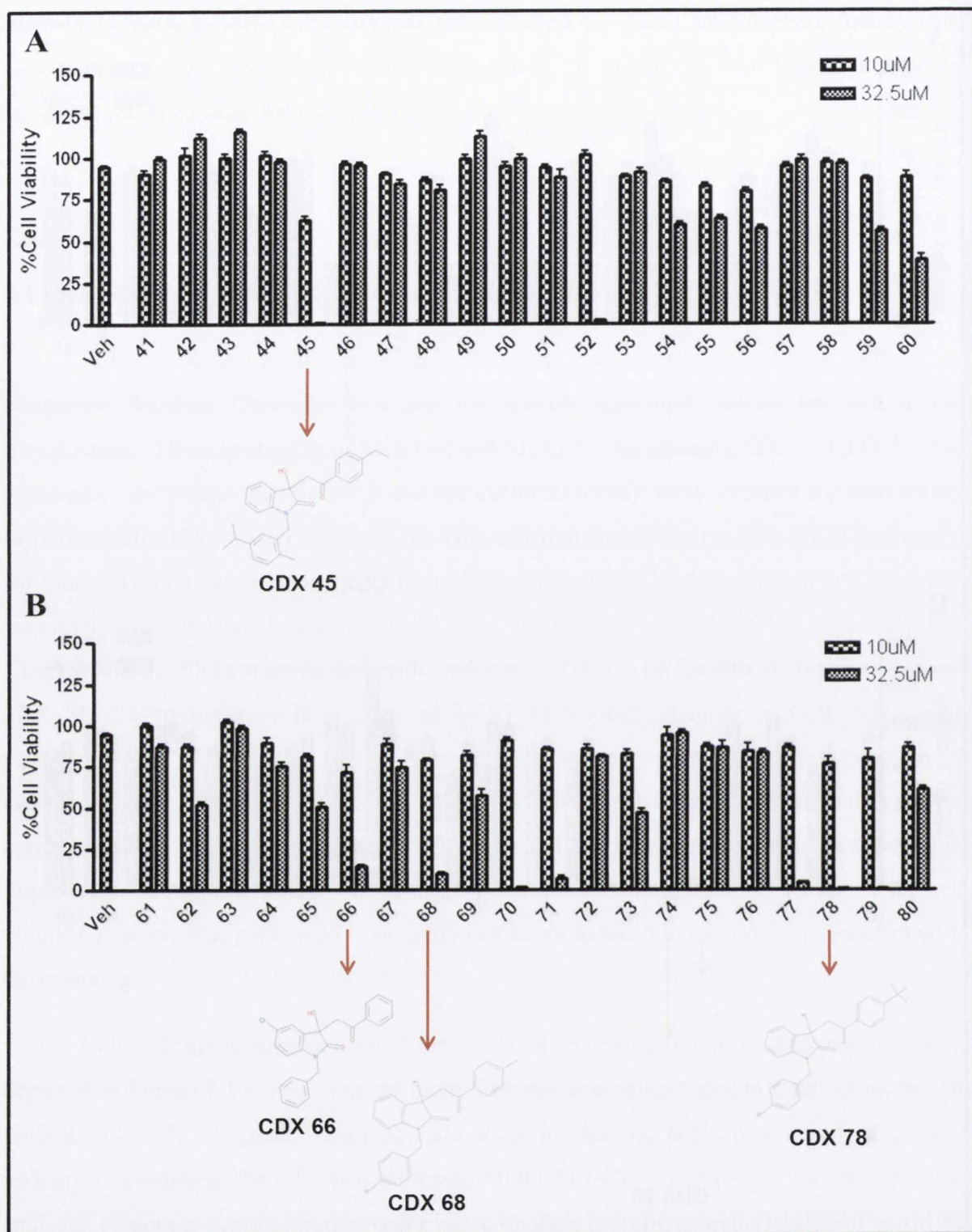


Figure 7.3: MDG 44 analogue screen: Cell viability results.

Cell viability results of MDG 44 analogues CDX 41 – 60 (**A**) and CDX 61 – 80 (**B**), tested at 10µM and 32.5µM in CRL 5915 cells. Molecular structures of analogues of interest are included. CRL 5915 cells were seeded at a density of 0.5×10^4 cells / well (200µl) of a 96 well plate and treated with indicated analogues for 48h. Cell viability was determined by Alamar Blue assay, as outlined in Section 2.6.

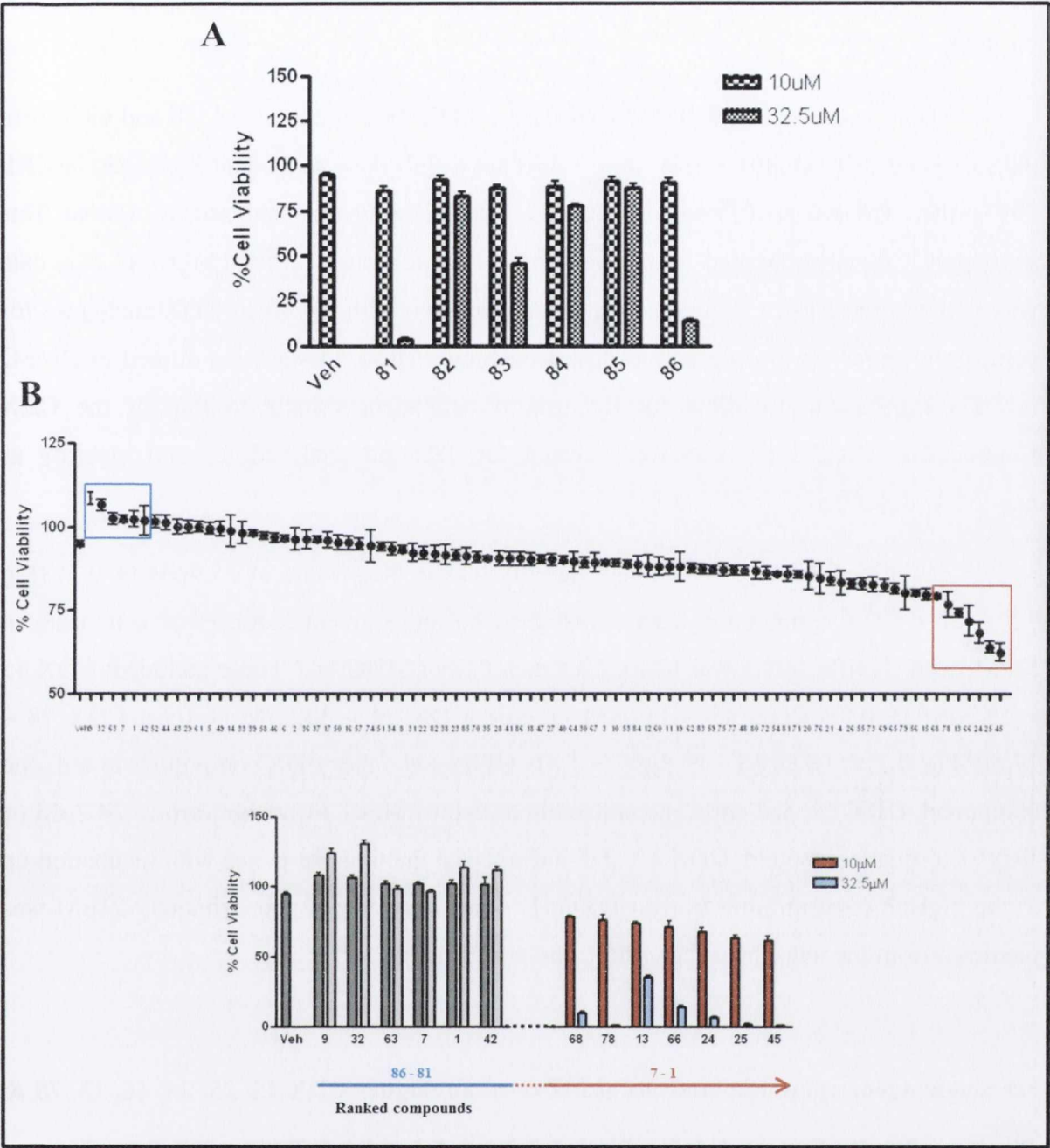


Figure 7.4: MDG 44 analogue screen: Cell viability results.

Cell viability results of MDG 44 analogues CDX 81 – 86 (A) tested at 10µM and 32.5µM in CRL 5915 cells. CRL 5915 cells were seeded at a density of 0.5×10^4 cells / well (200µl) of a 96 well plate and treated with indicated analogues for 48h. Cell viability was determined by Alamar Blue assay, as outlined in Section 2.6. The 86 MDG 44 analogues were ranked in terms of 10µM potency and ranked from highest to lowest cell viability at this concentration (B). The 7 top ranked analogues – CDX 45, 25, 24, 66, 13, 78, and 68 – were taken for further testing (□). Graph comparing 6 lowest ranked compounds is shown (□).

7.2 Cell viability dose response analysis of MDG 44 analogues CDX 45, 25, 24, 66, 13, 78 & 68

The top seven ranked CDX compounds – CDX 45, 25, 24, 66, 13, 78 and 68 – from the two-point cell viability screen were subjected to full dose dependence analysis in CRL 5915 cells. The effect of these compounds on cell viability was analysed by Alamar Blue assay and IC_{50} values plotted from seven-point, dose response curves. MDG 44 IC_{50} data was also generated in conjunction with experimentation with the seven CDX analogues for comparative analysis of drug and analogue potency. MDG 44 was also diluted in DMSO for this experiment, to allow for the use of equivalent vehicle to that of the CDX compounds. CRL 5915 cells were treated for 48h and analysed for cell viability as previously described (Section 2.6).

The effect of MDG 44 on cell viability had an IC_{50} value of $25.9\mu M (\pm 0.3)$ (Fig 7.5). Five CDX compounds were revealed to be more potent inducers of cell death in MPM, with significantly lower IC_{50} values than that of MDG 44. These included; CDX 45 – $10.2\mu M (\pm 0.5)$; CDX 25 – $11.6\mu M (\pm 0.6)$; CDX 24 – $14.0\mu M (\pm 0.6)$; CDX 78 – $17.5\mu M (\pm 0.2)$ & CDX 66 – $19.8\mu M (\pm 1.5)$. Of the two other CDX compounds tested, one compound, CDX 68, had an IC_{50} comparable to that of MDG 44, within error – $24.2\mu M (\pm 1.0)$. The final compound, CDX 13, did not induce a maximal response with incubation up to the highest concentration tested of $50\mu M$. An IC_{50} value of approximately $21\mu M$ was predicted from the non-linear regression, curve fit analysis.

7.3 Single-agent apoptotic analysis of MDG 44 analogues CDX 45, 25, 24, 66, 13, 78 & 68

Apoptotic induction was also investigated with the seven top-ranked CDX compounds in CRL 5915 cells. EC_{50} values for single-agent apoptotic induction were generated for each compound by incubation with increasing concentrations of drug for 24h and 48h time points. Dose dependant induction of apoptosis was quantified by PI cell-cycle Flow Cytometry analysis. Akin to cell viability experimentation, EC_{50} analysis of the reference molecule MDG 44 was also performed as a control for comparative analysis to data for CDX compounds. Dose dependant induction of apoptosis for all compounds is depicted in Figure 7.6.a and 7.6.b. Single-agent apoptosis EC_{50} values, and cell viability

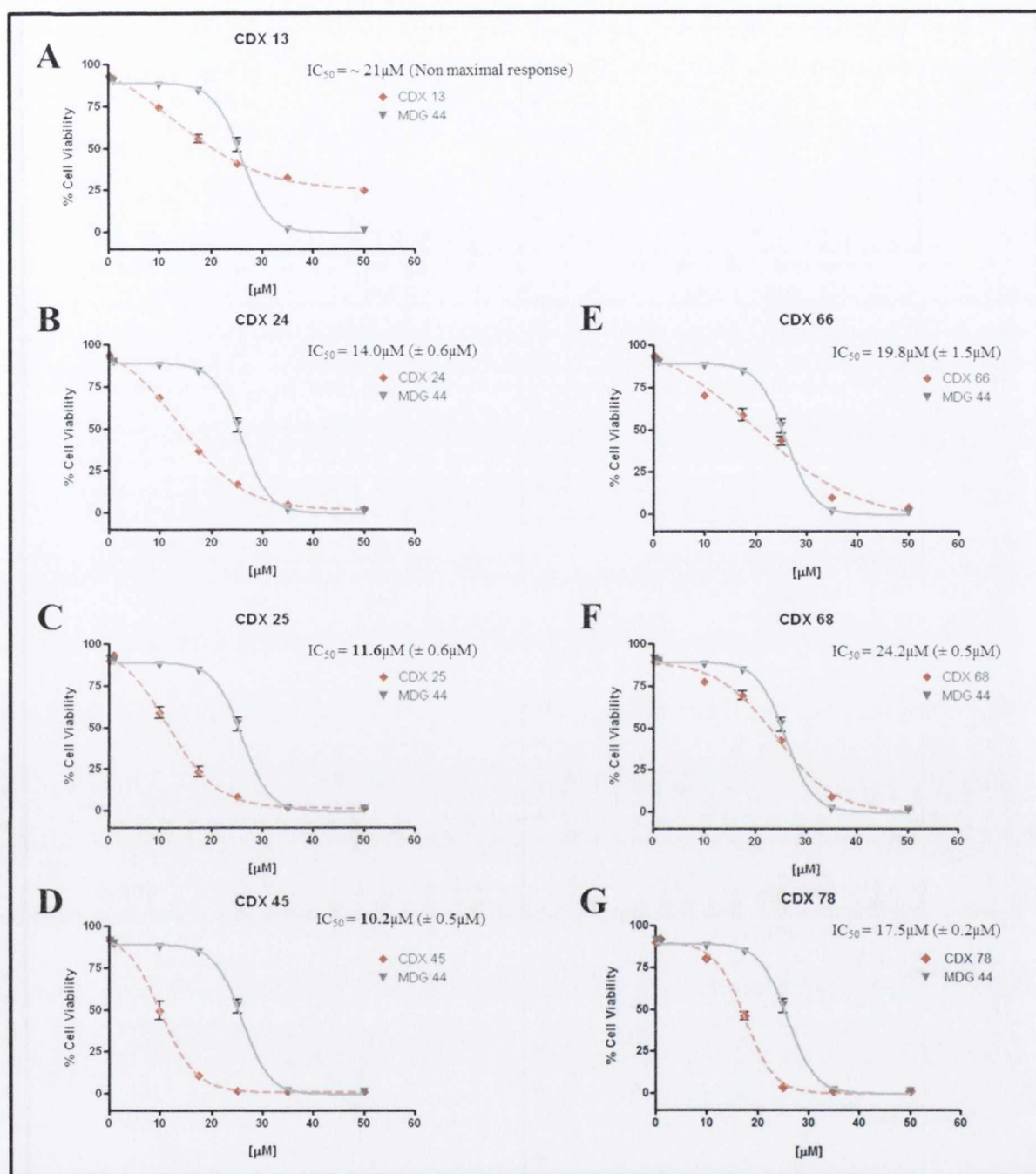


Figure 7.5: MDG 44 analogues CDX 45, 25, 24 & 78 were more potent inducers of cell death.

Dose response curves were generated for cell death induced by MDG 44 analogues CDX 13 (A), CDX 24 (B), CDX 25 (C), CDX 45 (D), CDX 66 (E), CDX 68 (F) & CDX 78 (G) – (◆) – and compared to the reference compound, MDG 44 (▼). CRL 5915 cells were seeded at a density of 0.5×10^4 cells / well (200μl) of a 96 well plate and treated at indicated concentrations for 48h. Cell viability was determined by Alamar Blue cell viability assay, as outlined in Section 2.6. Each data point is representative of the mean $\pm$ SEM of two independent triplicate experiments. Non-linear regression, variable slope curve fit analysis and IC_{50} values were generated using Graphpad Prism 4 statistical software.

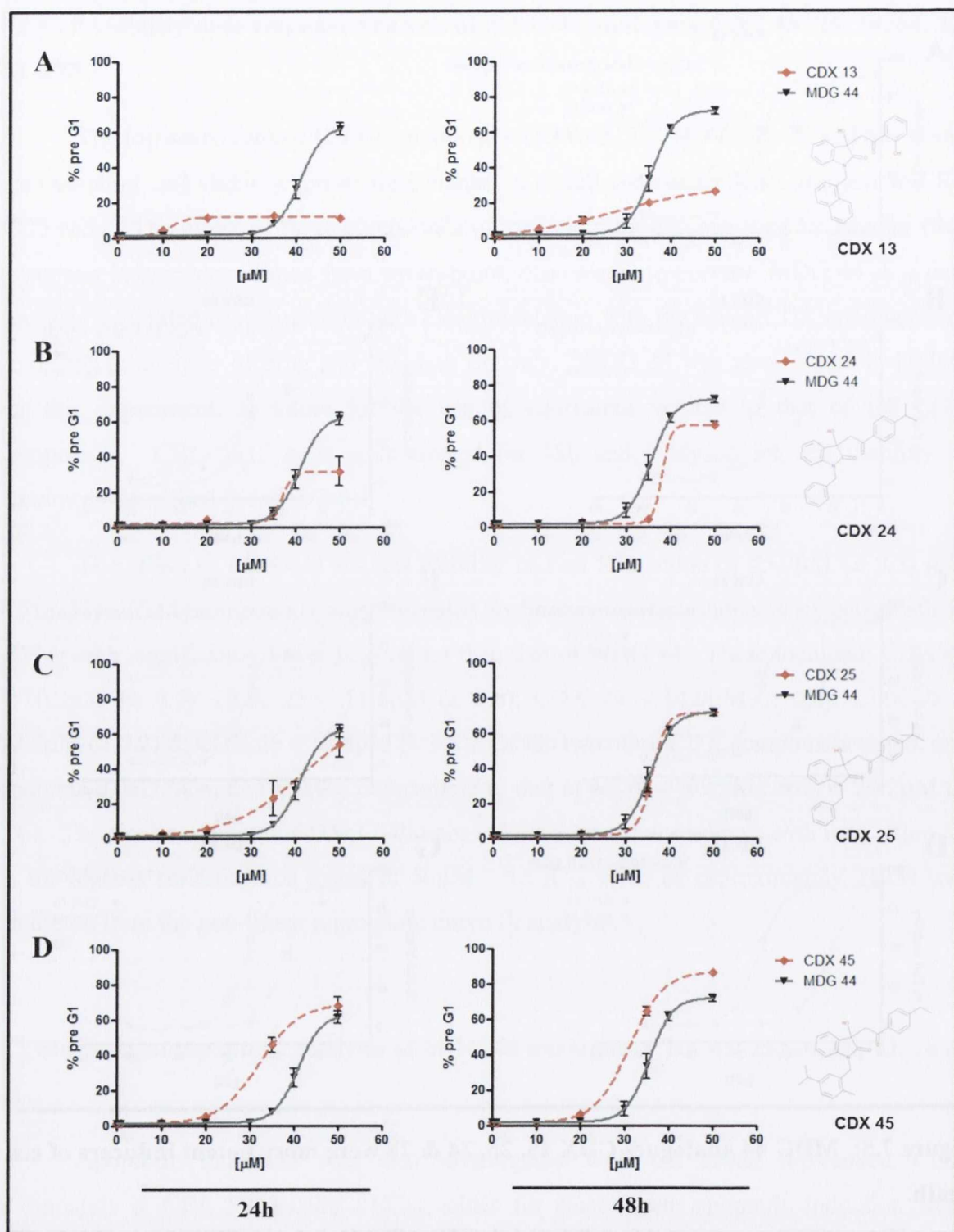


Figure 7.6.a: CDX 45 was a more potent inducer of apoptosis in CRL 5915 cells

CRL 5915 cells were treated with increasing concentrations of CDX 13 (A), CDX 24 (B), CDX 25 (C) and CDX 45 (D) (♦) for 24h and 48h – Results were compared to MDG 44 (▼) dose response treatments performed in parallel. CRL 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml). Apoptotic induction was determined by PI staining and cell-cycle Flow Cytometry analysis, as outlined in Section 2.7. Each data point is representative of the mean $\pm$ SEM of four independent experiments. Non-linear regression curve fit analysis and IC_{50} values were generated using Graphpad Prism 4 statistical software.

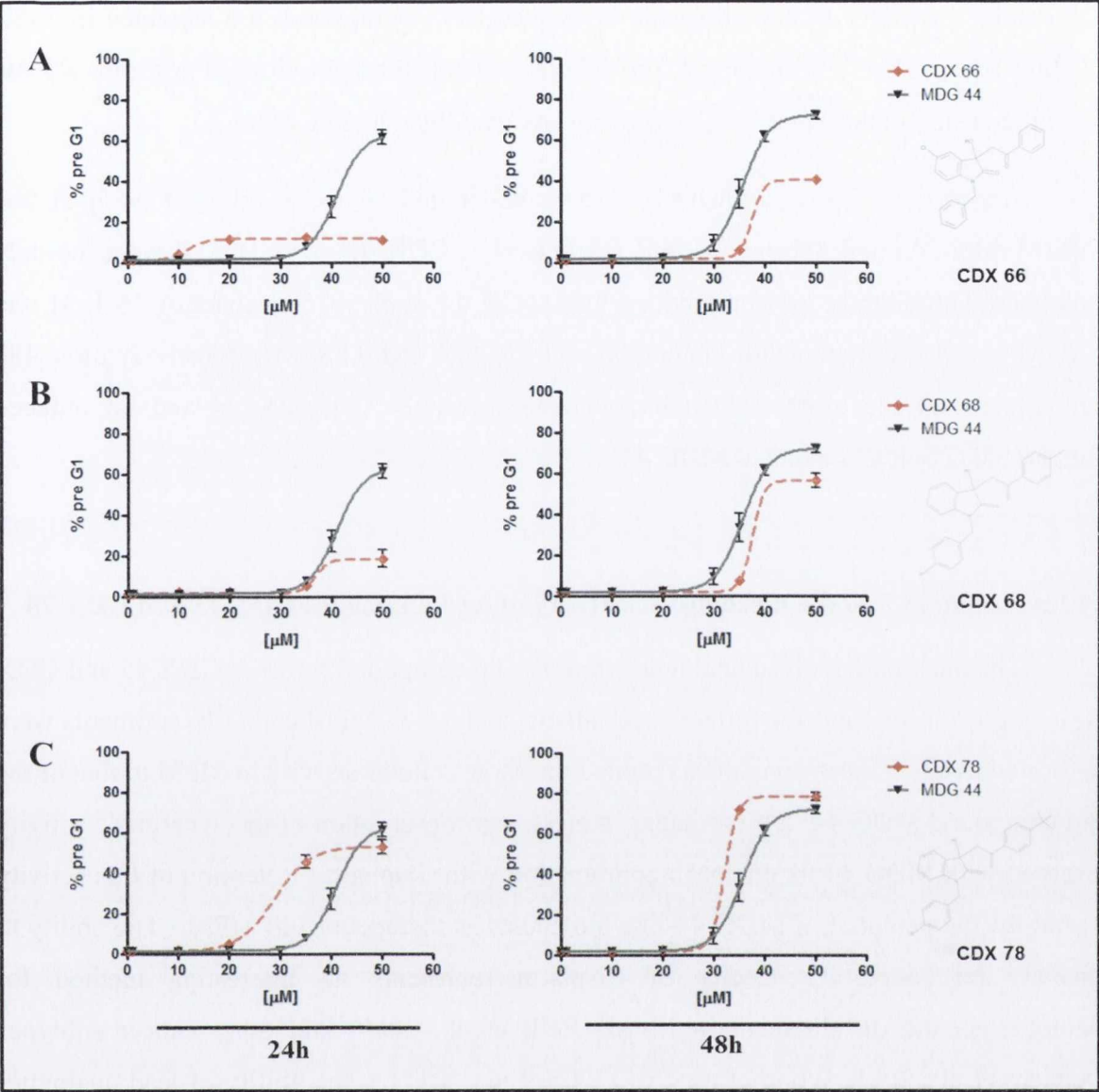


Figure 7.6.b: CDX 78 was a more potent inducer of apoptosis in CRL 5915 cells

CRL 5915 cells were treated with increasing concentrations of CDX 66 (A), CDX 68 (B) and CDX 78 (C) for 24h and 48h – Results were compared to MDG 44 (▼) dose response treatments performed in parallel. CRL 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml). Apoptotic induction was determined by PI staining and cell-cycle Flow Cytometry analysis, as outlined in Section 2.7. Each data point is representative of the mean $\pm$ SEM of four independent experiments. Non-linear regression curve fit analysis and IC₅₀ values were generated using Graphpad Prism 4 statistical software.

IC₅₀ results for MDG 44 and all seven top-ranked CDX compounds are tabulated in Table 7.1. For MDG 44 and its analogues, the different chemical substitutions at positions R1 and R2 of the basic 2-indolinone / MDG scaffold are also illustrated in Table 7.1.

Apoptotic analysis for MDG 44 revealed it had an EC₅₀ value of 44.5µM and 37.4µM after 24h and 48h incubations respectively. CDX 45 and CDX 78 were the only analogues with slightly greater potency than MDG 44, with EC₅₀ values of 36.4µM and 38.6µM respectively after 24h incubation and 31.9µM and 32.8µM respectively after 48h incubation. Of the other CDX compounds tested, only CDX 25, 24 and 68 induced comparable apoptotic results to MDG 44.

7.4 Investigation into the mechanistic activity of lead analogues CDX 45 and CDX 78

The most potent / lead analogues from the 86 compound screen – CDX 45 and CDX 78 – were taken forward for further mechanistic analysis in MPM cells. Experiments were performed to test if these analogues retained the same cellular activity in MPM to that of the lead compound MDG 44. Of particular interest was conservation of the synergistic activity observed with MDG 44 treatment in combination with Cisplatin. Retention of this activity is vital for the potential of MDG 44-like molecules as therapeutics in MPM. The ability to enhance the apoptotic capacity of Cisplatin represents an interesting method for chemotherapeutic development in MPM (Belli et al., 2009) and other cancer subtypes (Debaere et al., 2011, Wong et al., 2011, Xu et al., 2011). The ability of lead analogues CDX 45 and CDX 78 to synergise with Cisplatin and more potently induce cell death in MPM would represent a promising step forward in the drug development process. However, mechanistic analysis and Structural-Activity Relationship (SAR) enquires linking the type of structural substitutions present on analogues with their single-agent and synergistic activity, could inform the design of future generations of analogues.

As well as the ability of CDX 45 and CDX 78 to synergise with Cisplatin, their ability to induce the formation of large cytoplasmic vesicles within MPM cells was investigated. Formation of these vesicles, suspected to be autophagosomes from experimental characterisation in Chapter 6, was a specific response to MDG 44 incubation and thought to be indicative of a cellular stress mechanism through which PCD was initiated (Chapter 5, Fig. 5.17.a). Conservation of this activity in the analogues CDX 45 and CDX 78 was also of interest.

Name	Compound Substitutions			Cell Viability (IC ₅₀)	Apoptosis - PI FACS (EC ₅₀)	
	R1	R2	C		24h	48h
MDG 44				25.9μM (± 0.3μM)	44.5μM	37.4μM
CDX 13				~ 21μM (Non maximal response)	-	-
CDX 24				14.0μM (± 0.6μM)	-	39.8μM
CDX 25				11.6μM (± 0.6μM)	48.0μM	37.1μM
CDX 45				10.2μM (± 0.5μM)	36.4μM	31.9μM
CDX 66				19.8μM (± 1.5μM)	-	-
CDX 68				24.2μM (± 0.5μM)	-	39.8μM
CDX 78				17.5μM (± 0.2μM)	38.6μM	32.8μM

Table 7.1: MDG 44 analogues data – Compound substitutions, cell viability and apoptosis data for CDX compounds CDX 13, 24, 25, 45, 66, 68 & 78

7.4.1 CDX 45 and CDX 78 synergistically increased Cisplatin induced apoptosis in CRL 5915 cells

Synergy experiments for CDX 45 and CDX 78 in combination with Cisplatin were performed in the CRL 5915 cell line. Cells were treated with Cisplatin for 24h prior to incubation with CDX compounds for various time points up to 24h (+4h, +8h, +20h & +24h). The addition of Cisplatin and CDX compounds was staggered as a precaution, due to the DMSO solvent used to dissolve CDX 45 and CDX 78. Previous studies have recently reported that Cisplatin, in combination with DMSO, can rapidly bind and form DMSO adducts, reducing its ability to bind to double stranded DNA and induce toxicity in cancer cells (Fischer et al., 2008). The addition procedure was optimised prior to experimentation.

Results revealed that both CDX 45 and CDX 78 were able to induce synergistic increases in Cisplatin induced apoptosis. Treatment with 25 μ M CDX 45 and CDX 78 alone had no effect on apoptotic induction (2.0 % $\pm$ 0.8 and 2.2% $\pm$ 0.5 pre G1 +24h, respectively), however, in combination with Cisplatin, they significantly enhanced cisplatin induced apoptosis (Fig 7.7). Enhancement of apoptosis was detected at time points as early as 8h post incubation with CDX 45 – 8.5% ($\pm$ 0.1) apoptosis with Cisplatin alone v's 20.0% ($\pm$ 2.4) in combination with CDX 45 – and 20h post incubation with CDX 78 – 18.4% ($\pm$ 1.3) apoptosis with Cisplatin alone v's 27.6% ($\pm$ 1.8) in combination with CDX 78. Two-way ANOVA analysis was performed in order to confirm statistical difference between single and combinational treatments.

While synergism was detected with CDX 45 and CDX 78, comparison with the synergistic capacity of MDG 44, observed in experiments performed in parallel, revealed that the MDG 44 analogues were not more potent enhancers of Cisplatin induced apoptosis than the original lead compound (Fig 7.7.C).

7.4.2 CDX 45 and CDX 78 induce the formation of large Cytoplasmic vesicles in CRL 5915 cells

The effect of CDX 45 and CDX 78 treatment on the morphology of CRL 5915 cells was analysed by Cytospin and RapiDiff staining analysis. Results revealed that, similarly to MDG 44, its lead analogues CDX 45 and CDX 78 induced the formation of large cytoplasmic vesicles within treated cells (Fig 7.8). Cells were treated with high

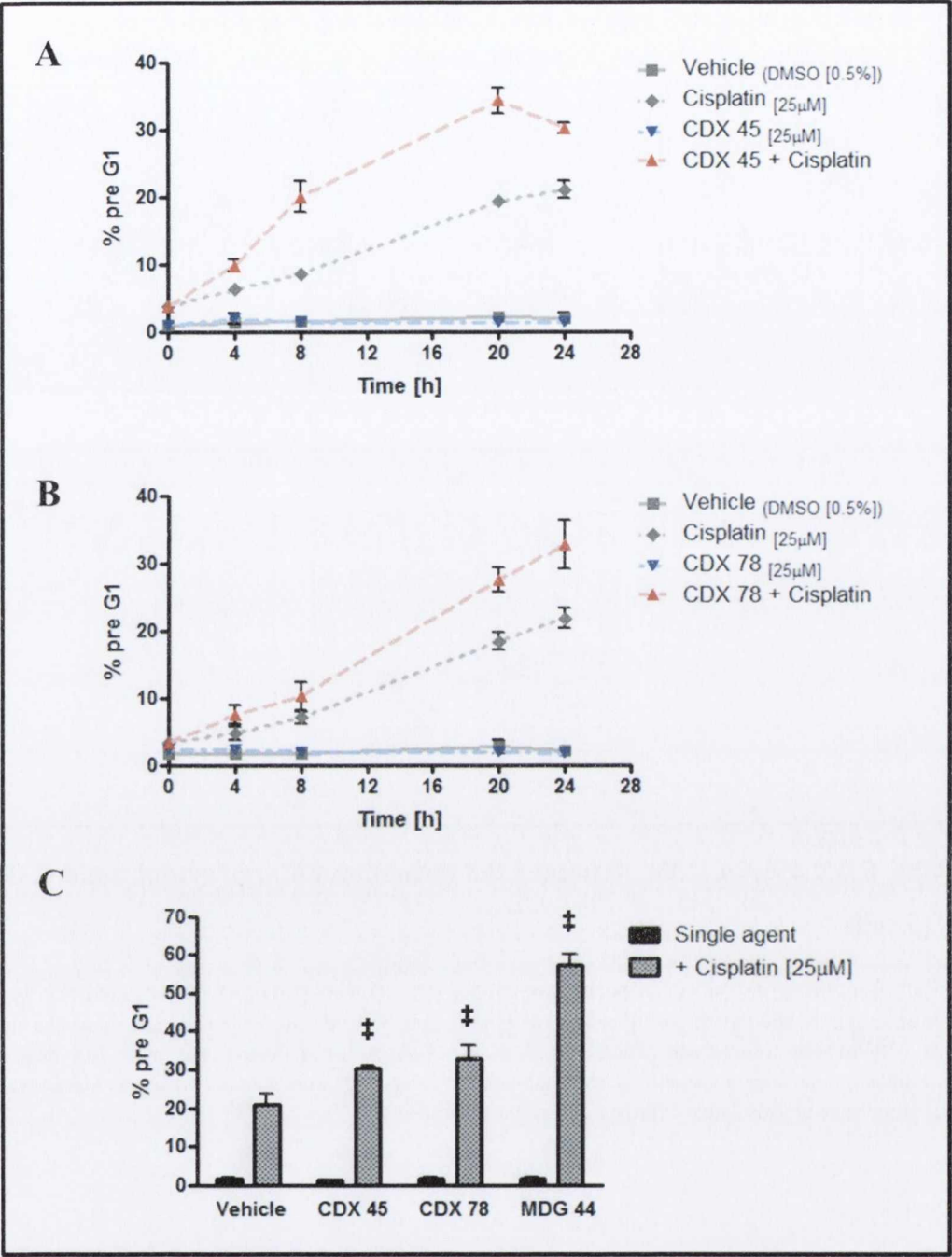


Figure 7.7: CDX 45 and CDX 78 induce synergistic increases in Cisplatin induced apoptosis in CRL 5915 cells

CRL 5915 cells were treated with CDX 45 [25μM] (A) and CDX 78 [25μM] (B) alone or in combination with Cisplatin [25μM] for indicated time points. CRL 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml). Cells were pre-treated with Cisplatin for 24h prior to treatment with CDX compounds for 0h, 4h, 8h, 20h and 24h. Apoptotic induction was determined by PI staining and cell-cycle Flow Cytometry analysis, as outlined in section 2.7. Synergistic increases in Cisplatin induced apoptosis by CDX 45 and CDX 78 treatment (+24h CDX compound treatment) compared to MDG 44 synergistic effects (C). Values are representative of mean $\pm$ SEM of three independent experiments. Synergism was determined by two-way analysis of variance (ANOVA) of the interaction effect of single agent and co-treatment response ($\ddagger$ P<0.01).

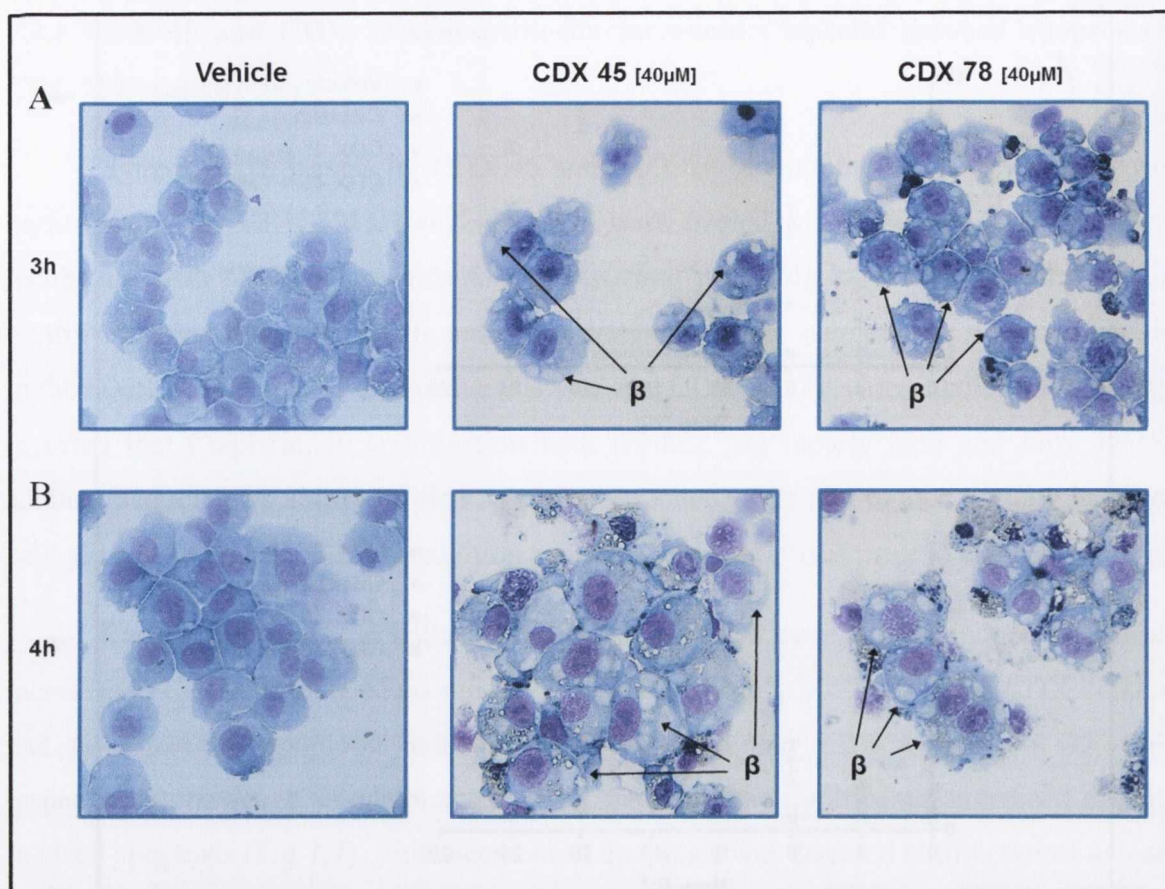


Figure 7.8: CDX 45 and CDX 78 induce the formation of large cytoplasmic vesicles in CRL 5915 cells

CRL 5915 cells were treated for 3h (A) or 4h (B) with Vehicle (DMSO 0.5%), CDX 45 [40μM] and CDX 78 [40μM]. Cells were seeded at a density of 0.225×10^6 cells / T25 (5ml). Cytospin slides were prepared and stained as outlined in Section 2.11. Slides were viewed and pictures taken using a Zeiss Axiovert microscope, under 20X magnification. Changes in cellular morphology in response to treatment with test compounds were noted: Formation of large cytoplasmic vesicles (β). Results are representative of three independent experiments.

concentrations of CDX compound (40 μ M) and vesicles formation was observed after only 3 – 4h incubations.

7.5 Investigation of a second series of MDG 44 analogues with increased predicted solubility

Subsequent to the original 86 analogue screen, a further series of MDG 44 analogues with increased predicted aqueous solubility, were tested in MPM. Compounds chosen for testing from a commercial compound database (ChemDiv) were analogues of MDG 44, containing the 2-indolinone / MDG basic scaffold, decorated with substitutions at positions R1, R2 and C, as previously described (Fig 7.1.C). In addition to this basic design framework, analogues containing hydroxyl and chlorine substitutions were specifically selected. Chlorine possesses lone pairs of electrons and can act as a hydrogen bond acceptor, while the hydroxyl moiety can act as both a hydrogen bond donor and acceptor, aiding the aqueous solubility of these organic small-molecules. Six molecules from the commercial compound database, designated CDX 87 – 92, fit the defined criteria and were tested for potency in MPM (see Appendix I for compound structures and full chemical name). Experiments were performed to classify the cell viability IC₅₀ values, single agent apoptotic EC₅₀ values and synergistic capacity of these compounds in MPM.

7.5.1 MDG 44 analogues CDX 87 – 92 were more potent inducers of cell death than lead compound MDG 44

Similarly to experimentation with the original panel of MDG 44 analogues, CRL 5915 cells were chosen as the test model for MPM. Cells were treated with increasing concentrations of all six MDG 44 analogues – CDX 87 - 92 – for 48h, with the effect of treatment on cell viability investigated by Alamar Blue assay. IC₅₀ values were determined through non-linear regression, curve fit analysis of seven-point dose response curves. Similarly to previous investigations, cells were treated with MDG 44 and IC₅₀ analysis performed in parallel with experimentation on the new series of analogues. Dose response curves and IC₅₀ values for MDG 44 and CDX 87 – 92 are depicted in Figure 7.9. MDG 44, in this experiment, had an IC₅₀ value of 21.5 μ M ($\pm$ 0.5), comparable with the reported IC₅₀ value of 25.9 μ M ($\pm$ 0.3) for MDG 44 in previous experiments in this Chapter (Table 7.1).

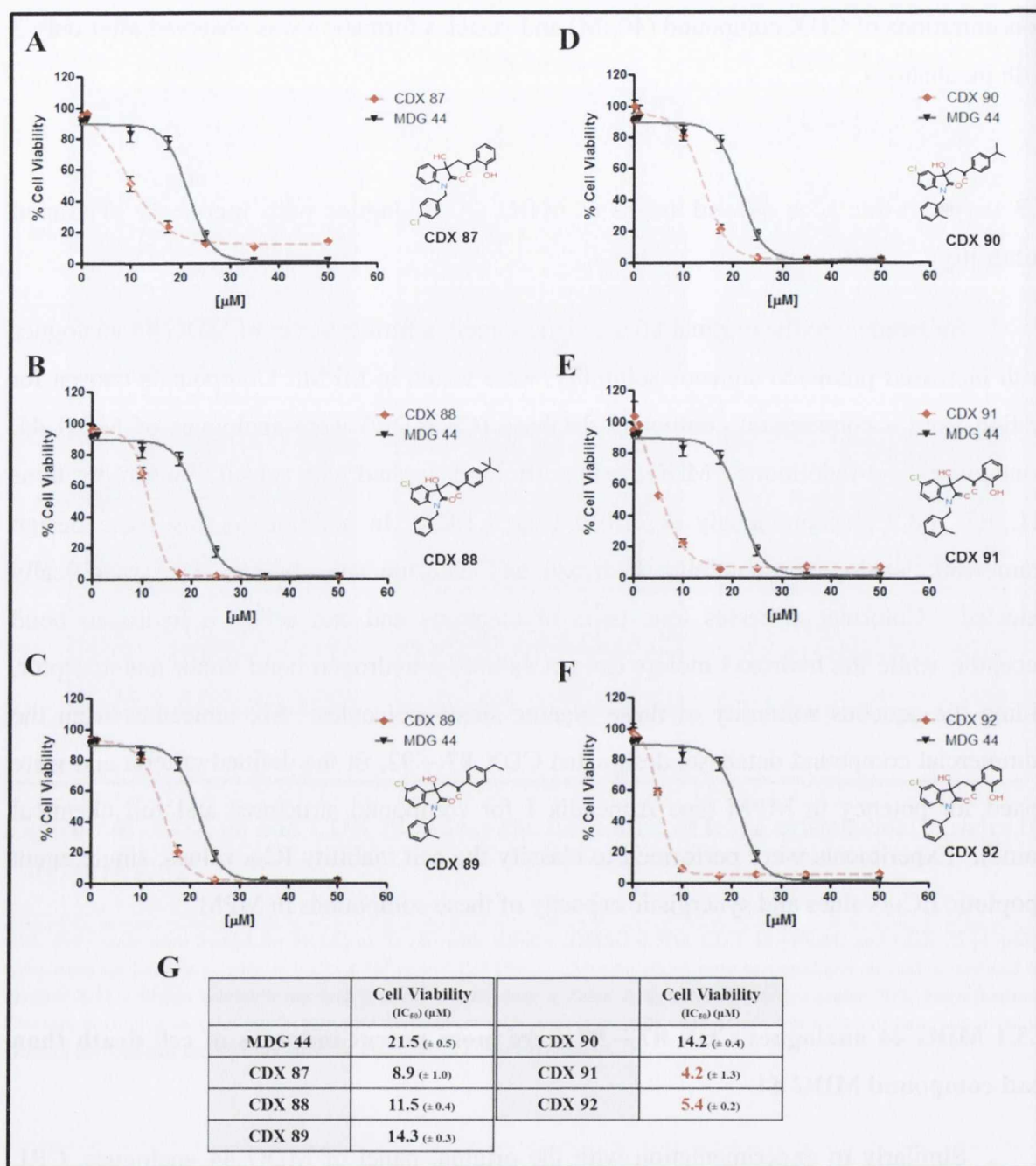


Figure 7.9: Analogues with predicted aqueous solubility – CDX 87 – 92 were more potent inducers of cell death in CRL 5915 cells

Dose response curves were generated for cell death induced by MDG 44 analogues CDX 87 (A), CDX 88 (B), CDX 89 (C), CDX 90 (D), CDX 91 (E) & CDX 92 (F) – (♦) – and compared to the reference compound, MDG 44 (▼). Table of IC₅₀ values shown (G). CRL 5915 cells were seeded at a density of 0.5 x 10⁴ cells / well (200μl) of a 96 well plate and treated at indicated concentrations for 48h. Cell viability was determined by Alamar Blue cell viability assay, as outlined in Section 2.6. Each data point is representative of the mean ± SEM of two independent triplicate experiments. Non-linear regression curve fit analysis and IC₅₀ values generated using Graphpad Prism 4 statistical software.

All six MDG 44 analogues tested showed significant improvement in IC_{50} value compared to that of MDG 44 (Fig 7.9.G). The most potent compounds of the series, CDX 91 and CDX 92, had low micromolar IC_{50} values of $4.2\mu M (\pm 1.3)$ and $5.4\mu M (\pm 0.2)$, respectively. This represented a significant improvement in cytotoxic activity of MDG 44 scaffold based molecules.

7.5.2 CDX 91 and 92 did not induce significant levels of single-agent or synergistic apoptosis in CRL 5915 cells

Of the six new analogues tested, CDX 91 and CDX 92 were chosen for apoptotic analysis in MPM. It was hoped the increased cytotoxic potency observed with these two analogues would translate as being more potent single-agent inducers of apoptosis and synergistic enhancers of Cisplatin mediated apoptosis. CRL 5915 cells treated with increasing concentrations of CDX 91 and CDX 92 alone showed low levels of apoptotic induction, analysed by PI cell-cycle analysis. A maximal apoptotic effect of $41.0\% (\pm 2.3)$ and $30.1\% (\pm 1.4)$ apoptosis was detected for CDX 91 and CDX 92, respectively, after $50\mu M$ incubation for 48h (Fig 7.10). EC_{50} values for CDX 91 and CDX 92 could not be predicted as 50% apoptotic induction was not reached with the highest concentration assayed [$50\mu M$]. Significant levels of apoptosis were detected after $20\mu M$ incubations with CDX 91 and 92 for 24h – $16.3\% (\pm 1.5)$ and $24.3\% (\pm 2.4)$, respectively – however, the percentage population of apoptotic cells did not significantly rise with increases in dose. The magnitude of apoptotic induction with CDX 91 and CDX 92 incubations did not compare with that of the potency of MDG 44 or lead analogues CDX 45 and CDX 78 (Table 7.2).

Synergistic analysis of CDX 91 and CDX 92 in combination with Cisplatin was also performed at analogue concentrations of $12.5\mu M$ and $25\mu M$ (Fig 7.10.C). Combinational treatments with Cisplatin and analogue at either concentration did not induce any synergistic increase in apoptosis.

Discussion

The design and testing of the second generation of MDG 44 structural analogues was a successful undertaking, helping identify new, more potent lead compounds for future

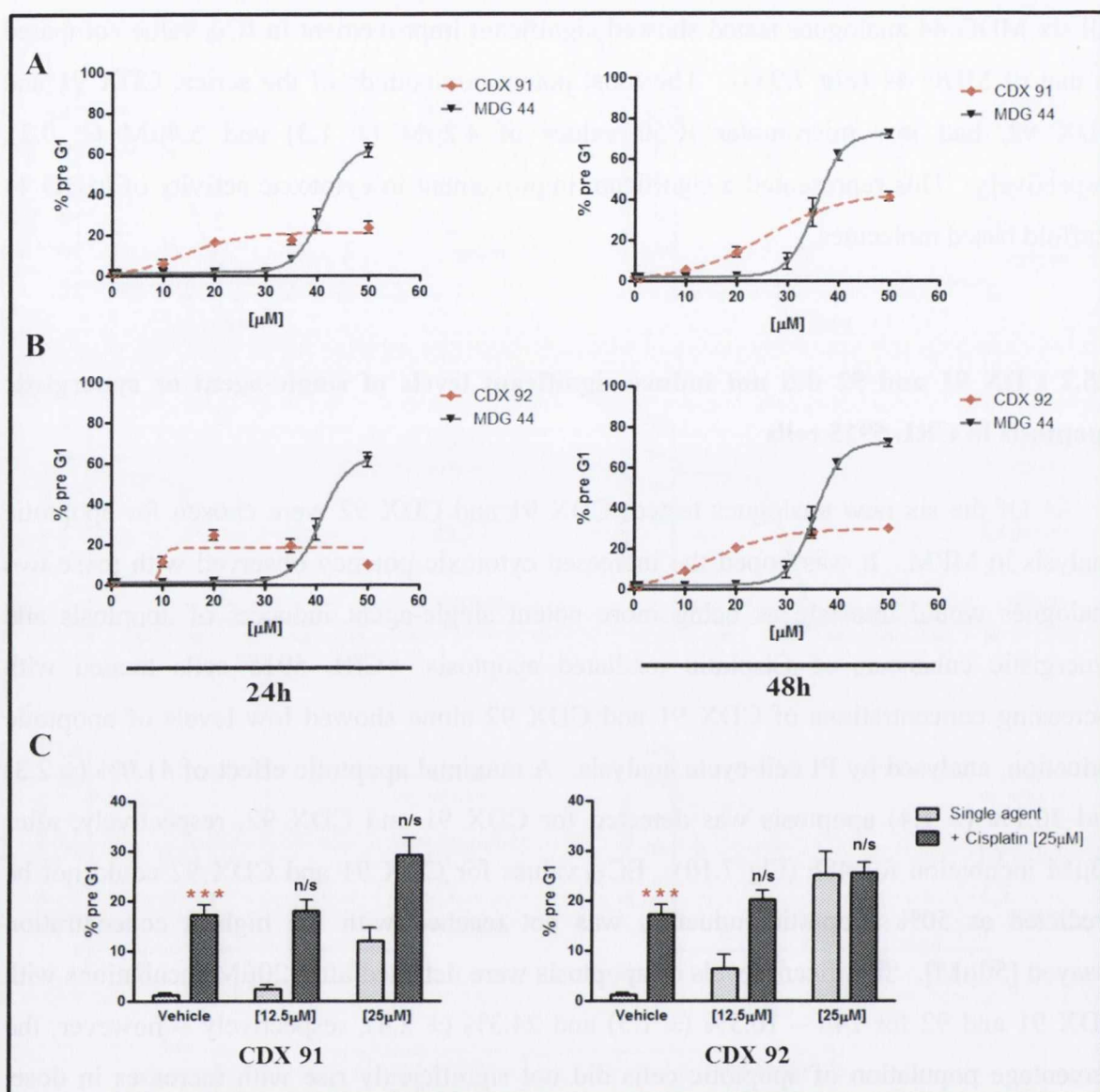


Figure 7.10: CDX 91 and 92 were not better than MDG 44 as single agent or synergistic inducers of apoptosis in CRL 5915

CRL 5915 cells were treated with increasing concentrations of CDX 91 (A) and CDX 92 (B) (♦) for 24h and 48h – Results were compared to MDG 44 (▼) dose response treatments performed in parallel. CRL 5915 cells were treated with indicated concentrations of CDX 91 or CDX 92 alone or in combination with Cisplatin [25μM] – cells were pre-treated for 24h with Cisplatin prior to treatment with CDX 91 and CDX 92 for a further 24h (C). CRL 5915 cells were seeded at a density of 0.225×10^6 cells / T25 (5ml). Apoptotic induction was determined by PI staining and cell-cycle Flow Cytometry analysis, as outlined in Section 2.6. Each data point is representative of the mean $\pm$ SEM of at least three independent experiments. Non-linear regression curve fit analysis and IC_{50} values were generated using Graphpad Prism 4 statistical software. Synergism was determined by two-way analysis of variance (ANOVA) of the interaction effect of single agent and co-treatment response (***) $P < 0.001$; ‡ $P < 0.01$; n/s – Not significant synergism effect).

Name	Compound Substitutions			Cell Viability (IC ₅₀)	Apoptosis - PI FACS (EC ₅₀)		Synergism (+ Cisplatin)	Large Cytoplasmic vacuoles
	R1	R2	C	48h	24h	48h		
MDG 44				21.5µM (± 0.5µM)	44.5µM	37.4µM	Yes	Yes
CDX 91				4.2µM (± 1.3µM)	-	-	No	-
CDX 92				5.4µM (± 0.2µM)	-	-	No	-
CDX 45				10.2µM (± 0.5µM)	36.4µM	31.9µM	Yes	Yes
CDX 78				17.5µM (± 0.2µM)	38.6µM	32.8µM	Yes	Yes

Table 7.2: MDG 44 analogues – Lead compound data

experimentation and providing a large data set for SAR analysis on the 2-indolinone / MDG scaffold class of compounds. Examination of the relationship between the structural substitutions and cytotoxic activity of the original 86 MDG 44 analogues screened revealed a number of correlations of note. SAR analysis was performed by ranking the 86 analogues by their anti-proliferative activity at both treatment concentrations – 32.5 μ M and 10 μ M. A general consensus in the ranking order of analogues was observed at both concentrations and a number of correlations between the class of structural substitution and the level of anti-proliferative activity could be inferred. A number of substitutions were seen to reduce or limit activity compared with that of MDG 44 – defined as anti-proliferative activity significantly greater than 50% with 32.5 μ M incubation. The presence of methoxyphenyl, bimethoxyphenyl, and ethoxyphenyl substitutions at the R1 position greatly reduced activity. The ortho, meta and para positioning of methoxymethane and methoxyethane moieties on phenyl groups at R1 all limited anti-proliferative activity. In addition to this, the presence of benzodioxin ether cycles, para-halogenated phenyl groups (chlorophenyl and fluorophenyl), pyrrolphenyl and other nitrogen containing ring structural substitutions at the R1 position all correlated with reduced anti-proliferative activity. At the R2 position, analogues containing the benzodioxol substitution also correlated with reduced activity.

SAR analysis also identified substitutions deemed essential for activity. Analogues containing phenyl substitutions with ethyl, 2-propyl and tetra-butyl moieties at the para position strongly correlated with anti-proliferative activity. This correlation was illustrated by the results reported in Table 7.1, indicating that of the seven top-ranked analogues taken for further analysis, the four most potent analogues identified (from IC₅₀ and EC₅₀ values) – CDX 45, CDX 78, CDX 25 and CDX 24 – all contained ethylbenzene, tetra-butylbenzene or propan-2-ylbenzene substitutions at R1. The seeming importance of this class of hydrophobic substitution is illustrated with direct comparison of structure and activity between CDX 24/25 and CDX 5. These analogues only differ at the R1 position, with the presence of 2-propyl and tetra-butyl moieties at the para-phenyl position of CDX 24 and CDX 25 respectively – CDX 5 contains a phenyl group at R1 and all contain an ethylbenzene group at R2. Cell viability results for CDX 5 incubations at 32.5 μ M and 10 μ M were 116.6% ($\pm$ 2.9) and 99.5% ($\pm$ 1.8), respectively. The absence of 2-propyl or tetra-butyl para-substitutions on CDX 5 completely abolished the potent anti-proliferative activity observed when these substitutions are present (CDX 24 and CDX 25).

A tetra-butylbenzene group is also present at position R1 on MDG 44 and MDG 42, and SAR analysis strongly backed up initial suspicions that this type of hydrophobic moiety was essential for activity. This coupled with the observation that hydrophilic methoxyphenyl, benzodioxol, chlorophenyl and fluorophenyl substitutions at R1 limited anti-proliferative activity, strongly suggested that 2-indolinone scaffold compounds preferentially form a hydrophobic interaction with its target's binding pocket at the R1 position. Other positive correlations between analogue structure and activity identified by SAR included Naphthyl or ortho and para chlorophenyl substitutions at R2 and the addition of chlorine to the central benzene of the 2-indolinone scaffold (CDX 66).

While a large amount of SAR data on the 2-indolinone / MDG class of compounds was obtained, the primary objective of the MDG 44 analogue screen – the identification of more potent analogues – was of mixed success. Of the seven top-ranked analogues taken for further analysis, five showed greater anti-proliferative activity compared to that of MDG 44 in MPM. All five compounds contained either the ethyl-, propyl- or tetra-butyl-benzene R1 substitutions (CDX 24, 25, 45 and 78), para chlorophenyl substitution at R2 (CDX 78) or a chlorinated 2-indolinone scaffold (CDX 66), which were all reported to correlate with activity. Single-agent apoptotic analysis revealed however that only two of the analogues tested (CDX 45 and CDX 78) displayed marginal increases in apoptotic activity compared to that of MDG 44 (Table 7.1). Apoptosis EC_{50} values for CDX 45 and CDX 78, after 24h incubations, were 36.4 μ M and 38.6 μ M respectively, compared to 44.5 μ M with MDG 44 incubation.

Further analysis of the cellular effects induced by lead analogues CDX 45 and CDX 78 treatment in MPM revealed the conservation of vital activities observed with MDG 44 treatment in MPM. Both CDX 45 and CDX 78 induced the formation of large cytoplasmic vesicles in MPM cells, a cellular response previously observed with MDG 44 treatment (Chapter 5; Fig 5.17). CDX 45 and CDX 78 incubation also enhanced Cisplatin induced apoptosis in MPM. The retention of this activity in second-generation lead analogues of MDG 44 was essential. The ability of 2-indolinone / MDG scaffold compounds to synergise with the clinically approved and widely used chemotherapeutic Cisplatin represents a vital niche in this class of compounds future development. Importantly, the conservation of cytoplasmic vesicle formation and cisplatin synergism strongly suggested that lead analogues CDX 45 and CDX 78 were engaging the same molecular target as that of MDG 44. However, while synergism with Cisplatin was observed, in the assays

performed, CDX 45 and CDX 78 were observed to be less potent enhancers of Cisplatin induced apoptosis compared to the original lead compound MDG 44.

Testing of the second series of MDG 44 analogues with increased predicted aqueous solubility also produced mixed success. Cell viability IC_{50} analysis revealed all six analogues tested possessed significantly increased anti-proliferative activity in MPM compared to that of MDG 44. CDX 91 and CDX 92 were the most potent analogues of all 92 tested in the first and second screens, with IC_{50} values of $4.2\mu M (\pm 1.3)$ and $5.4\mu M (\pm 0.2)$ respectively. These results, compared to the IC_{50} value for cell viability of $21.5\mu M (\pm 0.5)$ for MDG 44, represented the identification of 2-indolinone analogues with significantly improved potency over the original lead molecule.

Both CDX 91 and CDX 92 structures contained chlorine substitutions to the central 2-indolinone scaffold and ortho- positioned hydroxyphenyl groups at R1. These substitutions were chosen to aid solubility by increasing the number of hydrogen bond donor and acceptor moieties, but could represent important substitutions aiding potency. Original SAR analysis identified that the addition of chlorine to the central 2-indolinone scaffold increased activity. This substitution was present in highly ranked CDX 86 and CDX 66, as well as all analogues of the second series, except CDX 87. Little was inferred about the importance of hydroxyphenyl substitutions from the original screen, but this moiety was present on CDX 13, one of the top seven ranked analogues chosen for further analysis. The lack of hydrophobic para substitutions at position R1 on CDX 91 and CDX 92 was noted. However, tetra-butyl-, ethyl-, and proan-2-yl-benzene groups were present on CDX 88, 89 and 90, respectively. Comparative potent anti-proliferative activity (IC_{50} values) was observed with these analogous and similarly substituted analogous identified in Table 7.1 (CDX 24, 25, 45 and 78). These results further confirmed that hydrophobic substitutions at R1 could confer anti-proliferative activity in MPM, but may not be indispensable.

While potent anti-proliferative activity was observed, single-agent and synergistic apoptotic analysis of CDX 91 and CDX 92 produced poor results. Both CDX 91 and CDX 92 were less potent inducers of apoptosis compared to MDG 44 and showed no capacity to synergistically enhance cisplatin induced apoptosis in MPM.

In conclusion, screening analysis of the second generation of MDG 44 analogues produced a large data set with regard to structure and activity relationship, identifying

moieties thought important for binding pocket affinity and cellular activity and greatly informing the future design of successive generation of MDG 44 analogues. A number of analogues with significantly increased anti-proliferative activity were identified. However, no lead analogues representing a significant increase in single-agent or synergistic apoptosis were found. Further analysis and testing of future generations of MDG 44 analogues will be required to significantly improve potency of this class of molecule.

Chapter 8

Biochemical analysis of putative Mitochondrial-Hexokinase dissociating compounds in Burkitt's Lymphoma and MPM cell lines

Introduction

Experimentation in Chapter 8 looked to investigate aerobic glycolysis (“The Warburg Effect”) as a mechanism of resistance in cancer (Warburg et al., 1927) and as a second target for rational drug design in the development of treatments for MPM. The increased glycolytic flux observed during aerobic glycolysis provides a number of metabolic and anti-apoptotic advantages in cancer. These advantages include the increased availability of carbon source for biosynthesis of cellular intermediates for cellular proliferation (amino acids, lipids, nucleic acids), and hexokinase (HK) mediated inhibition of MOMP, blocking initiation of the intrinsic apoptotic pathway (Vander Heiden et al., 2009, Abu-Hamad et al., 2008). The advantage this altered metabolic framework provides to fast proliferating malignancies makes it a good target for rational drug design. A number of genetic and epigenetic alterations in cancer cells help potentiate increased glycolytic flux (Moreno-Sanchez et al., 2007), however the most important and central control point is believed to be the association of HK with the mitochondrial outer membrane protein, VDAC (Voltage Dependent Anion Channel). HK localisation to the mitochondria (Mito) and complexing with VDAC and ANT allows it preferential access to Ox Phos generated ATP. This proximal localisation of HK to the Mito provides the advantage of abundant ATP availability for the initiation of the first committal step of glycolysis – the phosphorylation of Glucose to Glucose-6-phosphate – driving increased glycolytic flux in the cancer cell (Vander Heiden et al., 2009). HK binding also prevents Bax/Bak binding to VDAC and mitochondrial pore formation in response to apoptotic stimulus (Majewski et al., 2004b, Pastorino et al., 2002). Other alterations in cancers including increased VDAC, HK-I/II and Glucose transporter (GLUT 1/4) expression and increased IGFR/PI3K/Akt signalling all contribute to inducing and sustaining binding of HK to the Mito (Pastorino and Hoek, 2008). As such, sustained binding of HK to the Mito is thought to be an essential component for maintaining this aerobic glycolytic / anti-apoptotic phenotype in cancer. The identification of small molecules with the ability to force dissociation of HK from the Mito was the chosen target mechanism for rational drug design to inhibit this phenomenon in cancer.

Previous reports have illustrated that HK and Bax/Bak share a common binding site on the mitochondria, VDAC, and HK antagonises the apoptotic activity of Bax/Bak when bound to VDAC at the Mito (Pastorino et al., 2002). Dissociation of HK has been shown to result in loss of the metabolic advantage and resistant phenotype in cancer, allowing binding

of Bax/Bak to the Mito and making cells susceptible to apoptotic challenge (Pastorino et al., 2002). A number of different classes of compounds have been shown to promote the dissociation of HK and potently induce apoptosis in cancer (Chen et al., 2009, Chiara et al., 2008, Goldin et al., 2008, Penso and Beitner, 1998). Interestingly, whilst the prevailing theory for induction of apoptosis by forced dissociation of HK from the Mito is believed to be through a Bax/Bak dependent mechanism, HK dissociation is able to induce cell death in resistant Bax/Bak deficient cancer models (Majewski et al., 2004a). Cell death in this manner occurs with reduced kinetics due to the absence of Bax/Bak antagonism of HK and is speculated to occur due to the loss of Mito-bound HK inducing a “closed” conformation in the VDAC pore complex. This “closed” conformation prevents transport of metabolites between the matrix and cytoplasm and sustained dissociation culminates in mitochondrial inner membrane (MIM) swelling, rupture of the MOM and unregulated release of pro-apoptotic mitochondrial proteins (Robey and Hay, 2006). Apoptotic activity in Bax/Bak positive and resistant Bax/Bak deficient cancers makes targeting Mito-localised HK an attractive target for rational drug design.

In this Chapter, attempts were made to identify and develop new compounds able to dissociate mitochondrial-hexokinase and induce apoptosis in cancer cells. A ligand-based computational design approach was taken to develop compounds with HK-dissociating activity. Clotrimazole, an anti-fungal azole drug known to target and induce release of mitochondrial-hexokinase (Penso and Beitner, 1998), was the basis of the design of a pharmacophore used to identify potentially novel, “hit” compounds. Clotrimazole has been previously reported to induce dissociation of hexokinase from the mitochondria in a number of *in vitro* cancer models, including melanoma (B16), cervical cancer (HeLa) and mouse embryonic fibroblasts (MEF) (Chiara et al., 2008, Majewski et al., 2004a, Penso and Beitner, 1998), and is used as a model drug in the investigation of aerobic glycolysis and HK dissociation in cancer (Majewski et al., 2004a, Pastorino et al., 2002). Novel computationally designed compounds, predicted to have activity similar to Clotrimazole, were identified through Virtual-screening of structures from commercial compound databases (Specs, ChemDiv, Chembridge) onto the Clotrimazole pharmacophore. Scoring functions for the virtually screened structures were generated and compounds ranked based on their structural and chemical similarity to the computationally-defined Clotrimazole pharmacophore. The top five predicted “Virtual Hits” (A1 – A5) were purchased for biochemical analysis.

In attempts to identify further potentially novel HK-dissociating compounds, it was proposed to investigate compounds of a similar chemical class to that of Clotrimazole for activity as HK-dissociating compounds. Initial experiments investigated the anti-fungal azole compounds, Econazole and Miconazole. Both compounds are of the same chemical class (diazole) as Clotrimazole and have similar chemical structure, including the presence of an imidazole ring and mono- / di-chlorobenzene moieties on each compound. Like Clotrimazole, Econazole and Miconazole are commonly used as topical anti-fungal agents and are known inhibitors of cytochrome P450-dependent steroidogenic enzymes (Ayub and Levell, 1989). Importantly, Econazole and Miconazole have also previously been shown to be inhibitors of the peripheral-type benzodiazepine receptor (PBR), a binding partner of VDAC in the MPTP and essential in the uptake of cholesterol into the mitochondrion (Zisterer and Williams, 1997). PBR activation and cholesterol uptake is a rate-limiting step in steroidogenesis, but has also been implicated in promoting HK localisation to the Mito and inhibition of apoptosis. *Baggetto et al* report that cancer cells can contain a between two and ten-fold increase in the cholesterol content of the MOM, which was proposed to facilitate increased binding of HK to VDAC (Baggetto et al., 1992). Various other studies have reported that increased mitochondrial cholesterol content inhibits Bax/Bak pore formation and permeabilisation of the Mito, which prevents induction of the intrinsic apoptotic pathway (Pastorino and Hoek, 2008, Lucken-Ardjomande et al., 2008). Due to the PBR being a component of the MPTP-HK complex, the proposed involvement of the PBR in promoting HK binding to the Mito and the chemical similarity of Econazole and Miconazole to Clotrimazole, these PBR inhibitors were tested for activity as proposed mitochondrial HK-dissociating compounds.

Clotrimazole, the PBR ligands Econazole and Miconazole, and the novel, computationally-designed Clotrimazole pharmacophore compounds were screened in Burkitt's Lymphoma "Bax/Bak null" and Bax/Bak positive" cell models. Experiments were performed to test the difference in efficacy of treatment between apoptotic susceptible (Bax/Bak positive) and resistant (Bax/Bak negative) cell lines. Compounds were also screened in MPM cell lines to test if targeting aerobic glycolysis and HK-Mito dissociation was a viable treatment strategy in this resistant form of cancer. The ultimate aim of this study was to identify new drugs with HK dissociating activity, develop novel compounds through computational drug design and assess the efficacy of these compounds in the treatment of MPM. In this Chapter, initial experimentation into this study investigated the

cytotoxic and apoptotic activity of test compounds in the panel of Burkitt Lymphoma and MPM cell lines.

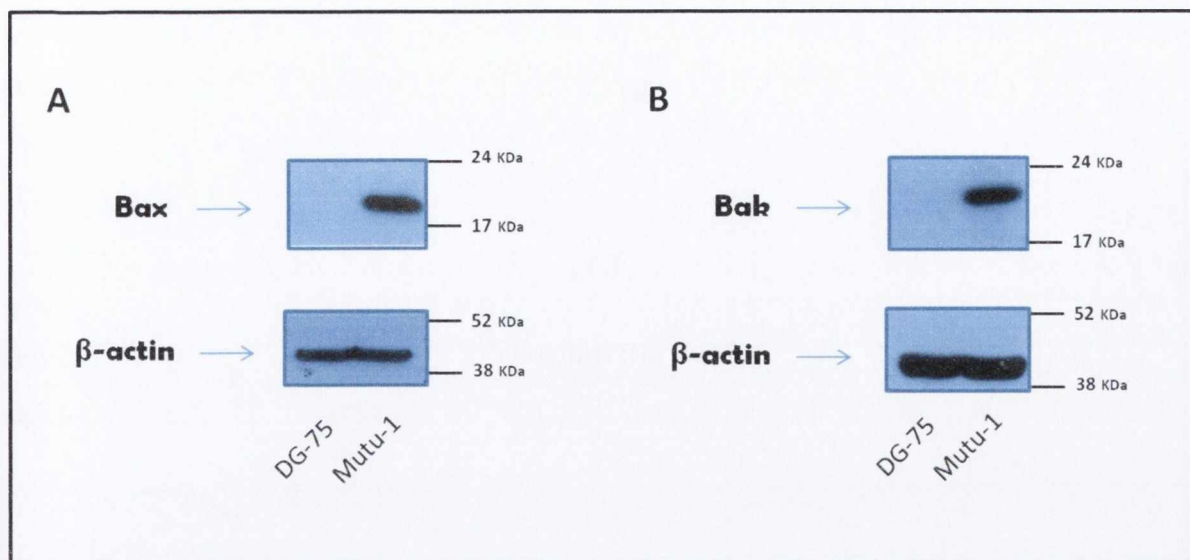
Results

8.1 Mutu-1, but not DG-75 BL cell lines express pro-apoptotic BCL-2 family proteins Bax and Bak

The protein expression levels of the BCL-2 family proteins Bax and Bak were analysed in the Mutu-1 and DG-75 Burkitt's Lymphoma (BL) cell lines by way of Western Blot. Analysis of protein expression of these VDAC associating pro-apoptotic proteins was performed in order to confirm the "Bax/Bak positive" and "Bax/Bak null" BL cell model for apoptotic testing of putative HK dissociating compounds. Whole cell lysates from sub-confluent cultures of Mutu-1 and DG-75 cell lines were prepared for experimentation. Western blot analysis confirmed that the Mutu-1 cell lines exclusively expressed both Bax and Bak with no basal expression of either detected in DG-75 cells (Fig 8.1). These results confirmed that Mutu-1 cells represent a "Bax/Bak positive" cell model and DG-75 cells a "Bax/Bak null" cell model for testing the differential effects of Bax and Bak expression on the apoptotic efficacy of HK dissociating compounds.

8.2 Examination of the effect of anti-fungal azole compounds on induction of cell death and apoptosis in BL and MPM cell lines

Three anti-fungal azole compounds, Clotrimazole, Econazole and Miconazole were analysed for their anti-proliferative and apoptotic effects in Burkitt Lymphoma and MPM cancer models. An original study by *Penso et al* reported that Clotrimazole and its imidazole derivative, Bifonazole, could potentially induce mitochondrial-HK dissociation and cell death in B16 melanoma cells (Penso and Beitner, 1998). Subsequent studies confirmed the efficacy of Clotrimazole as an apoptotic, HK-dissociating compound in numerous other cell lines (Majewski et al., 2004a, Chiara et al., 2008). Clotrimazole is a diazole compound containing an imidazole, a mono-chlorobenzene and two phenyl moieties (Fig 8.2). In this study two other anti-fungal diazole compounds, Econazole and Miconazole, which are structurally similar to Clotrimazole and contain imidazole and chlorobenzene moieties, were investigated for their ability to act as HK dissociating compounds in BL and MPM cell lines



Figures 8.1: Mutu-1 cells, but not DG-75 cells, express pro-apoptotic Bcl-2 proteins, Bax and Bak

Protein expression of BCL-2 family proteins, Bax (A) and Bak (B), investigated in Mutu-1 and DG-75 Burkitt's Lymphoma cell lines. Sub-confluent cultures of each cell line were taken for preparation of whole cell lysates. Proteins (20 μ g) were resolved on a 12% SDS-PAGE gel and transferred to PVDF membrane, as outlined in Section 2.5. Blots were blocked in 5% Marvel TBS-Tween and probed using primary antibodies anti-Bax (Primary Ab – 1:500) or anti-Bak (Primary Ab – 1:1,000). Blots were stripped and reprobed for expression of anti- β -actin (Primary Ab – 1:10,000) as an experimental loading control, as outlined in Section 2.5.6. Results are representative of three independent experiments.

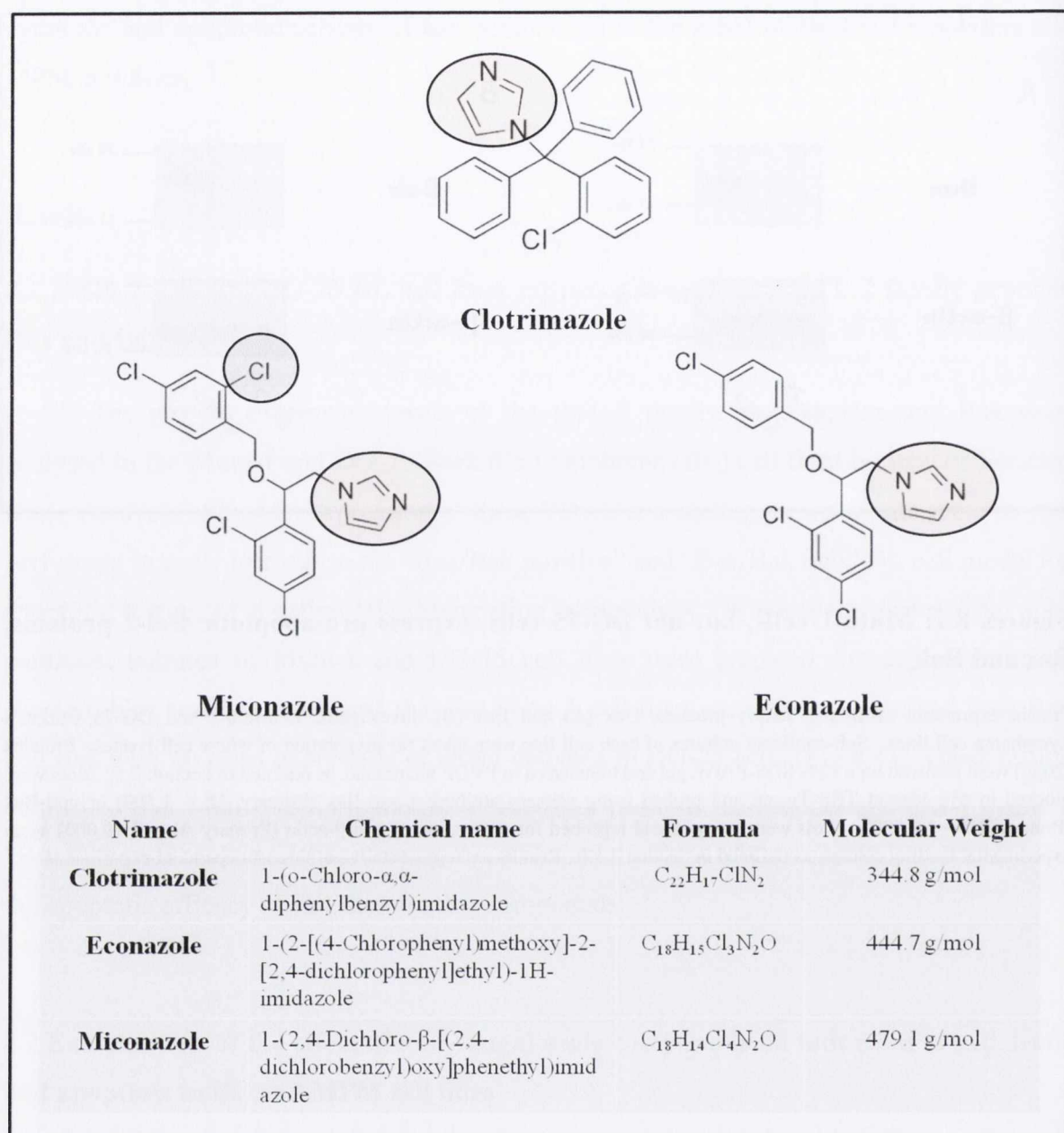


Figure 8.2: Chemical structure, name and formula of Clotrimazole and anti-fungalazole compounds

Diagrams illustrate the chemical structure of the three imidazole (diazole) test compounds – Clotrimazole, Econazole and Miconazole. Compounds are also similar in the presence of mono and dichlorobenzene moieties.

(Fig 8.2). Econazole and Miconazole are analogues of each other, the only difference being a dichlorobenzene, as opposed to a monochlorobenzene group, present at a distal position on Miconazole. As well as structural similarity to Clotrimazole, these two compounds are also known inhibitors of PBR (Zisterer and Williams, 1997), a component of the MPTP with suspected activity in promoting HK association to the mitochondria (Pastorino and Hoek, 2008). Initial experiments were performed to test the ability of Clotrimazole and the two similar imidazole compounds, Econazole and Miconazole, to induce cytotoxicity and apoptosis in the “Bax/Bak positive” Mutu-1 and “Bax/Bak null” DG-75 BL cell lines.

8.2.1 Azole compounds induced dose dependent cell death in Mutu-1 and DG-75 cell lines

Mutu-1 and DG-75 cells were treated with Clotrimazole, Econazole and Miconazole in a dose dependent manner for 24h and 48h incubations (Fig 8.3). Dose dependent induction of cell death was analysed by Alamar Blue cell viability assay. Clotrimazole treatment induced a differential response in “Bax/Bak positive” and “Bax/Bak null” cell lines. A significant difference in susceptibility to Clotrimazole was observed between Mutu-1 and DG-75 cells, with Mutu-1 cells significantly more sensitive to Clotrimazole treatment than DG-75 cells. After 24h incubation, an IC_{50} value for cell viability of $24.5\mu M$ (± 2.1) was observed with Clotrimazole treatment, compared to negligible levels of cell death induced in DG-75 cells (Table 8.1). Similar responses were observed after 48h incubations with IC_{50} values of $21.8\mu M$ (± 2.0) and approximately $48\mu M$ in Mutu-1 and DG-75 cells respectively.

Treatment of Mutu-1 and DG-75 cells with both Econazole and Miconazole induced IC_{50} values for cell viability in the same low-micromolar range to Clotrimazole incubations (Table 8.1). However, contrary to results observed with Clotrimazole, no significant difference in sensitivity to Econazole and Miconazole incubation was observed between Mutu-1 and DG-75 cell lines.

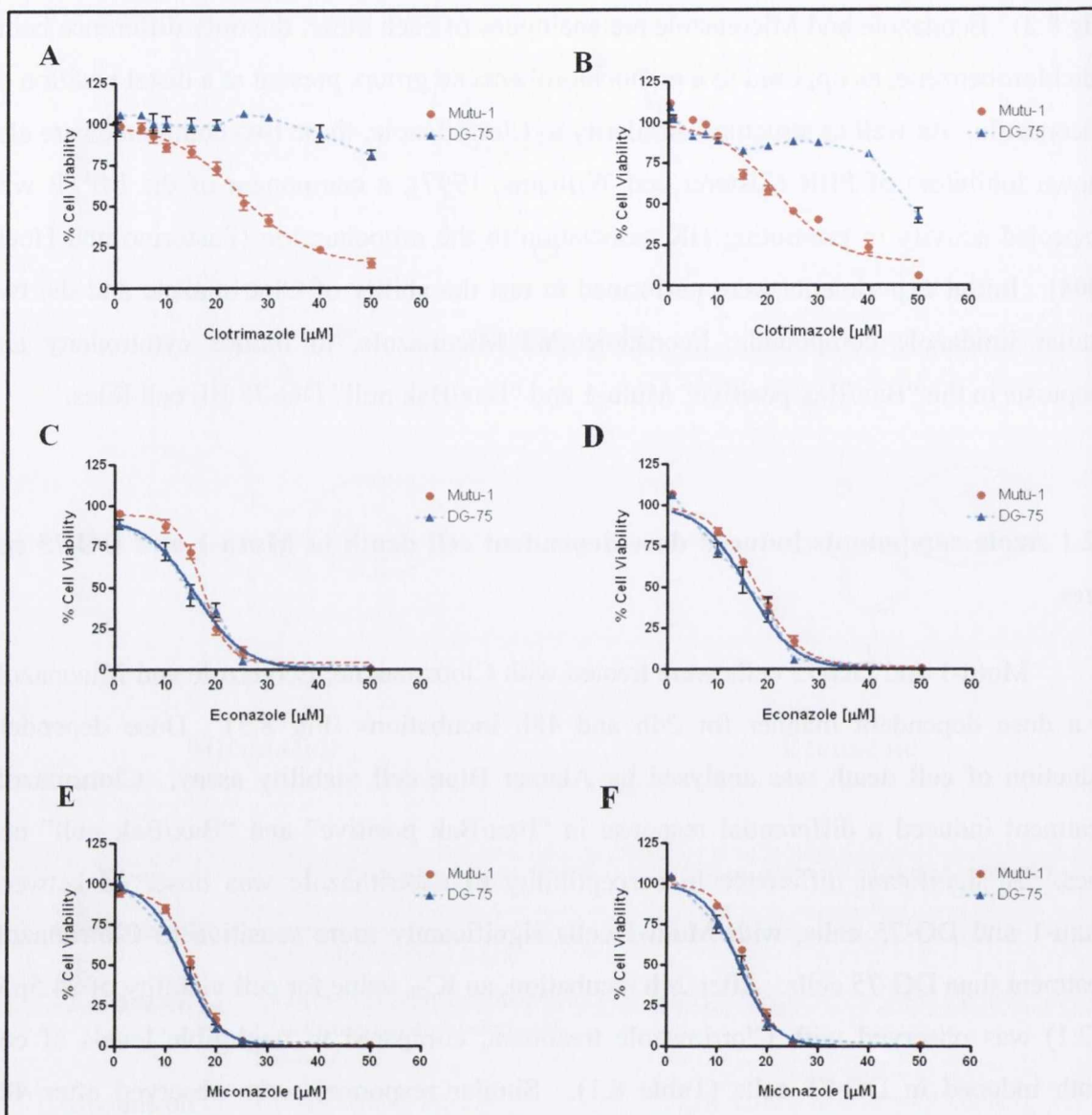


Figure 8.3: Azole compounds induced dose dependent cell death in Mutu-1 and DG-75 Burkitt's Lymphoma cell lines

Burkitt's Lymphoma cell lines, Mutu-1 (●) and DG-75(▲), were treated with anti-fungal azole compounds, Clotrimazole (A-B), Econazole (C-D) and Miconazole (E-F), for 24h (A, C & E) and 48h (B, D & F) and analysed for reduction in cell viability. Cells were seeded on 96 well plates at a density of 1.5×10^5 cells per well (Mutu-1) and 0.5×10^5 cells per well (DG-75) containing 200 μl media. Cells were treated at indicated concentrations of azole compounds for 24h and 48h. Cell viability was analysed by Alamar Blue assay, as outlined in Section 2.6. Results are representative of the mean ($\pm\text{SEM}$) of three independent triplicate experiments. Non-linear regression curve fit analysis was performed using Graphpad Prism 4 statistical software.

Mutu-1		Clotrimazole	Econazole	Miconazole
Cell Viability	(EC ₅₀ – 24h)	24.5µM (± 2.1)	17.5µM (± 0.9)	15.5µM (± 0.8)
	(EC ₅₀ – 48h)	21.8µM (± 2.0)	17.7µM (± 1.2)	15.9µM (± 0.5)
Apoptosis	(EC ₅₀ – 24h)	13.2µM	18.8µM	17.1µM
Time Dependence	(EC ₅₀ – 24h)	4.0 h	9.9 h	4.3 h

DG-75		Clotrimazole	Econazole	Miconazole
Cell Viability	(EC ₅₀ – 24h)	—	15.9µM (± 2.5)	13.6µM (± 1.1)
	(EC ₅₀ – 48h)	~ 48 µM	15.8µM (± 2.5)	14.4µM (± 1.3)
Apoptosis	(@ 50µM – 72h)	41.1% (± 10.8)	17.0% (± 4.1)	60.2% (± 1.5)

Table 8.1: Cell viability and Apoptosis results for azole treatment in Burkitt’s Lymphoma cell lines

Summary of cell viability (Fig 8.3) and apoptosis (Fig 8.4) results for azole treatment in Mutu-1 and DG-75 cell lines.

8.2.2 Mutu-1 cells were more susceptible to apoptosis induced by anti-fungal azole compounds than DG-75 cells

After examining induction of cell death in response to azole treatment in BL cell lines, their ability to specifically induce apoptosis was investigated. Mutu-1 and DG-75 cells were incubated with increasing concentrations of Clotrimazole, Econazole and Miconazole and apoptotic induction was determined by PI cell-cycle analysis after 24h incubation. Results revealed that Mutu-1 cells were more susceptible to azole induced apoptosis inducing significant dose-dependent apoptosis with low micromolar EC₅₀ values – Clotrimazole - 13.2μM; Econazole - 18.8μM and Miconazole - 17.1μM (Fig 8.4.A – C & Table 8.1).

In comparison, DG-75 cells were completely insensitive to treatment. In line with their “Bax/Bak null” apoptotic resistant phenotype, incubation with azole compounds in DG-75 cells induced no significant increase in apoptosis after 24h (Fig 8.4.A – C).

8.2.3 Azole compounds induced dose and time dependent apoptosis in Mutu-1 and DG-75 cell lines

The temporal dependence of azole induced apoptosis was investigated in both Mutu-1 and DG-75 cell lines. As outlined previously, time-dependent induction of cell death is a hallmark of PCD and an indication of drug potency. In Mutu-1 cells, maximal induction of apoptosis was induced after 24h incubations with azole compounds at concentrations as low as 20 - 25μM. Temporal dependence was investigated at time points between 2 and 24h. Cells were incubated with vehicle or 25μM Clotrimazole, Econazole or Miconazole for indicated time points (Fig 8.4.D). Results indicated that each compound induced time dependent activation of apoptosis, with significant levels of apoptotic induction detected with Clotrimazole, Econazole and Miconazole incubations at time points as short as 4h – 51.1% (± 1.1), 27.3% (± 1.8) and 47.9% (± 1.7) respectively (Fig 8.4.D). EC₅₀ analysis of time dependent induction of apoptosis indicated that both Clotrimazole and Miconazole possessed similar potency with EC₅₀ values of 4.0h and 4.3h respectively. Econazole was considered to be a significantly less potent inducer of apoptosis with induction of 50% apoptosis detected after a much longer incubation period 9.9h (Table 8.1).

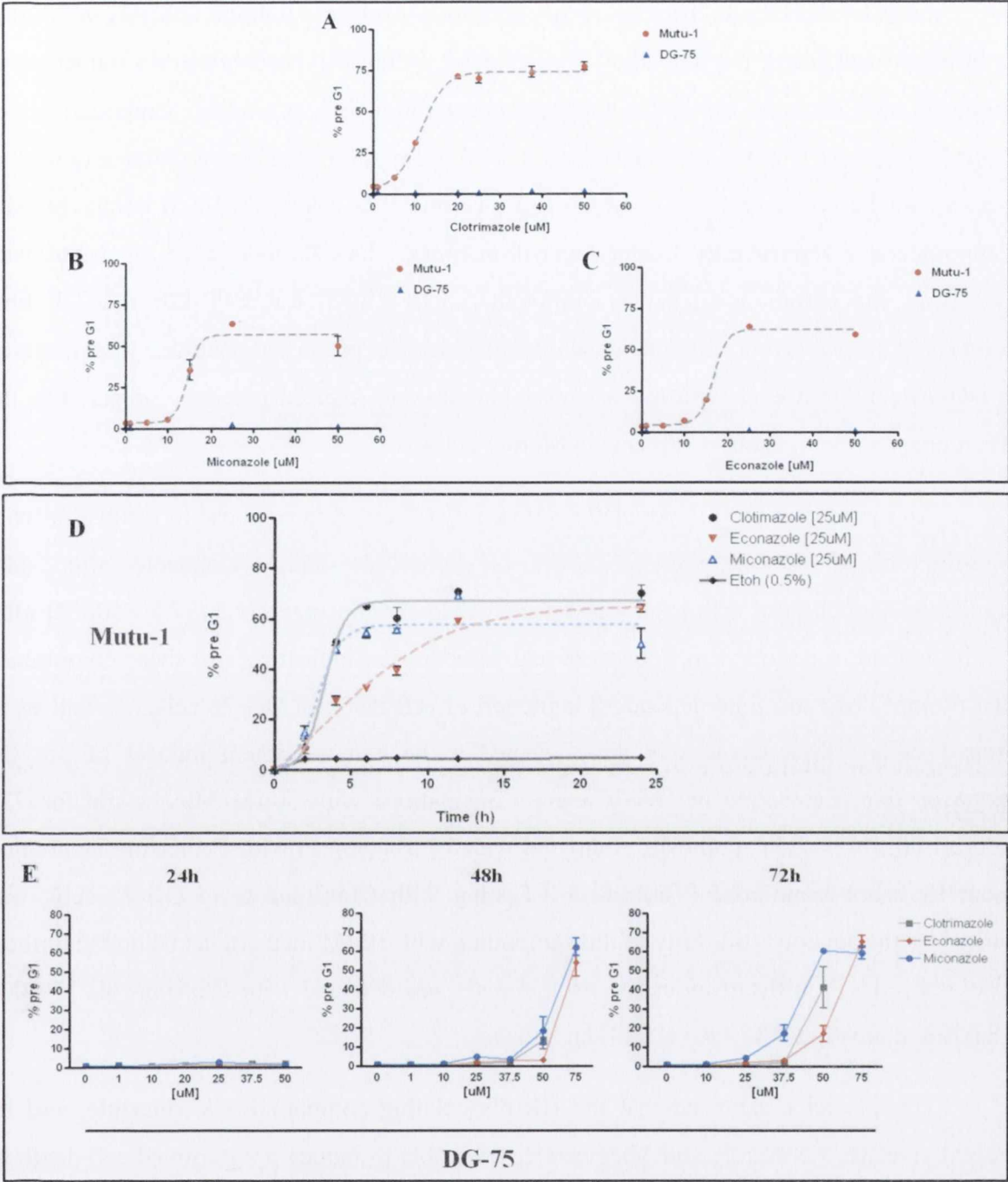


Figure 8.4: Azole compounds induced time and dose dependent apoptosis in Mutu-1 and DG-75 cell lines

Mutu-1 (A-D) and DG-75 (A-C & D) cell lines were treated with Clotrimazole, Econazole and Miconazole in a dose and time dependent manner and analysed for induction of apoptosis. (A-C) Mutu-1 (●) and DG-75 (◆) cells were treated with azole compounds at indicated concentrations for 24h and analysed for induction of apoptosis by PI cell cycle analysis, as outlined in Section 2.7. (D) Mutu-1 cells were treated with Vehicle (EtOH 0.5%) or 25 μ M of azole compound for increasing time points and analysed for apoptotic induction by PI cell cycle analysis. (E) DG-75 cells were treated with indicated concentration of azole compound and analysed for apoptotic induction after 24h, 48h and 72h incubations. Results in each case are representative of the mean ($\pm$ SEM) of at least three independent experiments. Non linear regression curve fit and statistical analysis (Student's t-test) was performed using Graphpad Prism 4 statistical software.

In DG-75 cells, the original assay conditions used for apoptotic analysis in Mutu-1 cells were insufficient for detection of apoptosis. After 24h incubation, no induction of apoptosis was detected up to the highest concentration of each azole compound tested (50 μ M) (Fig 8.4.E). Apoptotic analysis in this “Bax/Bak null” resistant cell line required an increase in the concentrations at which azole compounds were tested and treatment with compounds for significantly longer incubation times. DG-75 cells were incubated with increasing concentrations of azole compound (up to 75 μ M) for both 48h and 72h time points (Fig 8.4.E). Time and dose-dependent increases in pre-G1 populations was observed in DG-75 cells, however with much slower kinetics and reduced potency compared to the effect observed with azole treatments in Mutu-1 cells.

An increase in pre-G1 populations was only observed in response to incubation with high-micromolar concentrations (75 μ M) of Econazole and Miconazole after 48h. Significant induction of apoptosis was detected at lower concentrations (37.5 - 50 μ M) after a 72h incubation period with Econazole and Miconazole, indicating that these compounds also prompt dose and time dependent induction of cell death in DG-75 cells, as well as in Mutu-1 cells. Miconazole was also detected to be a more potent inducer of pre G1 apoptosis than Econazole in DG-75 cells. Incubations with 50 μ M Miconazole for 72h induced 60.2% ($\pm$ 1.5) apoptosis, compared with 17.0% ($\pm$ 4.1) with Econazole incubation under the same conditions (Table 8.1). Testing with Clotrimazole in DG-75 cells was limited by the aqueous solubility of the compound, with 50 μ M incubations being the highest dose assayed. Significant induction of apoptosis was detected with Clotrimazole [50 μ M] after 72h incubation – 41.1% ($\pm$ 10.8) apoptosis.

These results indicated that the HK-dissociating compound, Clotrimazole, and its azole derivative, Econazole and Miconazole, were able to induce programmed cell death in both “Bax/Bak” positive Mutu-1 cells and resistant, “Bax/Bak null” DG-75 cell lines. However, a significant difference in the dose and incubation time required for induction of cell death was observed. Incubation times of up to 72h were required for detection of cell death in DG-75 cells. Experimental results also indicated a significant difference in potency between the two analogues, Econazole and Miconazole. These two compounds differ only in the presence of a dichlorobenzene substitution in Miconazole compared to a monochlorobenzene moiety in Econazole (Fig 8.2).

8.2.4 Azole compounds induced PARP cleavage in Mutu-1, but not DG-75 cell lines

In order to confirm that an apoptotic programme of cell death was induced by azole compound treatment in BL cells, induction of PARP cleavage was investigated in response to treatment in Mutu-1 and DG-75 cell lines. Cells were incubated with Clotrimazole, Econazole, Miconazole (Mutu-1 and DG-75 treated at 25 μ M and 50 μ M, respectively) or vehicle for 24h. Mutu-1 cells were incubated with 25 μ M azole compound, while DG-75 cells were incubated with 50 μ M of each azole compound. Nocodazole, a microtubule targeting agent, was used as a positive assay control for induction of apoptosis and the cleavage of PARP (Beswick et al., 2006). PARP cleavage was examined by Western blot analysis. In Mutu-1 cells, the cleaved form of PARP (89 kDa) was specifically detected in all azole and Nocodazole treatment lysates, indicating induction of an apoptotic programme of cell death (Fig 8.5.A). The largest amount of cleaved PARP was observed in the Clotrimazole and Miconazole treated samples.

In contrast to Mutu-1 experimentation, no PARP cleavage was observed in DG-75 treated samples. PARP cleavage was investigated in DG-75 cells after 24h (Fig 8.5.A) and 72h (Fig 8.5.B) incubations. No 89 kDa cleaved band was detected in response to treatment with anti-fungal azole compounds, or with treatment with the apoptotic control, Nocodazole. Degradation of full length PARP was observed in DG-75 samples after 72h incubation with azole compounds, however, the absence of cleaved PARP bands suggests induction of cell death and degradation of cellular proteins through a method of PCD independent of caspase involvement (Fig 8.5.B).

8.2.5 Azole compounds induce Caspase-3 activation in Mutu-1, but not DG-75 cell lines

Further investigations were performed to determine whether the azole compounds could induce a caspase-dependent form of apoptotic PCD in Mutu-1 and DG-75 Burkitt's Lymphoma cell lines. The induction of caspase-3 activation in response incubations with Clotrimazole, Econazole and Miconazole in Mutu-1 and DG-75 cells was assayed. Again, Nocodazole was used as a positive assay control of apoptotic induction. In Mutu-1 cells, significant fold induction of caspase-3 activation was detected after 6h incubation with Clotrimazole, Econazole and Miconazole – 4.8 ($\pm$ 0.7), 5.0 ($\pm$ 0.8) and 5.4 ($\pm$ 0.4) fold

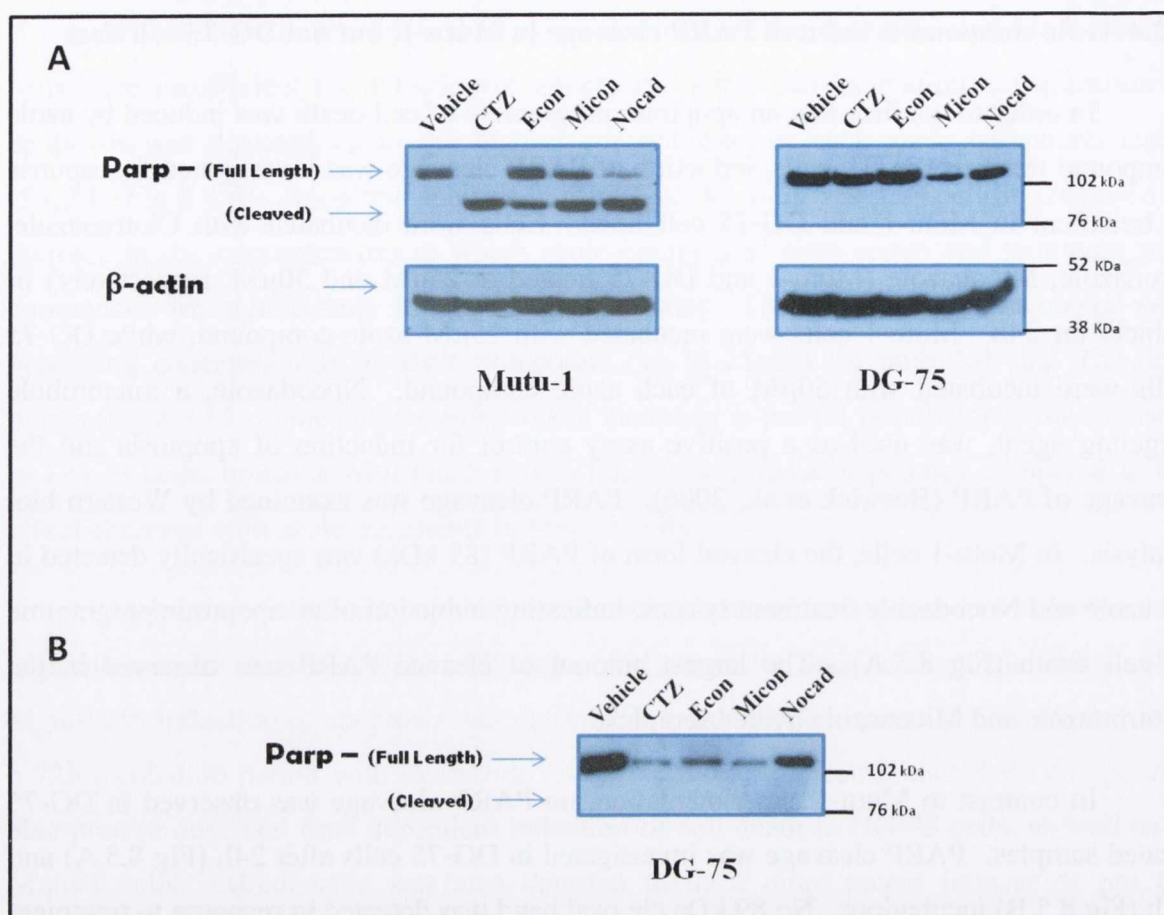


Figure 8.5: Azole compounds induced PARP cleavage in Mutu-1, but not DG-75 Burkitt's Lymphoma cell lines

PARP cleavage was analysed in Mutu-1 and DG-75 cell lines after 24h (**A**) and 72h (**B**) incubations with azole compounds. Cells were seeded at a density of 4×10^6 cells / 25ml in 75cm² tissue culture flasks and left for 18h. Mutu-1 cells were treated with 25μM azole compound, 5μM Nocodazole (Nocad) or vehicle for 24h. DG-75 cells were treated with 50μM azole compound, 5μM Nocodazole or vehicle for 24h and 72h. After incubation periods, cells were harvested and whole cell lysates prepared, as outlined in Section 2.5. Proteins (50μg) were resolved on an 8% SDS-PAGE gels and transferred to PVDF membrane. Blots were blocked in 5% Marvel TBS-Tween, and probed with anti-PARP (Primary Ab – 1:1,000). Blots were stripped and reprobed for expression of anti-β-actin (Primary Ab – 1:10,000) as an experimental loading control, as outlined in Section 2.5.6. Results are representative of three independent experiments.

increase, respectively (Fig 8.6.A). However, in DG-75 cells, incubations with azole compounds for 6h and 24h time points had no effect on caspase activation. The differential response between these two cell lines was mirrored with results for Nocodazole treatment. Incubation with Nocodazole for 24h induced a significant fold increase in caspase-3 activation in Mutu-1 cells (6.9 ± 0.7), but no inductive effect was observed with Nocodazole treatment in DG-75 cells (0.6 ± 0.1) (Fig 8.6.B).

8.2.6 Sub-lethal concentrations of azole compounds induced G1 cell cycle arrest in the DG-75 cell line

In the DG-75 cells, PI cell-cycle Flow Cytometry analysis was performed to investigate the effect of azole compounds on induction of the cell-cycle arrest, as opposed to apoptotic PCD, in this “Bax/Bak null” resistant cell line. Cells were treated with increasing concentrations of Clotrimazole, Econazole and Miconazole and the dose dependent accumulation of cells entering and arresting in the G1/G0 phase of the cell cycle was determined. Figure 8.7 illustrated how incubation of DG-75 cells with azole compounds induced the arrest of cells in the G1/G0 phase of the cell-cycle, at sub-lethal concentrations of drug. This effect was observed after both 48h and 72h time-points and in response to incubations with all azole compounds. Treatment of cells with 10 μ M Clotrimazole, Econazole and Miconazole, for 72h, induced a 30.3% (± 0.9), 16.7% (± 0.9) and 18.2% (± 1.2) increase in the G1/G0 population of cells, respectively, above vehicle incubations (Red arrow: $\longrightarrow$). This effect was maintained at drug concentrations prior to induction of cell death, denoted by increases in the pre G1 population of cells.

8.3 Azole compounds effect in MPM cell lines

Clotrimazole and the putative HK-dissociating compounds, Econazole and Miconazole, were also tested in the panel of four MPM cells lines; CRL 5915, CRL 5820, Ju77 and One 58 cells. Experiments were performed to examine the Warburg effect and specifically HK-dissociation from the mitochondria as a rational target for the treatment of resistant MPM. Results reported in Chapter 5 showed that all four MPM cell lines express both Bax and Bak proteins (Fig 5.1), suggesting that these cell lines may be susceptible to induction of apoptosis through HK dissociation from the mitochondria. Initial experiments

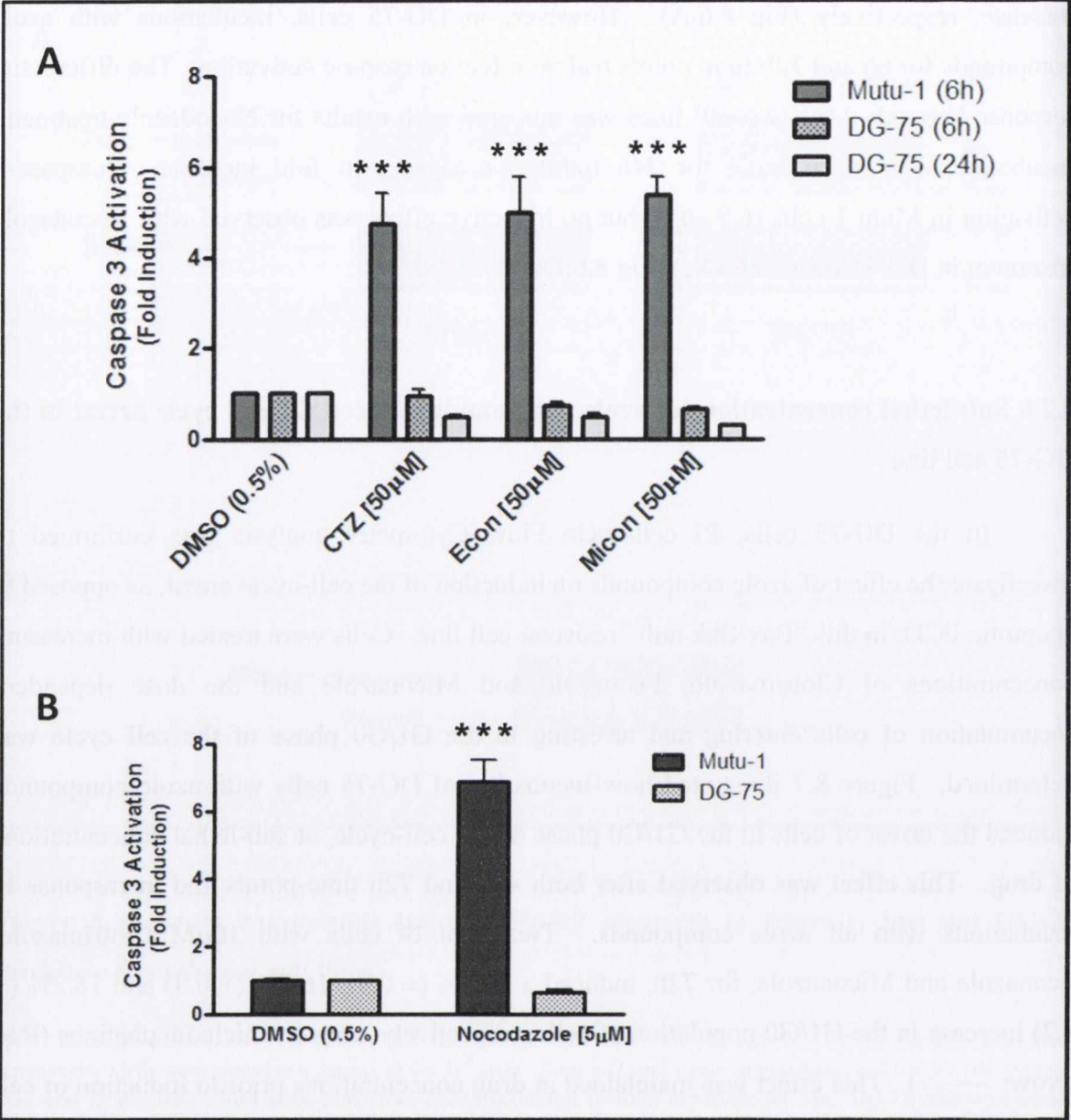


Figure 8.6: Azole compounds induce Caspase-3 activation in Mutu-1 cells, but not DG-75 Burkitt's Lymphoma cell lines

Mutu-1 and DG-75 cells treated with Vehicle (DMSO 0.5%) or azole compounds – Clotrimazole (CTZ), Econazole (Econ) and Miconazole (Micon) – at 50µM for 6h or 24h (A). Mutu-1 and DG-75 cells treated with Vehicle (DMSO 0.5%) or Nocodazole [5µM] for 24h (B). Mutu-1 and DG-75 cells were seeded at a density of 5×10^6 cells / T75 (20ml). At each experimental endpoint, cell lysates were prepared and caspase-3 activation was determined using a fluorogenic caspase-3 activation assay, as outlined in Section 2.8.3. Results represent the mean ($\pm$ SEM) of three independent experiments. *** $P < 0.001$ (Determined using Graphpad Prism 4 statistical software).

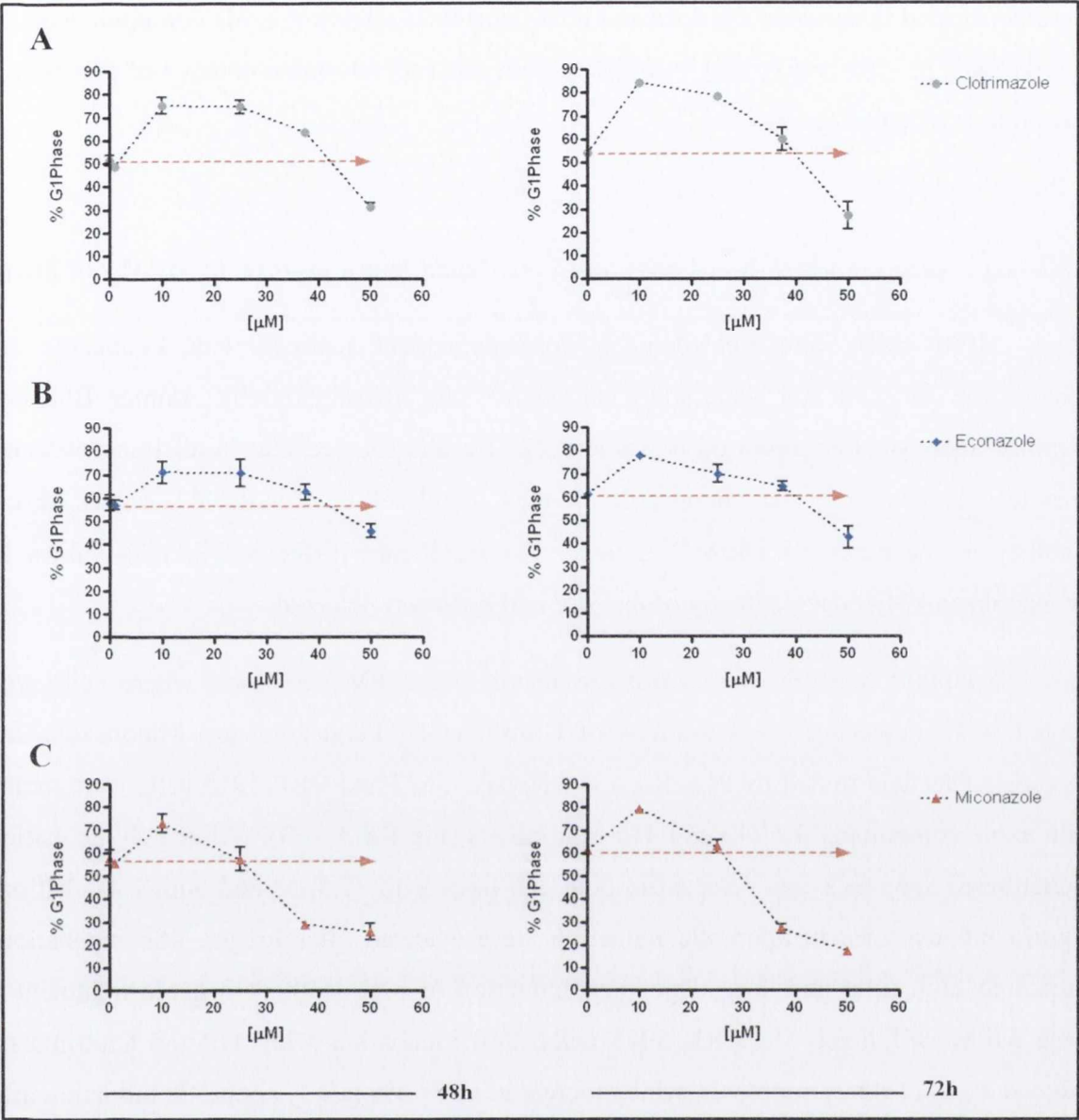


Figure 8.7: Sub-lethal concentrations of azole compounds induced G1 arrest in DG-75 cell line.

G1 cell cycle arrest was analysed in DG-75 cells treated with Clotrimazole (A), Econazole (B) and Miconazole (C) for 48h and 72h. Cells were seeded at a density of 0.75×10^6 cells per 25cm^2 tissue culture flask and left for 18h prior to treatment. Cells were treated with increasing concentrations of azole compound and cell cycle arrest was determined by PI staining and cell-cycle Flow Cytometry analysis, as outlined in Section 2.7. Results were representative of the mean ($\pm$ SEM) of three independent experiments.

were performed to examine the dose and time dependent effects of azole compounds on cell viability and apoptosis (Fig 8.8) in MPM cells in order to determine potency of this class of compounds in MPM cell lines.

8.3.1 Azole compounds induced dose and time dependent apoptosis in MPM cell lines

MPM cells were treated in a dose dependent manner with Econazole and Miconazole for 24h and 48h, and cytotoxicity was investigated by Alamar Blue cell viability assay (Results reported in Table 8.2). Results showed that in all four MPM cell lines tested, Econazole and Miconazole induced potent cell death with IC₅₀ values for cell viability in the range of 16µM - 25µM. No significant difference in IC₅₀ values for Econazole and Miconazole in any of the four cell lines was detected.

Apoptotic analysis was performed in all four MPM cell lines, where cells were treated with increasing concentrations of Clotrimazole, Econazole and Miconazole, and apoptosis was determined by PI cell-cycle analysis. Ju77 and CRL 5915 cells were treated with azole compounds for 24h and 48h time points (Fig 8.8.A – B). After 24h incubation, induction of apoptosis was detected in both cell lines with 37.5µM and 50µM incubations. Significant increases in apoptotic induction were detected after longer, 48h incubations, indicating both time and dose dependent induction of cell death with azole treatment in these MPM cell lines. In CRL 5915 cells, 24h incubation with 37.5µM Clotrimazole induced 10.3% (± 2.2) apoptosis, which increased to 77.4% (±2.3) apoptotic induction after 48h incubation (Table 8.2.A). Experimental results also revealed that both Clotrimazole and Miconazole were significantly more potent apoptotic inducers than Econazole in CRL 5915 and Ju77 cell lines. This point was illustrated by the significant difference in percentage apoptosis between Clotrimazole / Miconazole and Econazole after 48h incubation with 37.5µM drug (Table 8.2.A – B).

CRL 5820 and One 58 MPM cell lines were treated in a dose dependent manner with azole compounds for 24h, 48h and 72h time points (Fig 8.8.C – D). Resistant CRL 5820 cells were susceptible to azole treatment, inducing significant levels of apoptosis after 24h and increased with longer incubation times. No difference in potency was observed between the three azole compounds tested in CRL 5820 cells. In contrast, One 58 cells

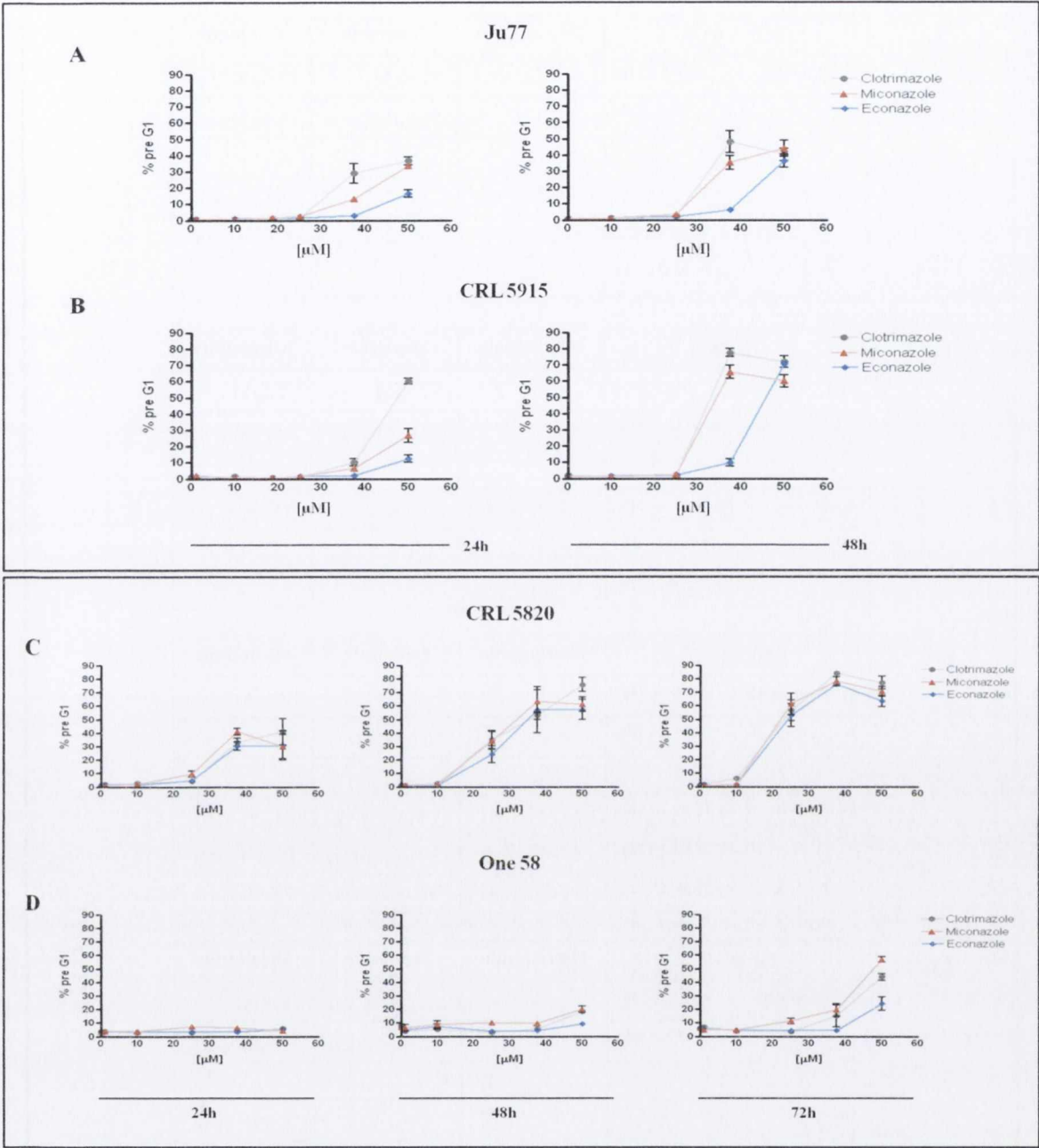


Figure 8.8: Azole compounds induced dose and time dependent apoptosis in MPM cell lines.

Dose and time dependent apoptotic induction was analysed in Ju77 (A), CRL 5915 (B), CRL 5820 (C) and One 58 (D) MPM cell lines treated with Clotrimazole (●), Econazole (◆) and Miconazole (▲). Cells were seeded at a density of 0.25×10^6 cells per 25cm^2 tissue culture flasks and left to adhere for 18h prior to treatment. Cells were treated with increasing concentrations of azole compound for 24h and 48h (72h for CRL 5820 and One 58 Fig C & D). Apoptosis was determined by PI staining and cell cycle Flow Cytometry analysis, as outlined in Section 2.7. Results are representative of the mean ($\pm$ SEM) of at least three independent experiments. Statistical analysis was performed using Graphpad Prism 4 statistical software.

A	CRL 5915	Clotrimazole	Econazole	Miconazole
	Cell Viability (EC ₅₀ – 24h)	-	21.9µM (± 0.6)	17.6µM (± 0.5)
	(EC ₅₀ – 48h)	-	22.3µM (± 0.2)	19.9µM (± 0.7)
	Apoptosis (@ 37.5µM – 24h)	10.3% (± 2.2)	2.0% (± 0.2)	6.8% (± 1.5)
	(@ 37.5µM – 48h)	77.4% (± 2.3)	9.7% (± 2.3)	65.7% (± 4.0)
B	Ju77	Clotrimazole	Econazole	Miconazole
	Cell Viability (EC ₅₀ – 24h)	-	24.7µM (± 0.4)	22.2µM (± 0.6)
	(EC ₅₀ – 48h)	-	24.9µM (± 0.3)	20.4µM (± 0.4)
	Apoptosis (@ 37.5µM – 24h)	28.9% (± 6.0)	3.2% (± 0.4)	13.3% (± 1.7)
	(@ 37.5µM – 48h)	48.0% (± 6.7)	6.1% (± 1.0)	35.3% (± 4.3)
C	CRL 5820	Clotrimazole	Econazole	Miconazole
	Cell Viability (EC ₅₀ – 24h)	-	19.8µM (± 0.6)	16.6µM (± 0.4)
	(EC ₅₀ – 48h)	-	20.6µM (± 0.8)	18.2µM (± 0.7)
	Apoptosis (@ 37.5µM – 24h)	33.3% (± 3.1)	30.6% (± 3.1)	41.1% (± 2.5)
	(@ 37.5µM – 48h)	52.8% (± 3.0)	57.1% (± 17.1)	63.3% (± 8.6)
D	One 58	Clotrimazole	Econazole	Miconazole
	Cell Viability (EC ₅₀ – 24h)	-	23.1µM (± 0.4)	18.9µM (± 0.3)
	(EC ₅₀ – 48h)	-	24.5µM (± 0.7)	21.9µM (± 0.5)
	Apoptosis (@ 37.5µM – 24h)	3.9% (± 0.5)	3.7% (± 0.4)	6.6% (± 0.4)
	(@ 37.5µM – 48h)	5.6% (± 1.4)	4.2% (± 1.0)	9.9% (± 1.8)
	(@ 37.5µM – 72h)	15.1% (± 8.1)	4.4% (± 0.5)	19.5% (± 4.6)
	↳ (@ 50µM – 72h)	43.8% (± 2.4)	24.0% (± 4.9)	56.7% (± 1.9)

Table 8.2: Cell viability and Apoptosis results for azole treatment in MPM cell lines.

Summary of cell viability (Graph not shown) and apoptosis (Fig 8.7) results for azole treatment in MPM cell lines. Cell viability assay: Cells were seeded on 96 well plates at a density of 0.5 x 10⁴ cells per well containing 200µl media and left to adhere for 18h prior to treatment. Cells were treated with Econazole and Miconazole in a dose dependent manner for 24 and 48h. Cell viability was analysed by Alamar Blue assay, as outlined in Section 2.6. Results are representative of the mean (±SEM) of three independent triplicate experiments.

were resistant to azole treatment. Significant levels of apoptosis were only observed after incubations with azole compounds at 50 μ M for a 72h time-point (Table 8.2.D).

8.3.2 Azole compounds induced PARP cleavage in all MPM cell lines

In a similar manner to experimentation performed with Burkitt's Lymphoma cell lines, specific induction of an apoptotic program of cell death in response to azole treatment was investigated in MPM. PARP cleavage was used as an indicator of apoptotic PCD and was investigated in all four MPM cell lines in response incubation with azole compounds. Cells were treated with vehicle or Clotrimazole, Econazole and Miconazole [50 μ M] for 24h and cleavage of full length PARP was examined by Western Blot analysis (Fig 8.9). Incubation with Cisplatin was used as a positive control for apoptotic induction (Eastman, 1990). In all four MPM cell lines, PARP cleavage was detected in response to incubation with all three azole compounds, indicating that these compounds specifically induce apoptotic program of cell death in MPM. Cleavage of PARP was also detected with Cisplatin incubation in all MPM cell lines. The degree of PARP cleavage detected in response to incubation with individual azole compounds correlated with previous apoptotic results reported in Figure 8.8 and Table 8.2. Clotrimazole and Miconazole incubations induced a greater degree of PARP cleavage than that of Econazole, in line with previous observations that Econazole was a less potent inducer of apoptosis in MPM and BL cell lines. This result was clearly illustrated in One 58, Ju77 and CRL 5915 Western Blot samples (Fig 8.9). In CRL 5820 cells, no difference in the level of PARP cleavage was observed between different azole compounds. This correlated with apoptotic results for CRL 5820 cells (Fig 8.8.C) in which no difference in potency of apoptotic induction was detected between all three azole compounds.

8.3.3 Sub-lethal concentrations of azole compounds induced G1 cell cycle arrest in MPM cell lines

Azole driven induction of cell cycle arrest was also investigated in MPM cell lines. In CRL 5915 cell line, arrest of cells in the G1 phase of the cell cycle was observed after incubation of cells with azole compounds for 24h and 48h. Cells were treated with various concentrations of Clotrimazole, Econazole and Miconazole and the dose dependent effects

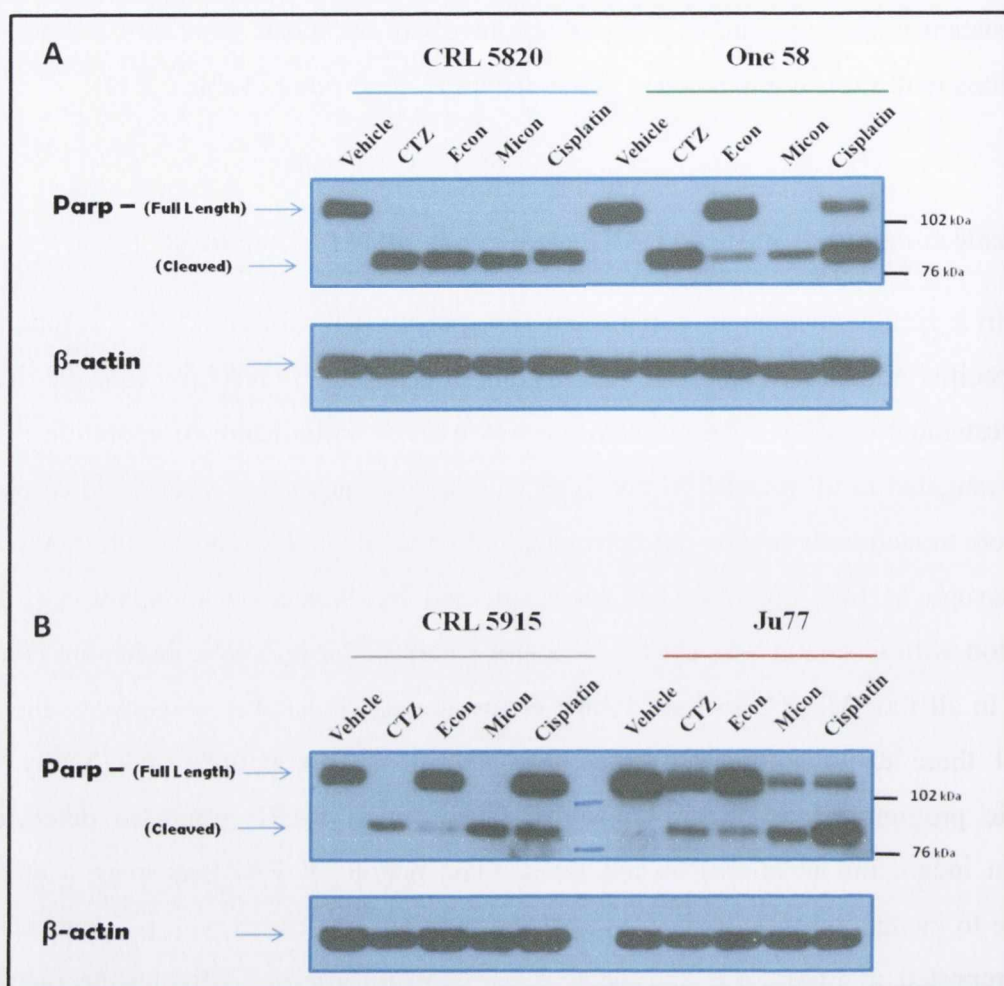


Figure 8.9: Azole compounds induced PARP cleavage in MPM cell lines.

PARP cleavage was analysed in CRL 5820 and One 58 (**A**) and CRL 5915 and Ju77 (**B**) cell lines after 24h incubations with azole compounds. Cells were seeded at a density of 1.5×10^6 cells / 25ml in 75cm² tissue culture flasks and left to adhere for 18h prior to treatment. Each cell line was treated with vehicle (0.5% DMSO), 50µM azole compound or 50µM Cisplatin for 24h. After incubation periods, cells were harvested and whole cell lysates prepared, as outlined in Section 2.5. Protein (50µg) was resolved on 8% SDS-PAGE gels and transferred to PVDF membrane. Blots were blocked in 5% Marvel TBS-Tween, and probed with anti-PARP (Primary Ab – 1:1,000). Blots were stripped and reprobed for expression of anti-β-actin (Primary Ab – 1:10,000) as an experimental loading control, as outlined in Section 2.5.6. Results are representative of three independent experiments.

on G1 and pre-G1 populations were illustrated in Figure 8.10. Results were comparable to the G1 cell cycle arrest observed with azole treatment in DG-75 BL cells (Fig 8.7). In MPM cell lines, low, sub-lethal concentrations of Clotrimazole (10 - 25 μ M), Econazole (10 - 37.5 μ M) and Miconazole (10 - 25 μ M) caused an accumulation of cells in the G1 phase of the cell cycle. Clotrimazole treatment induced an increase of as much as 22.6% (25 μ M) and 28.0% (18.75 μ M) in the G1 population after 24h and 48h incubations, respectively. Accumulation of cells in the G1 phase occurred at concentrations preceding activation of apoptosis.

8.4 Mito-HK-dissociation compounds were screened for apoptotic efficacy in BL and MPM cell lines

A novel series of computationally designed HK-dissociation compounds were developed by collaborators in the Molecular Design Group (MDG), Trinity College Dublin. Compounds were developed via a ligand-based design approach using Clotrimazole as a basis for a pharmacophore. Commercial compound databases were virtually screened onto this molecular feature map for the known HK-dissociating compound Clotrimazole and attempted to identify “Virtual-Hit” compounds, predicted to induce mitochondrial-HK dissociation. The computational-design approach identified five compounds (A1 – A5) with high scoring functions for structural and chemical similarity to the Clotrimazole pharmacophore. These five compounds (Fig 8.11) were chosen to be taken forward for *in vitro* analysis. Initial experimentation investigated the anti-proliferative activity of these five Clotrimazole pharmacophore compounds in the “Bax/Bak positive” and “Bax/Bak null” BL cell model and in the panel of four MPM cell lines

8.4.1 A4 and A5 induced dose dependent cell death in Mutu-1 and DG-75 BL cell lines

The anti-proliferative activity of Clotrimazole pharmacophore compounds A1 – A5 were initially screened at a single concentration of 50 μ M in Mutu-1 and DG-75 cells. Clotrimazole and Etoposide were used as positive controls in this assay. Cells were incubated with compounds for 24h and 48h time-points and cell viability was determined by Alamar Blue assay. In Mutu-1 cells, three Clotrimazole pharmacophore compounds showed

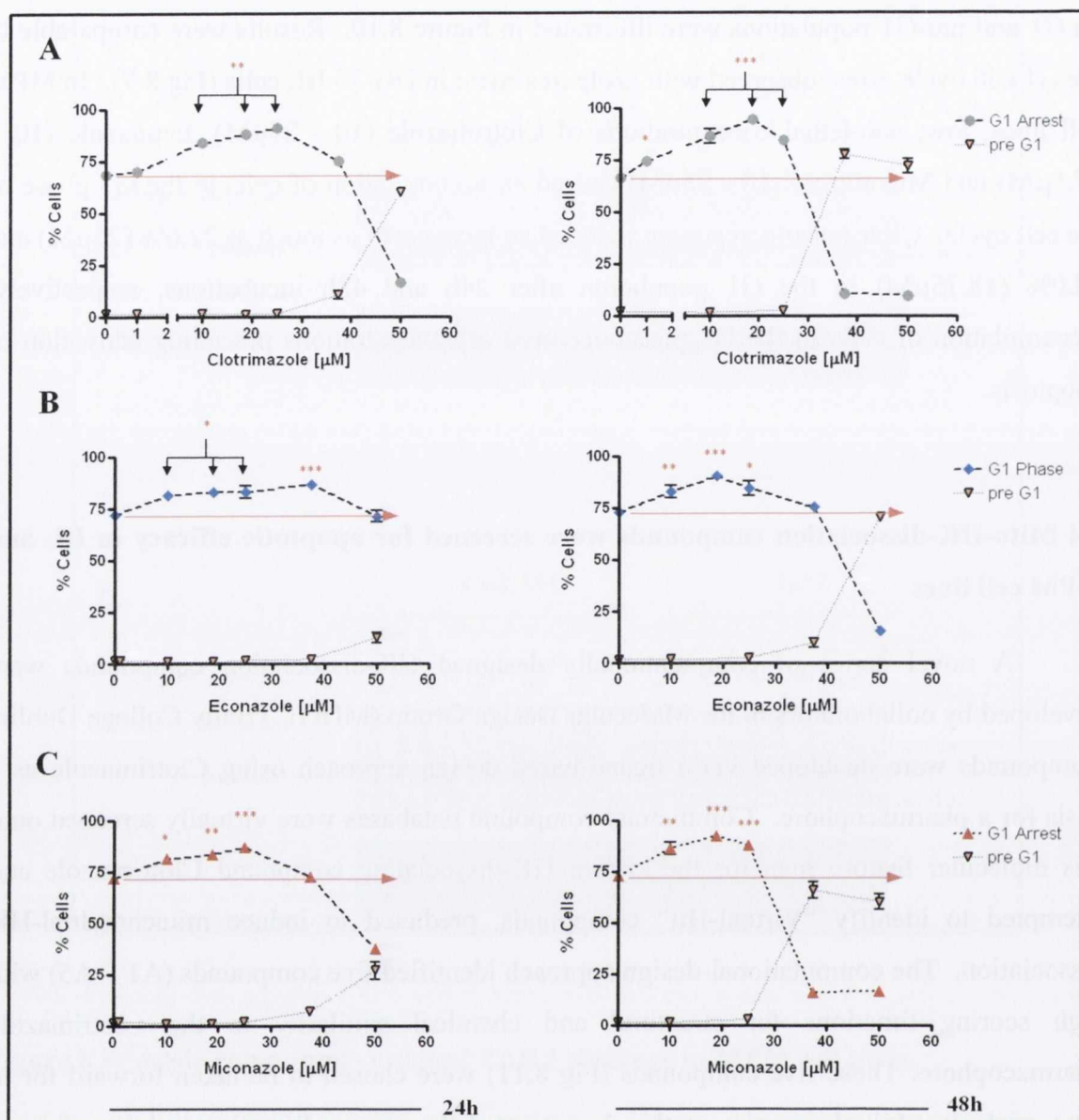
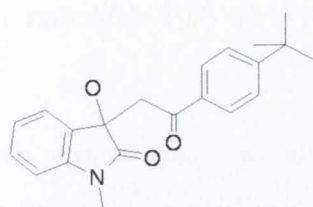


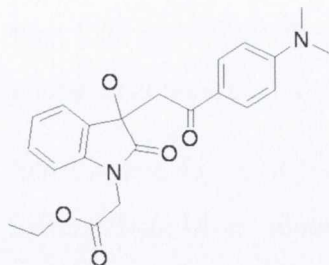
Figure 8.10: Azole compounds induced G1 arrest in MPM cell lines.

G1 cell cycle arrest was analysed in CRL 5915 cells treated with Clotrimazole (A), Econazole (B) and Miconazole (C) for 24h and 48h. Cells were seeded at a density of 0.25×10^6 cells per 25cm^2 tissue culture flasks and left to adhere for 18h prior to treatment. Cells were treated with increasing concentrations of azole compound and cell cycle arrest was determined by PI staining and cell cycle Flow Cytometry analysis, as outlined in Section 2.7. Results represent the mean ($\pm\text{SEM}$) of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Determined using Graphpad Prism 4 statistical software).



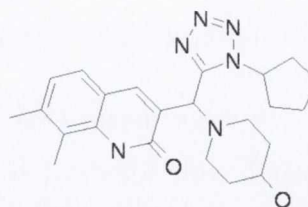
A1

(BAS 03084597)



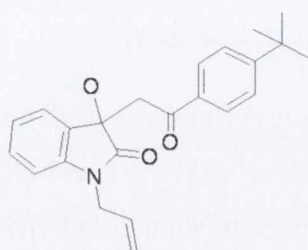
A2

(BAS 03549224)



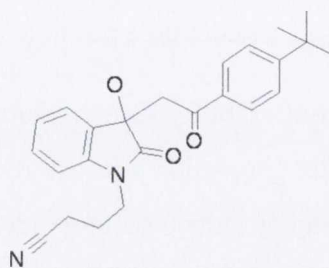
A3

(ASN 04187362)



A4

(BAS 04819119)



A5

(BAS 04819259)

Name	Chemical name	Formula	Molecular Weight
A1	3-[2-(4-tert-Butyl-phenyl)-2-oxo-ethyl]-3-hydroxy-1-methyl-1,3-dihydro-indol-2-one	C ₂₁ H ₂₃ N O ₃	337.41 g/mol
A2	{3-[2-(4-Dimethylamino-phenyl)-2-oxo-ethyl]-3-hydroxy-2-oxo-2,3-dihydro-indol-1-yl}-acetic acid ethyl ester	C ₂₂ H ₂₄ N ₂ O ₅	396.44 g/mol
A3	3-[(1-Cyclopentyl-1H-tetrazol-5-yl)-(4-hydroxy-piperidin-1-yl)-methyl]-7,8-dimethyl-1H-quinolin-2-one	C ₂₃ H ₃₀ N ₆ O ₂	422.53 g/mol
A4	1-Allyl-3-[2-(4-tert-butyl-phenyl)-2-oxo-ethyl]-3-hydroxy-1,3-dihydro-indol-2-one	C ₂₃ H ₂₅ N O ₃	363.45 g/mol
A5	4-{3-[2-(4-tert-Butyl-phenyl)-2-oxo-ethyl]-3-hydroxy-2-oxo-2,3-dihydro-indol-1-yl}-butyronitrile	C ₂₄ H ₂₆ N ₂ O ₃	390.48 g/mol

Figure 8.11: Chemical structure, name and formula of MDG computationally designed HK-Mitochondrial dissociation compounds.

significant anti-proliferative activity – A3, A4 and A5 (Fig 8.12.A – B). A4 was the most potent compound tested with only 92.0% (± 0.6) and 89.6% (± 0.2) reduction in cell viability observed after 24h and 48h incubations respectively.

In the resistant DG-75 cell line, A3 and A4 had similar anti-proliferative capacity compared with identical treatments in Mutu-1 cells. A4 was again the most potent compound tested, inducing a 96.4% (± 0.3) and 91.7% (± 0.3) reduction in cell viability after 24h and 48h incubations, respectively (Fig 8.12.C – D). Incubation with A5 [50 μ M] however induced no reduction in cell viability in DG-75 cells. In DG-75 cells, the anti-proliferative effect of A4 was much more significant than that of Clotrimazole, the basis for pharmacophore design, indicating a possible improvement in activity.

After the initial viability screen, compounds A3, A4 and A5 were taken for further testing of potency via generation of dose response curves in Mutu-1 and DG-75 cell lines (Fig 8.13). A4 treatment induced almost identical effects in Mutu-1 and DG-75 cells, with IC_{50} values of 25.9 μ M (± 0.5) and 26.5 μ M (± 0.9), respectively. Mutu-1 cells were much more susceptible to A5 induced cell death with an approximate 30 μ M difference in EC_{50} values between Mutu-1 (41.0 μ M) and DG-75 (predicted as $\sim$ 70 μ M) cell lines. A3 treatment was also more potent in Mutu-1 cells compared to DG-75 cells, but cytotoxicity did not reach a maximal response with only 53.1% cell death induced in Mutu-1 cells at the highest concentration of A3 assayed (100 μ M).

8.4.2 A4 induced potent cell death in all four MPM cell lines

The Clotrimazole pharmacophore compounds (A1 – A5) were also screened for anti-proliferative activity at 50 μ M in all four MPM cell lines (CRL 5915, CRL 5820, Ju77 and One 58). Similarly to experimentation in Burkitt's Lymphoma cell lines, A4 was the most potent compound assayed and the only Clotrimazole pharmacophore compound screened in MPM which induced significant decreases in cell viability (Fig 8.14). A4 induced up to a 90% reduction in cell viability in all four MPM cell lines after 24h and 48h time-points.

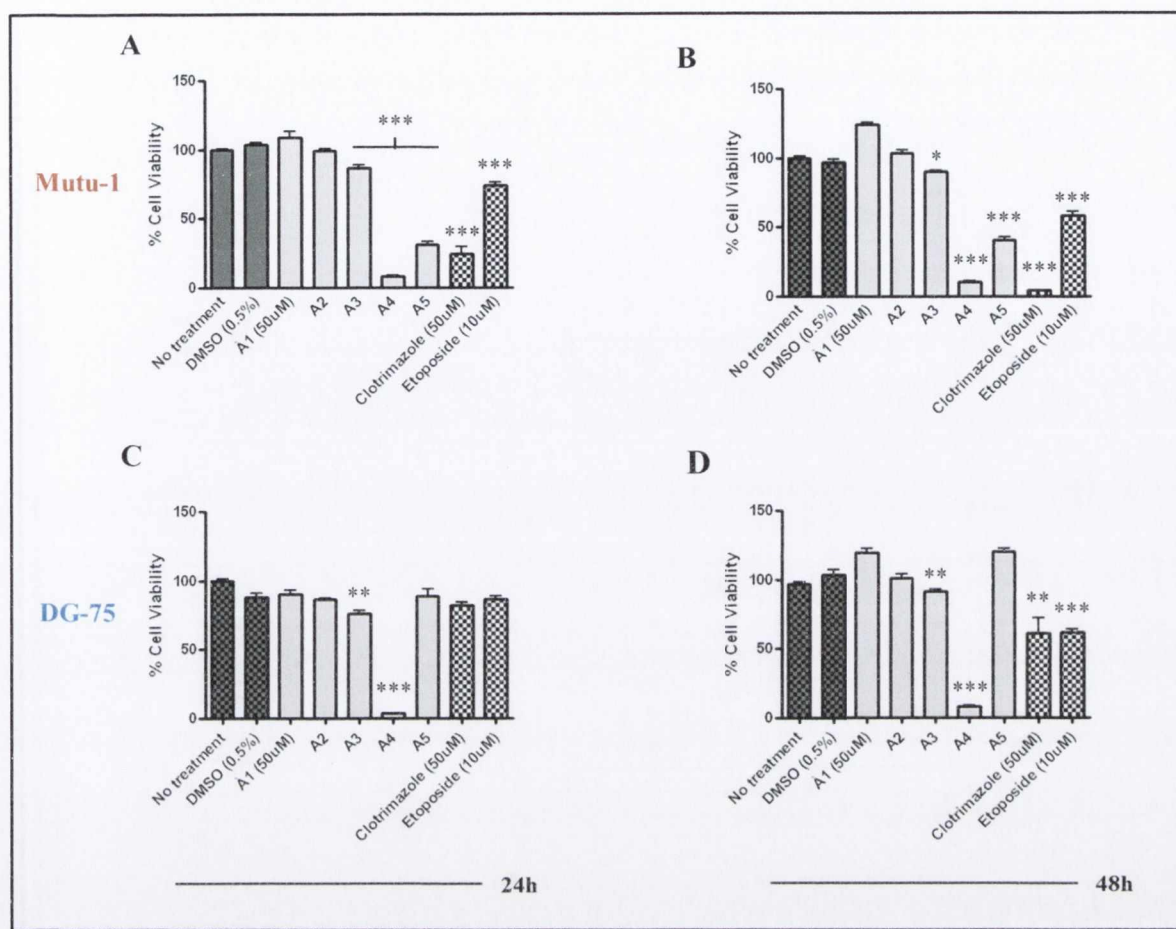


Figure 8.12: Select MDG Clotrimazole pharmacophore compounds induce cytotoxicity and apoptosis in Burkitt's Lymphoma cell lines.

Cell viability analysis of Mutu-1 and DG-75 cells treated with Clotrimazole pharmacophore compounds (A1 – A5) for 24h and 48h. (A – D) Cells were seeded on 96 well plates at a density of 1.5×10^5 cells per well (Mutu-1) and 0.5×10^5 cells per well (DG-75) containing 200 μ l media. Cells were treated with 50 μ M test compound (A1 – A5) for 24h and 48h. Cell viability was analysed by Alamar Blue assay, as outlined in Section 2.6. Results were representative of the mean ($\pm$ SEM) of two triplicate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Determined using Graphpad Prism 4 statistical software).

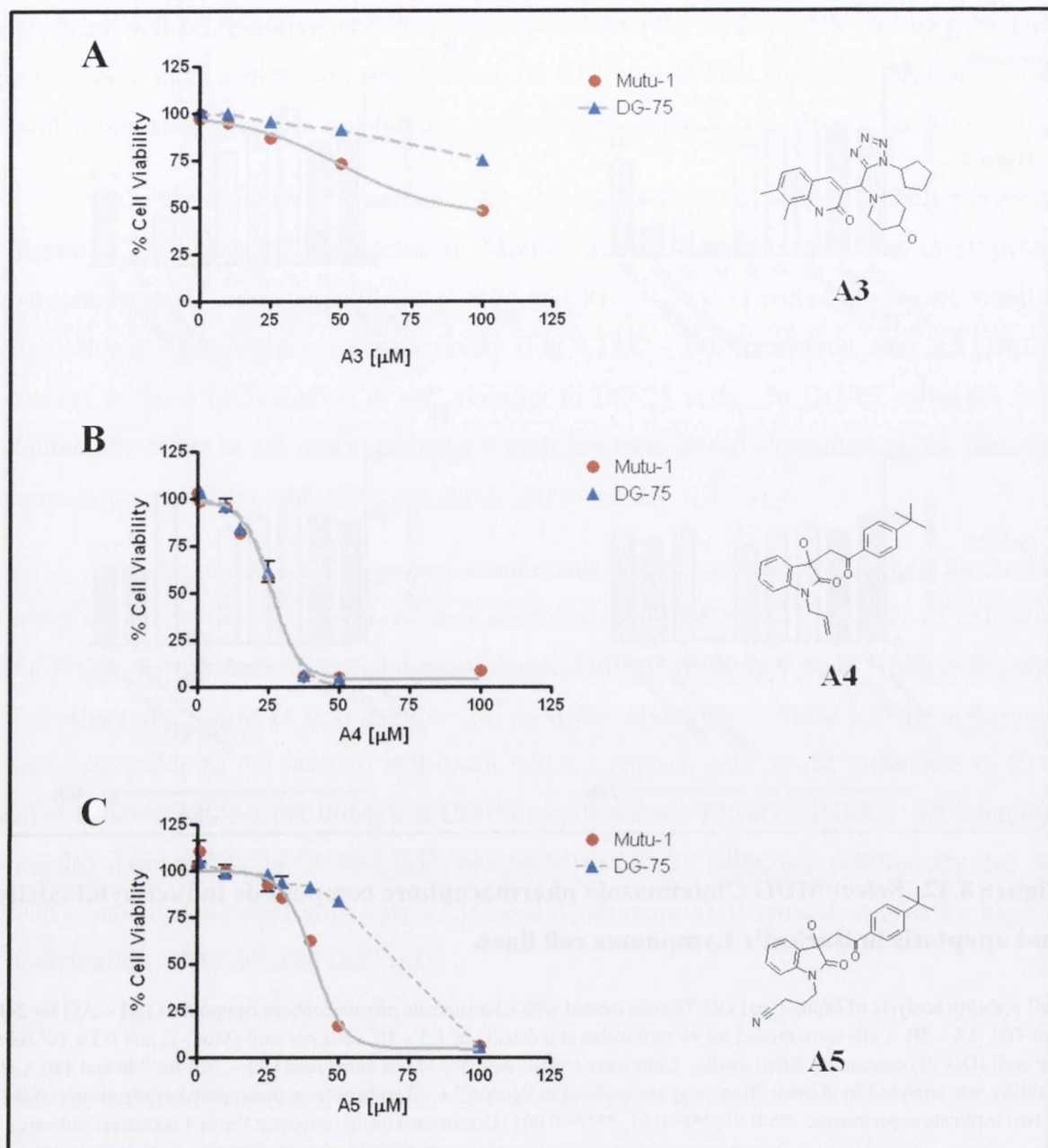


Figure 8.13: Compounds A3, A4 and A5 induced dose dependent cell death in Mutu-1 and DG-75 cell lines.

Cell viability analysis of Clotrimazole pharmacophore compounds – A3 (A), A4 (B) and A5 (C) – in Mutu-1 (●) and DG-75 (▲) cell lines. Cells were seeded on 96 well plates at a density of 1.5×10^5 cells per well (Mutu-1) and 0.5×10^5 cells per well (DG-75) containing 200 μl media. Cells were treated with increasing concentrations of compound for 24h and analysed for cell viability by Alamar Blue assay, as outlined in Section 2.6. Results were representative of the mean ($\pm$ SEM) of two independent triplicate experiments. Non-linear regression curve fit analysis was performed using Graphpad Prism 4 statistical software.

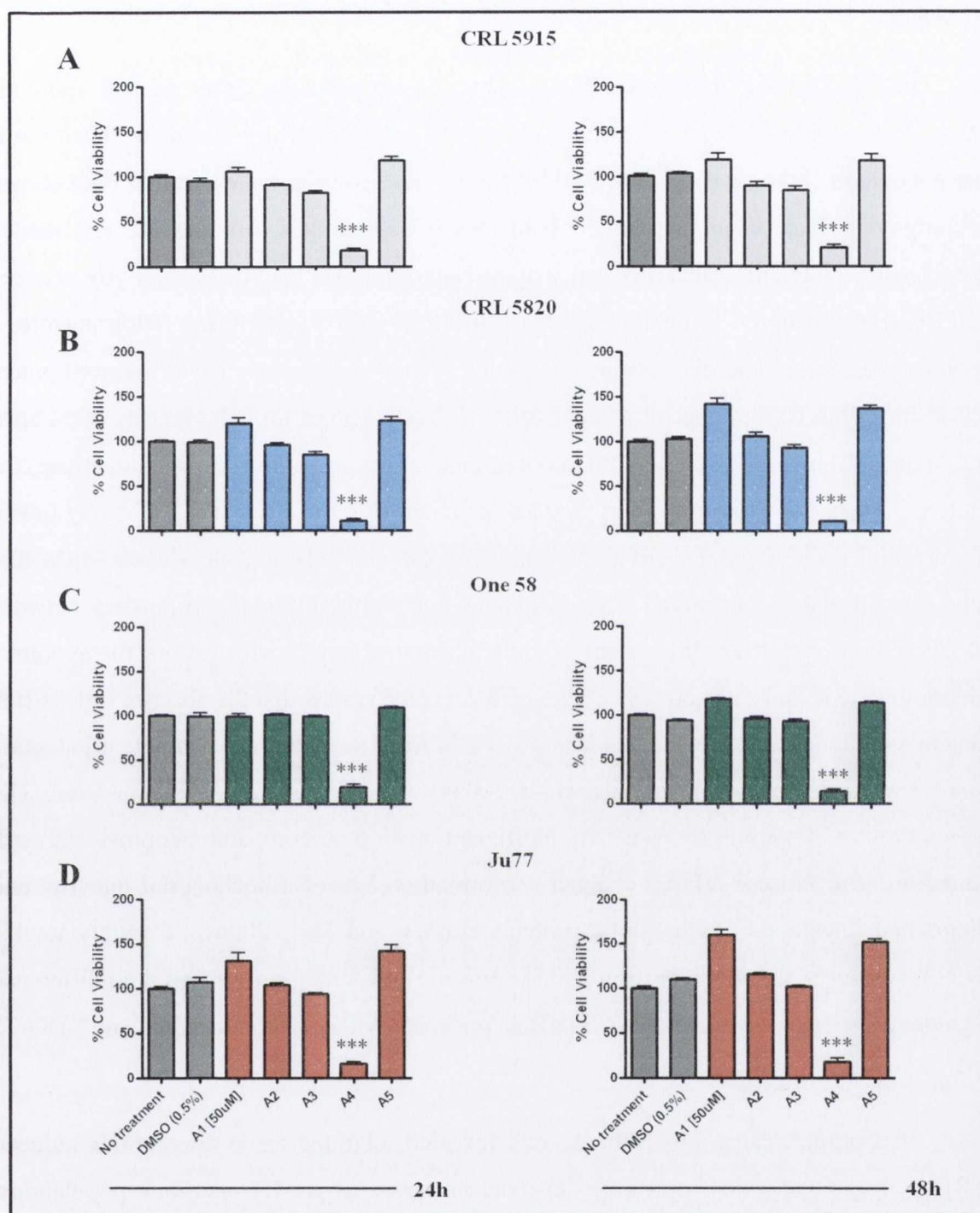


Figure 8.14: Cell viability screen of MDG Clotrimazole pharmacophore compounds in MPM cell lines.

Cell viability analysis of CRL 5915 (A), CRL 5820 (B), One 58 (C) and Ju77 (D) cells treated with Clotrimazole pharmacophore compounds (A1 – A5) for 24h and 48h. Cells were seeded on 96 well plates at a density of 0.5×10^4 cells per well containing 200μl media and left to adhere for 18h prior to treatment. Cells were treated with 50μM test compound (A1 – A5) for 24h and 48h. Cell viability was analysed by Alamar Blue assay, as outlined in Section 2.6. Results are representative of the mean ($\pm$ SEM) of two independent triplicate experiments. ***P<0.001 (Determined using Graphpad Prism 4 statistical software).

Discussion

In this Chapter, initial experiments have investigated the cytotoxic and apoptotic potential of anti-fungal azole compounds and novel, computationally-designed Clotrimazole pharmacophore compounds in BL and MPM cell lines. Results indicated that both classes of compounds had potent activity in both cancer cell models. In the BL test model, Clotrimazole treatment results revealed a significant difference in cytotoxic activity between the “Bax/Bak positive” Mutu-1 and “Bax/Bak null” DG-75 cell lines. Clotrimazole, a previously identified HK-dissociating compound (Penso and Beitner, 1998), induced potent cell death in Bax/Bak competent Mutu-1 cells, with IC_{50} values for cell viability of $24.5\mu M$ (± 2.1) and $21.8\mu M$ (± 2.0), after 24h and 48h incubations respectively. In comparison, the cytotoxic activity of Clotrimazole in Bax/Bak deficient DG-75 cells was significantly lower, with no significant cytotoxic activity detected after 24h and only an approximate $48\mu M$ IC_{50} value for cell viability observed after 48h incubation. This difference in potency between “Bax/Bak null” and “Bax/Bak positive” cell lines corresponds with results for apoptotic induction, PARP cleavage and was in line with expected results that the absence of Bax/Bak antagonism elicits less efficient dissociation of HK from the Mito and less potent induction of cell death and apoptosis (Majewski et al., 2004a). In cells deficient of Bax and Bak, HK dissociation is proposed to result in inefficient cyto c release and apoptosis through generation of a “closed” VDAC channel conformation, loss of mitochondrial integrity and unregulated release of pro-apoptotic proteins (Robey and Hay, 2006). Contrary to this model mechanism, cytotoxicity results for Econazole and Miconazole showed no difference in potency between treatments in “Bax/Bak positive” Mutu-1 or “Bax/Bak null” DG-75 cells.

Apoptotic analysis in Mutu-1 cells revealed all three azole compounds induced PARP cleavage and a dose and time dependent increase in pre-G1 apoptotic populations, with EC_{50} values at low micromolar concentrations and 4 – 10h incubations (Table 8.1). Treatment of Mutu-1 cells with all three azole compounds also induced significant fold increases in caspase-3 activation. These results strongly suggest induction of a caspase-dependent, apoptotic form of PCD in the Mutu-1 cell line (Konopleva et al., 1999).

In DG-75 cells, increases in pre-G1 populations were observed in response to all azole treatments, but induction occurred with much slower kinetics compared with responses observed in Mutu-1 cells. Significant apoptotic induction at $50\mu M$ was not

observed until after a 72h incubation period, compared with low micromolar EC₅₀ values in Mutu-1 cells after only 24h incubations – 13.2μM, 18.8μM and 17.1μM EC₅₀ values for Clotrimazole, Econazole and Miconazole incubation in Mutu-1 cells, respectively. While DG-75 cells were resistant to apoptotic induction, treatment with azole compounds did affect cellular homeostasis causing significant accumulation of cells in the G1 phase of the cell cycle. At sub-lethal concentrations of azole compounds, cells arrested in the G1 phase as opposed to going through apoptosis.

Investigations into the mechanism of PCD induced in DG-75 cells in response to azole compounds and specifically the dependence on caspases for the execution of an apoptotic program of cell death in this cell line, revealed alternate results to that observed with experimentation in Mutu-1 cells. The involvement of caspases was shown to be absent in the execution of cell death in DG-75 cells. No induction of Parp cleavage or activation of caspase-3 was detected with treatment with all three azole compounds in DG-75 cells, even with significantly longer incubation times assayed compared to that in Mutu-1 cells. Incubation with the positive control Nocodazole, a known inducer of apoptotic cell death (Beswick et al., 2006), induced PARP cleavage and caspase-3 activation in Mutu-1 cells. However, even with the use of a positive apoptotic control in DG-75 cells, no caspase dependent activity was detected.

Previous studies have reported on the necessity of Bax and Bak for mediating induction of apoptotic cell death in response to intrinsic apoptotic pathway stimulus and deficiency in Bax and Bak expression has been associated with a defective apoptotic phenotype in cancer (Konopleva et al., 1999). Specifically in “Bax / Bak null” DG-75 cells, chemotherapeutic resistance in this cell line has been attributed to the absent expression of Bax and Bak (Gutierrez et al., 1999). This epigenetic alteration has been reported to inhibit induction of mitochondrial outer membrane permeabilisation, caspase activation and downstream execution of apoptosis in response to chemotherapeutic treatment (Cloonan and Williams, 2010). Without Bax and Bak expression, DG-75 cells are unable to successfully engage an apoptotic response, making them highly resistant to chemotherapy. Chemotherapeutic challenge in DG-75 cells has previously been reported to induce delayed kinetics in the detection of cell death, with ultimate engagement of a type II, autophagic form of cell death as opposed to apoptosis (Cloonan and Williams, 2010). Similar delayed kinetics in the induction of cell death, coupled with the absence of caspase dependence, indicated by the lack of PARP cleavage and caspase activation, strongly indicate that azole

compounds were also unable to induce apoptosis in DG-75 cells, but rather cell death occurred through an alternate, autophagic form of PCD. Despite the impaired apoptotic network and resistance to cell death in DG-75 cells, Clotrimazole, Econazole and Miconazole were able to induce cell death through a Bax/Bak independent mechanism, indicating the potential of HK-dissociating drugs as a treatment strategy even in resistant cancer cell types.

Experiments in MPM cell lines also revealed that azole compounds had potent cytotoxic and apoptotic effects in these cell lines. Cell viability dose response curves for Econazole and Miconazole produced IC_{50} values of approximately 15 - 25 μ M in MPM cell lines. Apoptotic analysis revealed induction of PARP cleavage and dose and time dependent increases in pre-G1 apoptotic populations of cells in all MPM lines, with the most potent effects observed in Ju77, CRL 5915 and CRL 5820 cell lines. One 58 cells were much more resistant to treatment with the most potent drug effects only approaching 50% apoptosis with 50 μ M incubation for 72h (Clotrimazole – 43.8% ($\pm$ 2.4); Econazole – 24.0% ($\pm$ 4.9) and Miconazole – 56.7% ($\pm$ 1.9)). Sub-lethal concentrations of azole compound also induced G1 arrest of cells prior to induction of apoptosis, a similar response to treatment observed in DG-75 BL cells. The ability of Clotrimazole to inhibit cell cycle progression and specifically induce arrest in the G0/G1 phase of the cell cycle has previously been reported in other cancer models including non-small cell lung carcinoma (NSCLC) and colon adenocarcinoma (Al-Qawasmeh et al., 2004). Arrest of cells in the G0/G1 phase of the cell cycle preceded apoptotic induction in glioblastoma cells via forced dissociation of Mito-HK (Liu et al., 2010). These results indicate that Clotrimazole and the proposed HK-dissociating azole compounds were able to potently induce apoptosis in resistant MPM cells and mitochondrial-localised HK may represent a viable anti-cancer target in MPM.

Cellular results in BL and MPM cells indicated a significant difference in potency between the two azole analogues, Econazole and Miconazole. Miconazole was shown to be the more potent compound with lower EC_{50} values for dose and time dependent apoptosis in BL and certain MPM cell lines (CRL 5915, Ju77 and One 58). The results reported in Table 8.1 and Table 8.2 illustrated the dose and time dependent difference in potency between Econazole and Miconazole incubations in BL and MPM, respectively. The reason for such a difference in potency between azole analogues with a single alteration in the presence of a mono-chlorobenzene moiety on Econazole, as opposed to a di-chlorobenzene moiety

present on Miconazole was unknown, but may represent an essential structural alteration for increased forced dissociation of HK from the mitochondria.

Before such assertions could be made with regard to the apoptotic activity of azole compounds being mediated specifically through dissociation of Mito-HK, “On-Target” validation for this effect in BL and MPM cells would be required. Clotrimazole, Econazole and Miconazole have all been reported to possess diverse functions in cells, including activity as antifungal agents, targeted inhibitors in mammalian cells and potential chemotherapeutic agents. The activity of antifungal azoles is attributed to the inhibition of the fungal cytochrome P450 monooxygenase, CYP51 (lanosterol-14 α -demethylase), disrupting steroid biosynthesis and fungal cell membrane integrity (Trosken et al., 2006). Imidazole compounds have also been reported as inhibitors of the mammalian cytochrome P450 enzyme, aromatase (CYP19), which is involved in estradiol steroidogenesis (Trosken et al., 2004). Aromatase and its inhibition are of clinical significance in cancer, with the suppression of estradiol biosynthesis by azole compounds an effective treatment strategy in hormone sensitive breast cancer. The azole derivative Letrozole is a current clinical treatment option in breast cancer (Janni and Hepp, 2010).

Clotrimazole has been reported to possess a wide array of cellular targets in mammalian cells, besides cytochrome P450 and dissociation of Mito-HK in cancer. Clotrimazole has recently been reported to regulate an alternate member of the glycolytic pathway, 6-phosphofructo-1-kinase (PFK), displacing it from the cytoskeleton (Zancan et al., 2007), disrupting glycolytic flux and cell viability in breast cancer cells (Meira et al., 2005). Clotrimazole has also shown activity as a calmodulin antagonist (Hegemann et al., 1993), the ability to affect intracellular Ca²⁺ homeostasis via targeting of the sarcoplasmic reticulum Ca²⁺ ATPase and voltage dependent Ca²⁺ channels (Snajdrova et al., 1998, Wu et al., 1999), and even regulate zinc (Zn²⁺) and cadmium (Cd²⁺) membrane permeability in rat thymocytes (Matsui et al., 2008, Oyama et al., 2007). A number of triphenylmethyl scaffold containing compounds, a motif present in Clotrimazole, have also been associated with mediating anti-cancer activity through diverse mechanisms, including cell cycle arrest and inhibition of microtubule polymerisation, on top of Mito-HK dissociation and calcium channel regulation observed with Clotrimazole (Palchaudhuri et al., 2008). While Clotrimazole does possess numerous targets and provoke diverse activity in mammalian cells, it is widely reported its anti-cancer activity is mediated through Mito-HK dissociation (Liu et al., 2010, Penso and Beitner, 1998).

Econazole and Miconazole also have diverse cellular functions reported, including calmodulin antagonism (Hegemann et al., 1993) and affecting Ca^{2+} homeostasis (Zhang et al., 2006). Both azole compounds are reported inhibitors of Ca^{2+} channels, TRPV5/6 (Landowski et al., 2011), and Econazole has been shown to deplete endoplasmic reticulum (ER) calcium stores and block Ca^{2+} influx in mammalian cells (Jan et al., 1999). Disrupting ER Ca^{2+} homeostasis adversely affects protein synthesis, resulting in accumulation of misfolded proteins and initiation of apoptosis through ER stress / the unfolded protein response (UPR) (Soboloff and Berger, 2002). The cytostatic and anti-proliferative activity of Econazole and Miconazole via affecting ER stress, mitochondrial ROS generation / ER stress (Zhang et al., 2006) and cell cycle arrest have been reported in numerous studies, including leukemic and colon cancer models (Ho et al., 2005, Wu et al., 2002).

As outlined above, azole compounds are promiscuous binders in mammalian cells, eliciting diverse mechanisms of anti-cancer activity. Confirming that the apoptotic activity of Clotrimazole in BL and MPM was mediated specifically through Mito-HK dissociation and identifying this novel activity in Econazole and Miconazole was of vital importance. In this study, a number of “On Target” experimental approaches were taken to qualify and quantify the anti-apoptotic activity of azole compounds as being mediated through Mito-HK dissociation. Initial experiments employed Western Blot detection of Hexokinase-II (HK-II) in cytoplasmic and mitochondrial fractions, isolated by differential centrifugation (Method outlined in Section 2.20). The loss of HK-II in the mitochondrial fraction and subsequent increase in the cytoplasmic fraction in response to incubation with azole compounds in cells was assayed. A Spectrophotometric enzyme assay was used in an attempt to quantify the reduction in Hexokinase activity in the mitochondrial fraction in response to incubation with azole compounds (Method outlined in Section 2.19.3). Azole incubations were performed on isolated mitochondria and a coupled enzyme reaction to that of Hexokinase was used to detect activity spectrophotometrically (Glucose-6-phosphate Dehydrogenase). Glucose-6-phosphate (G-6-P), a negative feedback inhibitor of Hexokinase and inducer of Mito-HK dissociation, was used as a positive control. Extensive work was performed to optimise both Western Blot and spectrophotometric “On Target” assays, however, detection of induced Mito-HK dissociation using these methods was unsuccessful. Dissociation was not observed in response to treatment with Econazole, Miconazole or the known inducers of Mito-HK dissociation, Clotrimazole and G-6-P. As

such, “On Target” validation of azole compounds mediating apoptosis through Mito-HK dissociation could not be confirmed to date.

Screening of novel Computationally-designed Clotrimazole pharmacophore compounds in BL and MPM cells revealed a number of compounds with potent cytotoxic effects in these cell lines. In a 50 μ M screen of these novel drugs, test compound A4 was revealed to be the most active, inducing approximately a 90% reduction in cell viability in all BL and MPM cell lines tested. Cell viability dose response analysis was performed on A4 and test compounds A3 and A5, which had significant, yet less potent single point cytotoxic effects in BL cells. Analysis revealed A4 to be the most potent compound with similar IC₅₀ values for cell viability in Mutu-1 (25.9 μ M) and DG-75 (26.5 μ M) cell lines. Dose response analysis for A3 and A5 showed differential responses for cytotoxicity in “Bax/Bak positive” Mutu-1 and “Bax/Bak null” DG-75 cell lines. A3 and A5 were significantly more potent in the Mutu-1 cell line, a result more in line with expected effects for HK-dissociating compounds in this test model.

In this Chapter we report on the potent apoptotic effects of known and potentially novel HK-dissociating compounds. Clotrimazole, its imidazole derivatives Econazole and Miconazole, and the novel series of computationally-designed HK-dissociating compounds were shown to be able to induce apoptosis in “Bax/Bak positive” BL cells and induce cell death in “Bax/Bak null” BL cells, the latter suspected to occur through an alternate, Type II autophagic, form of PCD. Azole compounds and select computationally-designed compounds were also able to induce cell death in a panel of MPM cell lines. Further cellular and “on target” analysis of these compounds is required in order to validate the mechanism of action of Econazole, Miconazole and the novel computationally designed compounds, however results with the known HK dissociating compound Clotrimazole suggests mitochondrial-localised HK may be a viable target for rational drug design and the development of treatments for MPM.

Concluding Remarks

&

Future Perspectives

Concluding remarks and future perspectives

At the conclusion of this study, a diverse set of molecular targets and mechanisms of action had been investigated in the development of therapies for Malignant Pleural Mesothelioma. Initially, two separate rational computational design programs had been undertaken, targeting the conserved anti-cancer targets of Inhibitor of apoptosis family proteins (IAPs) and aerobic glycolysis / mitochondrial-associated Hexokinase. Initial work validated MPM as a model for SMAC mimetic design showing IAP overexpression mediated apoptotic resistance, which could be alleviated by treatment with SMAC mimetic therapy. Computational design of novel small-molecule SMAC mimetics was undertaken, but after biochemical evaluation it was revealed that the approach was unsuccessful in identifying compounds with affinity for IAPs. While lead structures (MDG 44) with alternate proposed mechanisms of action were identified from this class of compounds and taken forward for further investigation, SMAC mimetic therapy still represented a potential effective treatment strategy in MPM. IAP overexpression has been clinically associated with poor prognosis and chemotherapeutic resistance in MPM (Gordon et al., 2002, Gordon et al., 2007, Kleinberg et al., 2007). Results in this study had confirmed this association in *in vitro* MPM models and shown the capacity of IAP inhibition to alleviate apoptotic resistance and synergistically enhance the apoptotic activity of conventional chemotherapy (Cisplatin) in MPM. Future work is proposed to revisit this target in MPM and attempt to develop novel and effective SMAC mimetic therapies.

Comprehensive apoptotic and mechanistic analysis on lead compound MDG 44 was performed in an attempt to characterise the nature of its cellular activates. Experimentation revealed MDG 44 specifically induced a caspase-dependant apoptotic program of cell death in MPM cells. Its activity was characterised by both single-agent and synergistic apoptotic activity. At sub-lethal single-agent concentrations, MDG 44 was able to synergistically enhance cisplatin induced apoptosis in the CRL 5915 MPM cell line. This synergistic apoptotic activity was confirmed through PI cell cycle analysis, PARP cleavage, Caspase activation and Annexin V / Pi staining assays. This result was of particular interest for the potential clinical development of this compound, as Cisplatin combinational therapies is still the standard of care for MPM patients (Belli et al., 2009). Synergism with Cisplatin represents a promising clinical rational for MDG 44. For this reason MDG 44 was taken forward for further *in vitro* analysis. Key requirements for further drug development were both target identification and development of more potent analogues of MDG 44.

Target analysis for MDG 44 ruled out a number of putative targets, but was successful in identifying a promising target for future analysis. Targets were considered for investigation from a patent database search, serendipitous discoveries of structural commonalities between MDG 44 and another experimental compound of known target and consideration of the cellular effects induced by MDG 44 in MPM. The target identified showing most promise was that involving autophagy and the ubiquitin-proteasome system, specifically the 26S proteasome. The reported cellular activities of proteasome inhibitors and the synergistic activity in combination with platinum-based therapies suggested the proteasome as a rational molecular target for MDG 44. The mechanistic link between proteasome inhibition and autophagy induction also fit observations with MDG 44, i.e. the formation of large cytoplasmic vesicles in MPM. Autophagy experiments were performed and identified that MDG 44 induced increased expression of autophagosome marker LC3-II in CRL 5915 cells and induced the formation of LC3 positive structures in LC3-GFP transfected macrophages. However, autophagic flux experiments indicated no increased lysosomal turnover of autophagosome cargo. This possibly suggested induction of defective autophagic flux. Continuing experiments are proposed to further investigate the induction of autophagosomal formation in response MDG 44. Experiments are underway to investigate the formation of autophagosomes directly in MPM cells using LC3 immunofluorescence and confocal microscopy. While LC3 positive structures have already been identified in a stably transfected LC3-GFP macrophage line, confocal detection of LC3 positive structures (autophagosomes) specifically in an MPM cell model is desired. Transmission electron microscopy (TEM) is the golden standard for detection of autophagosomes (visualisation of double-membraned vesicles). It is proposed to use this technique to confirm the formation of autophagosomes (Nadal and Gold, 2012).

Analysis of proteasome targeting revealed MDG 44 induced dose-dependent inhibition of both NF κ B activation and I κ B degradation. However, MDG 44 treatment failed to induce accumulation of poly-ubiquitinated in MPM. This result suggested involvement of the UPS pathway, but not direct inhibition of the proteasome. Inhibition of the ubiquitination of I κ B would inhibit its targeting to the proteasome for degradation and prevent NF κ B activation. This mechanism of action would also not affect direct proteasomal activity or induce accumulation of poly-ubiquitinated proteins. It was proposed that elements of the ubiquitination pathway may be molecular targets for MDG 44 and future experimentation is proposed to investigate this target. Of particular interest are the

E3 Ubiquitin ligases SCF^{βTrCP} (Skp1–cullin-1–F-box protein) and MDM2 (murine double minute). SCF^{βTrCP} is the F-box E3-ubiquitin ligase enzyme which regulates NFκB activation via ubiquitination of IκB. The βTrCP component of the complex can recognise the double phosphorylated motif of IκB and Lys48 ubiquitin tag it for proteasomal degradation (Kroll et al., 1999). This complex is of interest because inhibition of IκB proteasomal degradation was directly assayed in this study.

The second target is that of oncogene MDM2, a RING E3 Ubiquitin ligase which is responsible for regulating proteasomal degradation of the tumour suppressor gene, p53 (Guedat and Colland, 2007). p53 is a transcription factor activated in response cellular stress mechanisms, in particular detection of DNA damage. Activation of p53 results in the induced expression of numerous target genes involved in induction of cell cycle arrest and apoptosis, including p21 (Bunz et al., 1998) and Bax (Zhang et al., 2000). In normal cells, cellular protein levels of p53 are regulated at the proteasomal level via ubiquitin tagging by MDM2. In certain malignant tumours, MDM2 expression has been reported to be amplified, resulting in excessive ubiquitination and proteasomal degradation of p53 (Toledo and Wahl, 2006). Diminished protein expression of the tumour suppressor gene facilitates a resistant phenotype in MDM2 overexpressed cancers. For this reason the MDM2-p53 interaction is an attractive target for drug development. A number of diverse classes of compounds have been reported to be able to inhibit this interaction, including Nutlins (Vassilev et al., 2004) and benzodiazepinedine derivatives (Grasberger et al., 2005). A class of compound of particular interest for this study are isoindolinone scaffold compounds reported in numerous recent studies by *Hardcastle et al* (Hardcastle et al., 2005, Hardcastle et al., 2006, Hardcastle et al., 2011, Watson et al., 2011). These compounds have been shown to directly inhibit the interaction between MDM2 and p53 and this class of compound has been optimised over numerous generations of analogues. The basic scaffold for these compounds (isoindolinone) is very similar in structure to the 2-indolinone basic scaffold of MDG 44.

These observations make the MDM2-p53 interaction a very interesting target for investigation with MDG 44. Results to date suggest ubiquitination enzymes are a plausible target for MDG 44. The target interaction itself, preventing p53 suppression, provides a strong rationale for the mechanism of synergism with Cisplatin observed in MPM cells. The structural association between the isoindolinone inhibitors of MDM2 and the 2-indolinone scaffold of MDG 44 also suggests this target is a reasonable target to investigate. It is

proposed to perform *in vitro* ubiquitination experiments using the ubiquitin ligases of interest. The ability of MDG 44 to inhibit the ubiquitination of target proteins will be assessed to validate “On Target” activity.

Analogue generation and screening identified a number of compounds with increased anti-proliferative activity to that of MDG 44. The most potent analogues identified were CDX 91 and CDX 92, possessing IC₅₀ values for cell viability of 4.2µM (± 1.3) and 5.4µM (± 0.2), respectively. CDX 92 has been chosen for investigation as a single-agent therapeutic in an MPM mouse model. The mouse model will be performed in collaboration with Prof. Fallon of the Institute of Molecular Medicine, St. James’ Hospital, Dublin. A xenograph model has been designed using a Ren MPM cell line stably transfected with a luciferase reporter gene. Analysis and quantification of tumour size and growth will be performed using live *in vivo* imaging techniques.

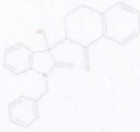
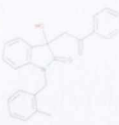
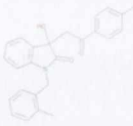
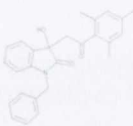
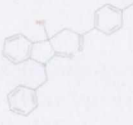
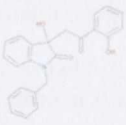
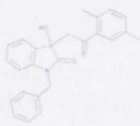
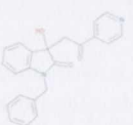
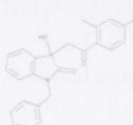
Experimentation in Chapter 8 proposed to investigate the activity of potentially novel Mito-HK dissociating compounds. The apoptotic and anti-proliferative activities of these compounds was reported on, however, full optimisation of an “On Target” assay was unsuccessful. Extensive lengths were taken in attempts to develop an effective assay to detect forced dissociation of Mito-HK. Compound treatments in both live cells and isolated mitochondria were performed. Western Blot analysis and Spectrophotometric enzyme assays were used to assess Mito-HK dissociation, however optimisation in both cases was unsuccessful. Future experimentation on this project requires “On target” validation and, as such, further attempts will be made to optimise an “On Target” assay.

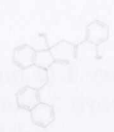
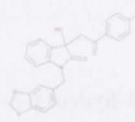
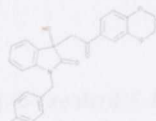
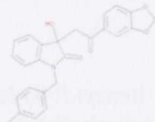
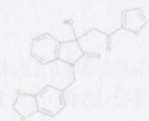
Appendix – 1

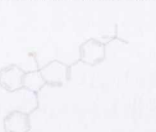
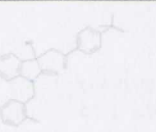
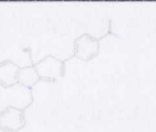
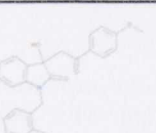
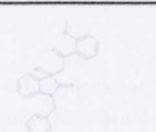
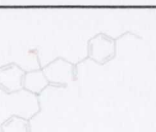
MDG 44 analogues:

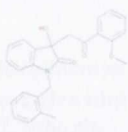
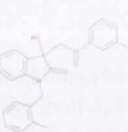
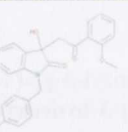
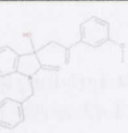
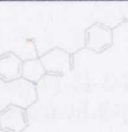
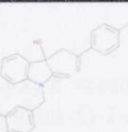
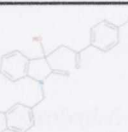
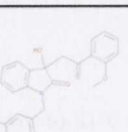
Chemical Name and Structure

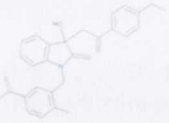
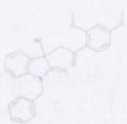
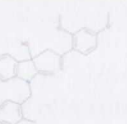
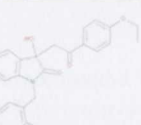
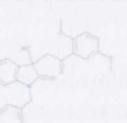
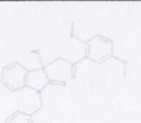
CDX 1 - 92

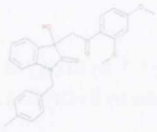
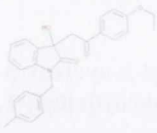
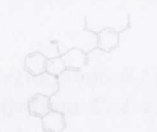
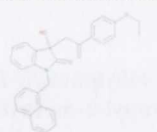
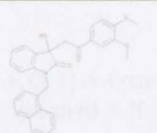
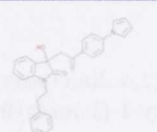
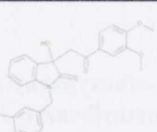
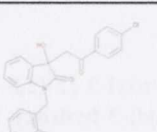
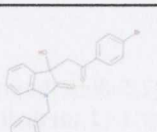
Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-1		AK-778/43206442	C25H21NO3	1-benzyl-3-hydroxy-3-(1-oxo-1,2,3,4-tetrahydro-2-naphthalenyl)-1,3-dihydro-2H-indol-2-one
cdx-2		AK-778/40789044	C24H21NO3	3-hydroxy-1-(2-methylbenzyl)-3-(2-oxo-2-phenylethyl)-1,3-dihydro-2H-indol-2-one
cdx-3		AK-778/40789045	C25H23NO3	3-hydroxy-1-(2-methylbenzyl)-3-[2-(4-methylphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-4		AK-778/40985013	C26H25NO3	1-benzyl-3-hydroxy-3-(2-mesityl-2-oxoethyl)-1,3-dihydro-2H-indol-2-one
cdx-5		AK-778/40688008	C23H19NO3	1-benzyl-3-hydroxy-3-(2-oxo-2-phenylethyl)-1,3-dihydro-2H-indol-2-one
cdx-6		AK-778/40789018	C24H21NO3	1-benzyl-3-hydroxy-3-[2-(4-methylphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-7		AK-778/40789025	C23H19NO4	1-benzyl-3-hydroxy-3-[2-(2-hydroxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-8		AK-778/40985014	C25H23NO3	1-benzyl-3-[2-(2,5-dimethylphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-9		AK-778/41314733	C22H18N2O3	1-benzyl-3-hydroxy-3-[2-oxo-2-(4-pyridinyl)ethyl]-1,3-dihydro-2H-indol-2-one
cdx-10		AK-778/40985009	C25H23NO3	1-benzyl-3-[2-(2,4-dimethylphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one

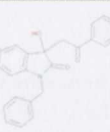
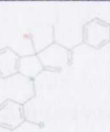
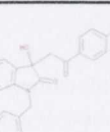
Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-11		AK-778/40789063	C28H27NO3	3-hydroxy-1-(2-methylbenzyl)-3-[2-oxo-2-(5,6,7,8-tetrahydro-2-naphthalenyl)ethyl]-1,3-dihydro-2H-indol-2-one
cdx-12		AK-778/40984980	C28H23NO3	3-hydroxy-3-[2-(4-methylphenyl)-2-oxoethyl]-1-(1-naphthylmethyl)-1,3-dihydro-2H-indol-2-one
cdx-13		AK-778/40984989	C27H21NO4	3-hydroxy-3-[2-(2-hydroxyphenyl)-2-oxoethyl]-1-(1-naphthylmethyl)-1,3-dihydro-2H-indol-2-one
cdx-14		AK-778/40958948	C24H19NO5	1-(1,3-benzodioxol-5-ylmethyl)-3-hydroxy-3-(2-oxo-2-phenylethyl)-1,3-dihydro-2H-indol-2-one
cdx-15		AK-778/40984979	C27H21NO3	3-hydroxy-1-(1-naphthylmethyl)-3-(2-oxo-2-phenylethyl)-1,3-dihydro-2H-indol-2-one
cdx-16		AK-778/41050986	C25H21NO5	1-(1,3-benzodioxol-5-ylmethyl)-3-hydroxy-3-[2-(4-methylphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-17		AK-778/41314757	C26H23NO5	3-[2-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-oxoethyl]-3-hydroxy-1-(4-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-18		AK-778/43206312	C25H21NO5	3-[2-(1,3-benzodioxol-5-yl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-19		AK-778/43206332	C25H21NO5	3-[2-(1,3-benzodioxol-5-yl)-2-oxoethyl]-3-hydroxy-1-(4-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-20		AK-778/43206374	C22H17NO6	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(2-furyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one

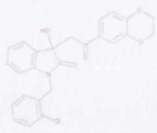
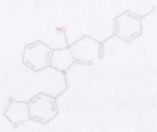
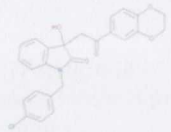
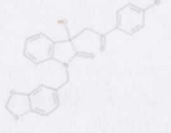
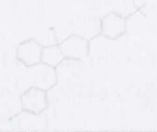
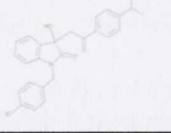
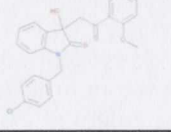
Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-21		AK-778/43206376	C ₂₆ H ₂₃ NO ₅	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(2,5-dimethylphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-22		AK-778/43206436	C ₂₄ H ₁₉ NO ₅	3-[2-(1,3-benzodioxol-5-yl)-2-oxoethyl]-1-benzyl-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-23		AK-778/43206370	C ₂₆ H ₂₁ NO ₇	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-24		AK-778/40789020	C ₂₆ H ₂₅ NO ₃	1-benzyl-3-hydroxy-3-[2-(4-isopropylphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-25		AK-778/40789021	C ₂₇ H ₂₇ NO ₃	1-benzyl-3-[2-(4-tert-butylphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-26		AK-778/40789048	C ₂₈ H ₂₉ NO ₃	3-[2-(4-tert-butylphenyl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-27		AK-778/40985010	C ₂₅ H ₂₄ N ₂ O ₃	1-benzyl-3-{2-[4-(dimethylamino)phenyl]-2-oxoethyl}-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-28		AK-778/40789054	C ₂₅ H ₂₃ NO ₄	3-hydroxy-3-[2-(4-methoxyphenyl)-2-oxoethyl]-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-29		AK-778/40688033	C ₂₄ H ₂₁ NO ₄	1-benzyl-3-hydroxy-3-[2-(2-methoxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-30		AK-778/40789019	C ₂₅ H ₂₃ NO ₃	1-benzyl-3-[2-(4-ethylphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one

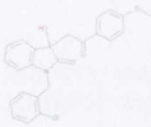
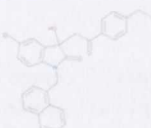
Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-31		AK-778/41051036	C25H23NO4	3-hydroxy-3-[2-(2-methoxyphenyl)-2-oxoethyl]-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-32		AK-778/41314707	C24H21NO4	1-benzyl-3-hydroxy-3-[2-(3-methoxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-33		AK-778/41314713	C25H23NO4	3-hydroxy-3-[2-(3-methoxyphenyl)-2-oxoethyl]-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-34		AK-778/43206318	C25H23NO4	3-hydroxy-3-[2-(4-methoxyphenyl)-2-oxoethyl]-1-(4-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-35		AK-778/43206330	C25H23NO4	3-hydroxy-3-[2-(2-methoxyphenyl)-2-oxoethyl]-1-(4-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-36		AK-778/43206331	C25H23NO4	3-hydroxy-3-[2-(3-methoxyphenyl)-2-oxoethyl]-1-(4-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-37		AK-778/40789026	C24H21NO4	1-benzyl-3-hydroxy-3-[2-(4-methoxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-38		AK-778/43206360	C26H23NO5	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(4-ethylphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-39		AK-778/43206367	C25H21NO6	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(4-methoxyphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-40		AK-778/43206378	C25H21NO6	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(2-methoxyphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one

Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-41		AK-778/43206443	C27H22N2O3	1-benzyl-3-hydroxy-3-{2-oxo-2-[4-(1H-pyrrol-1-yl)phenyl]ethyl}-1,3-dihydro-2H-indol-2-one
cdx-42		AK-778/43206444	C27H22N2O3	1-benzyl-3-hydroxy-3-{2-oxo-2-[3-(1H-pyrrol-1-yl)phenyl]ethyl}-1,3-dihydro-2H-indol-2-one
cdx-43		AK-778/40789027	C25H23NO5	1-benzyl-3-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-44		AK-778/40789055	C26H25NO5	3-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-45		AK-778/41507493	C29H31NO3	3-[2-(4-ethylphenyl)-2-oxoethyl]-3-hydroxy-1-(5-isopropyl-2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-46		AK-778/40789040	C25H23NO5	1-benzyl-3-[2-(2,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-47		AK-778/40789067	C26H25NO5	3-[2-(2,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-48		AK-778/41051035	C26H25NO4	3-[2-(4-ethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-49		AK-778/41314711	C25H23NO5	1-benzyl-3-[2-(2,5-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-50		AK-778/41314717	C26H25NO5	3-[2-(2,5-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one

Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-51		AK-778/43206323	C ₂₆ H ₂₅ NO ₅	3-[2-(2,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(4-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-52		AK-778/43206329	C ₂₆ H ₂₅ NO ₄	3-[2-(4-ethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(4-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-53		AK-778/40985015	C ₂₅ H ₂₃ NO ₄	1-benzyl-3-[2-(4-ethoxyphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-54		AK-778/40985003	C ₂₉ H ₂₅ NO ₅	3-[2-(2,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(1-naphthylmethyl)-1,3-dihydro-2H-indol-2-one
cdx-55		AK-778/40985008	C ₂₉ H ₂₅ NO ₄	3-[2-(4-ethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(1-naphthylmethyl)-1,3-dihydro-2H-indol-2-one
cdx-56		AK-778/40984991	C ₂₉ H ₂₅ NO ₅	3-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1-(1-naphthylmethyl)-1,3-dihydro-2H-indol-2-one
cdx-57		AK-778/43114931	C ₂₈ H ₂₄ N ₂ O ₃	3-hydroxy-3-{2-oxo-2-[4-(1H-pyrrol-1-yl)phenyl]ethyl}-1-(2-phenylethyl)-1,3-dihydro-2H-indol-2-one
cdx-58		AK-778/43206368	C ₂₆ H ₂₃ NO ₇	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-59		AK-778/40789050	C ₂₄ H ₂₀ ClNO ₃	3-[2-(4-chlorophenyl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-60		AK-778/40789051	C ₂₄ H ₂₀ BrNO ₃	3-[2-(4-bromophenyl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one

Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-61		AN-655/40687925	C22H17ClN2O3	1-(2-chlorobenzyl)-3-hydroxy-3-[2-oxo-2-(2-pyridinyl)ethyl]-1,3-dihydro-2H-indol-2-one
cdx-62		AK-778/40688024	C23H18BrNO3	1-benzyl-3-[2-(4-bromophenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-63		AK-778/40789022	C23H18FNO3	1-benzyl-3-[2-(4-fluorophenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-64		AK-778/40789049	C24H20FNO3	3-[2-(4-fluorophenyl)-2-oxoethyl]-3-hydroxy-1-(2-methylbenzyl)-1,3-dihydro-2H-indol-2-one
cdx-65		AK-778/41182513	C24H20ClNO3	1-(2-chlorobenzyl)-3-hydroxy-3-[2-(4-methylphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-66		AK-778/41182532	C23H18ClNO3	1-benzyl-5-chloro-3-hydroxy-3-(2-oxo-2-phenylethyl)-1,3-dihydro-2H-indol-2-one
cdx-67		AK-778/43206349	C24H20ClNO3	1-(2-chlorobenzyl)-3-hydroxy-3-(1-methyl-2-oxo-2-phenylethyl)-1,3-dihydro-2H-indol-2-one
cdx-68		AK-778/15446017	C24H20ClNO3	1-(4-chlorobenzyl)-3-hydroxy-3-[2-(4-methylphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-69		AK-778/40789023	C23H18ClNO3	1-benzyl-3-[2-(4-chlorophenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-70		AK-778/15445009	C27H24ClNO3	1-(4-chlorobenzyl)-3-hydroxy-3-[2-oxo-2-(5,6,7,8-tetrahydronaphthalen-2-yl)ethyl]-1,3-dihydro-2H-indol-2-one

Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-71		AK-778/15446027	C ₂₇ H ₂₀ ClNO 3	1-(4-chlorobenzyl)-3-hydroxy-3-[2-(2-naphthyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-72		AK-778/40958944	C ₂₅ H ₂₀ ClNO 5	1-(2-chlorobenzyl)-3-[2-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-73		AK-778/40984985	C ₂₇ H ₂₀ FNO 3	3-[2-(4-fluorophenyl)-2-oxoethyl]-3-hydroxy-1-(1-naphthylmethyl)-1,3-dihydro-2H-indol-2-one
cdx-74		AK-778/41050987	C ₂₄ H ₁₈ FNO 5	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(4-fluorophenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-75		AK-778/40958950	C ₂₅ H ₂₀ ClNO 5	1-(4-chlorobenzyl)-3-[2-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-76		AK-778/43206363	C ₂₄ H ₁₈ ClNO 5	1-(1,3-benzodioxol-5-ylmethyl)-3-[2-(4-chlorophenyl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-77		AK-778/15445008	C ₂₉ H ₂₂ ClNO 3	1-(4-chlorobenzyl)-3-[2-(1,2-dihydroacenaphthyl-5-yl)-2-oxoethyl]-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-78		AK-778/15445005	C ₂₇ H ₂₆ ClNO 3	3-[2-(4-tert-butylphenyl)-2-oxoethyl]-1-(4-chlorobenzyl)-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-79		AK-778/40789042	C ₂₆ H ₂₄ ClNO 3	1-(4-chlorobenzyl)-3-hydroxy-3-[2-(4-isopropylphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-80		AK-778/41051044	C ₂₄ H ₂₀ ClNO 4	1-(4-chlorobenzyl)-3-hydroxy-3-[2-(2-methoxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one

Name	Structure	Specs ID	Chemical Formula	Chemical Name
cdx-81		AK-778/41182516	C ₂₇ H ₂₆ ClNO 3	3-[2-(4-tert-butylphenyl)-2-oxoethyl]-1-(2-chlorobenzyl)-3-hydroxy-1,3-dihydro-2H-indol-2-one
cdx-82		AK-778/40687917	C ₂₄ H ₂₀ ClNO 4	1-(2-chlorobenzyl)-3-hydroxy-3-[2-(2-methoxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-83		AK-778/41314719	C ₂₄ H ₂₀ ClNO 4	1-(4-chlorobenzyl)-3-hydroxy-3-[2-(3-methoxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-84		AK-778/43206343	C ₂₄ H ₂₀ ClNO 4	1-(2-chlorobenzyl)-3-hydroxy-3-[2-(4-methoxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-85		AK-778/43206354	C ₂₄ H ₂₀ ClNO 4	1-(2-chlorobenzyl)-3-hydroxy-3-[2-(3-methoxyphenyl)-2-oxoethyl]-1,3-dihydro-2H-indol-2-one
cdx-86		AK-778/41507448	C ₂₉ H ₂₄ ClNO 3	5-chloro-3-[2-(4-ethylphenyl)-2-oxoethyl]-3-hydroxy-1-(1-naphthylmethyl)-1,3-dihydro-2H-indol-2-one

Name	Structure	Chem Div. ID	Chemical Formula	Chemical Name
cdx-87		3817-0053	C23 H18 Cl N O4	1-(((4-chlorophenyl)methyl)-3-hydroxy-3-[2-(2-hydroxyphenyl)-2-oxoethyl]-2,3-dihydro-1H-indol-2-one
cdx-88		5056-0105	C27 H26 Cl N O3	1-benzyl-3-[2-(4-tert-butylphenyl)-2-oxoethyl]-5-chloro-3-hydroxy-2,3-dihydro-1H-indol-2-one
cdx-89		5056-0118	C26 H24 Cl N O3	5-chloro-3-[2-(4-ethylphenyl)-2-oxoethyl]-3-hydroxy-1-[(2-methylphenyl)methyl]-2,3-dihydro-1H-indol-2-one
cdx-90		5056-0119	C27 H26 Cl N O3	5-chloro-3-hydroxy-1-[(2-methylphenyl)methyl]-3-[2-oxo-2[4-(propan-2-yl)phenyl]ethyl]-2,3-dihydro-1H-indol-2-one
cdx-91		5510-1412	C24 H20 Cl N O4	5-chloro-3-hydroxy-3-[2-(2-hydroxyphenyl)-2-oxoethyl]-1-[(2-methylphenyl)methyl]-2,3-dihydro-1H-indol-2-one
cdx-92		5510-1405	C23 H18 Cl N O4	1-benzyl-5-chloro-3-hydroxy-3-[2-(2-hydroxyphenyl)-2-oxoethyl]-2,3-dihydro-1H-indol-2-one

Bibliography

Bibliography

- ABD-ELRAHMAN, I., HERSHKO, K., NEUMAN, T., NACHMIAS, B., PERLMAN, R. & BEN-YEHUDA, D. 2009. The inhibitor of apoptosis protein Livin (ML-IAP) plays a dual role in tumorigenicity. *Cancer Res*, 69, 5475-80.
- ABU-HAMAD, S., ZAID, H., ISRAELSON, A., NAHON, E. & SHOSHAN-BARMATZ, V. 2008. Hexokinase-I protection against apoptotic cell death is mediated via interaction with the voltage-dependent anion channel-1: mapping the site of binding. *J Biol Chem*, 283, 13482-90.
- AHN, K. S., SETHI, G. & AGGARWAL, B. B. 2007. Embelin, an inhibitor of X chromosome-linked inhibitor-of-apoptosis protein, blocks nuclear factor-kappaB (NF-kappaB) signaling pathway leading to suppression of NF-kappaB-regulated antiapoptotic and metastatic gene products. *Mol Pharmacol*, 71, 209-19.
- AL-ALEM, L., SOUTHARD, R. C., KILGORE, M. W. & CURRY, T. E. 2011. Specific thiazolidinediones inhibit ovarian cancer cell line proliferation and cause cell cycle arrest in a PPARgamma independent manner. *PLoS One*, 6, e16179.
- AL-QAWASMEH, R. A., LEE, Y., CAO, M. Y., GU, X., VASSILAKOS, A., WRIGHT, J. A. & YOUNG, A. 2004. Triaryl methane derivatives as antiproliferative agents. *Bioorg Med Chem Lett*, 14, 347-50.
- ALESHIN, A. E., ZENG, C., BARTUNIK, H. D., FROMM, H. J. & HONZATKO, R. B. 1998. Regulation of hexokinase I: crystal structure of recombinant human brain hexokinase complexed with glucose and phosphate. *J Mol Biol*, 282, 345-57.
- ALTIERI, D. C. 2003. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer*, 3, 46-54.
- AMBROSINI, G., ADIDA, C. & ALTIERI, D. C. 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med*, 3, 917-21.
- AMBROSINI, G., ADIDA, C., SIRUGO, G. & ALTIERI, D. C. 1998. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J Biol Chem*, 273, 11177-82.
- ANTONSSON, B., CONTI, F., CIAVATTA, A., MONTESSUIT, S., LEWIS, S., MARTINOU, I., BERNASCONI, L., BERNARD, A., MERMOD, J. J., MAZZEI, G., MAUNDRELL, K., GAMBALE, F., SADOUL, R. & MARTINOU, J. C. 1997. Inhibition of Bax channel-forming activity by Bcl-2. *Science*, 277, 370-2.
- ANTONSSON, B., MONTESSUIT, S., SANCHEZ, B. & MARTINOU, J. C. 2001. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem*, 276, 11615-23.
- ARORA, V., CHEUNG, H. H., PLENCHETTE, S., MICALI, O. C., LISTON, P. & KORNELUK, R. G. 2007. Degradation of survivin by the X-linked inhibitor of apoptosis (XIAP)-XAF1 complex. *J Biol Chem*, 282, 26202-9.
- ARZOINE, L., ZILBERBERG, N., BEN-ROMANO, R. & SHOSHAN-BARMATZ, V. 2009. Voltage-dependent anion channel 1-based peptides interact with hexokinase to prevent its anti-apoptotic activity. *J Biol Chem*, 284, 3946-55.
- AYUB, M. & LEVELL, M. J. 1989. Inhibition of human adrenal steroidogenic enzymes in vitro by imidazole drugs including ketoconazole. *J Steroid Biochem*, 32, 515-24.
- AZOULAY-ZOHAR, H., ISRAELSON, A., ABU-HAMAD, S. & SHOSHAN-BARMATZ, V. 2004. In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. *Biochem J*, 377, 347-55.

- BAGGETTO, L. G., CLOTTES, E. & VIAL, C. 1992. Low mitochondrial proton leak due to high membrane cholesterol content and cytosolic creatine kinase as two features of the deviant bioenergetics of Ehrlich and AS30-D tumor cells. *Cancer Res*, 52, 4935-41.
- BASU, A. & KRISHNAMURTHY, S. 2010. Cellular responses to Cisplatin-induced DNA damage. *J Nucleic Acids*, 2010.
- BEDFORD, L., LOWE, J., DICK, L. R., MAYER, R. J. & BROWNELL, J. E. 2011. Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. *Nat Rev Drug Discov*, 10, 29-46.
- BEERS, R. F., JR. & SIZER, I. W. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem*, 195, 133-40.
- BELLI, C., FENNELL, D., GIOVANNINI, M., GAUDINO, G. & MUTTI, L. 2009. Malignant pleural mesothelioma: current treatments and emerging drugs. *Expert Opin Emerg Drugs*, 14, 423-37.
- BEN-BASSAT, H., GOLDBLUM, N., MITRANI, S., GOLDBLUM, T., YOFFEY, J. M., COHEN, M. M., BENTWICH, Z., RAMOT, B., KLEIN, E. & KLEIN, G. 1977. Establishment in continuous culture of a new type of lymphocyte from a "Burkitt like" malignant lymphoma (line D.G.-75). *Int J Cancer*, 19, 27-33.
- BERGER, N. A. 1985. Poly(ADP-ribose) in the cellular response to DNA damage. *Radiat Res*, 101, 4-15.
- BERTINO, P., CARBONE, M. & PASS, H. 2009. Chemotherapy of malignant pleural mesothelioma. *Expert Opin Pharmacother*, 10, 99-107.
- BERTRAND, M. J., DOIRON, K., LABBE, K., KORNELUK, R. G., BARKER, P. A. & SALEH, M. 2009. Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. *Immunity*, 30, 789-801.
- BERTRAND, M. J., MILUTINOVIC, S., DICKSON, K. M., HO, W. C., BOUDREAULT, A., DURKIN, J., GILLARD, J. W., JAQUITH, J. B., MORRIS, S. J. & BARKER, P. A. 2008. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol Cell*, 30, 689-700.
- BESWICK, R. W., AMBROSE, H. E. & WAGNER, S. D. 2006. Nocodazole, a microtubule de-polymerising agent, induces apoptosis of chronic lymphocytic leukaemia cells associated with changes in Bcl-2 phosphorylation and expression. *Leuk Res*, 30, 427-36.
- BIANCHI, G., OLIVA, L., CASCIO, P., PENGIO, N., FONTANA, F., CERRUTI, F., ORSI, A., PASQUALETTO, E., MEZGHRANI, A., CALBI, V., PALLADINI, G., GIULIANI, N., ANDERSON, K. C., SITIA, R. & CENCI, S. 2009. The proteasome load versus capacity balance determines apoptotic sensitivity of multiple myeloma cells to proteasome inhibition. *Blood*, 113, 3040-9.
- BJORKOY, G., LAMARK, T., BRECH, A., OUTZEN, H., PERANDER, M., OVERVATN, A., STENMARK, H. & JOHANSEN, T. 2005. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol*, 171, 603-14.
- BLAGOSKLONNY, M. V., WU, G. S., OMURA, S. & EL-DEIRY, W. S. 1996. Proteasome-dependent regulation of p21WAF1/CIP1 expression. *Biochem Biophys Res Commun*, 227, 564-9.
- BOCKBRADER, K. M., TAN, M. & SUN, Y. 2005. A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL- and etoposide-induced apoptosis in breast cancer cells. *Oncogene*, 24, 7381-8.
- BORCZUK, A. C., CAPPELLINI, G. C., KIM, H. K., HESDORFFER, M., TAUB, R. N. & POWELL, C. A. 2007. Molecular profiling of malignant peritoneal mesothelioma identifies the ubiquitin-proteasome pathway as a therapeutic target in poor prognosis tumors. *Oncogene*, 26, 610-7.

- BOULARES, A. H., YAKOVLEV, A. G., IVANOVA, V., STOICA, B. A., WANG, G., IYER, S. & SMULSON, M. 1999. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. *J Biol Chem*, 274, 22932-40.
- BOYA, P., GONZALEZ-POLO, R. A., CASARES, N., PERFETTINI, J. L., DESSEN, P., LAROCLETTE, N., METIVIER, D., MELEY, D., SOUQUERE, S., YOSHIMORI, T., PIERRON, G., CODOGNO, P. & KROEMER, G. 2005. Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol*, 25, 1025-40.
- BOYA, P., GONZALEZ-POLO, R. A., PONCET, D., ANDREAU, K., VIEIRA, H. L., ROUMIER, T., PERFETTINI, J. L. & KROEMER, G. 2003. Mitochondrial membrane permeabilization is a critical step of lysosome-initiated apoptosis induced by hydroxychloroquine. *Oncogene*, 22, 3927-36.
- BROWN, K. K., HENKE, B. R., BLANCHARD, S. G., COBB, J. E., MOOK, R., KALDOR, I., KLIEWER, S. A., LEHMANN, J. M., LENHARD, J. M., HARRINGTON, W. W., NOVAK, P. J., FAISON, W., BINZ, J. G., HASHIM, M. A., OLIVER, W. O., BROWN, H. R., PARKS, D. J., PLUNKET, K. D., TONG, W. Q., MENIUS, J. A., ADKISON, K., NOBLE, S. A. & WILLSON, T. M. 1999. A novel N-aryl tyrosine activator of peroxisome proliferator-activated receptor-gamma reverses the diabetic phenotype of the Zucker diabetic fatty rat. *Diabetes*, 48, 1415-24.
- BRUN, R. P., TONTONNOZ, P., FORMAN, B. M., ELLIS, R., CHEN, J., EVANS, R. M. & SPIEGELMAN, B. M. 1996. Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev*, 10, 974-84.
- BRUNNER, T., WASEM, C., TORGLER, R., CIMA, I., JAKOB, S. & CORAZZA, N. 2003. Fas (CD95/Apo-1) ligand regulation in T cell homeostasis, cell-mediated cytotoxicity and immune pathology. *Semin Immunol*, 15, 167-76.
- BUENO, R., APPASANI, K., MERCER, H., LESTER, S. & SUGARBAKER, D. 2001. The alpha folate receptor is highly activated in malignant pleural mesothelioma. *J Thorac Cardiovasc Surg*, 121, 225-33.
- BUNZ, F., DUTRIAUX, A., LENGAUER, C., WALDMAN, T., ZHOU, S., BROWN, J. P., SEDIVY, J. M., KINZLER, K. W. & VOGELSTEIN, B. 1998. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*, 282, 1497-501.
- BURKE, S. P., SMITH, L. & SMITH, J. B. 2010. cIAP1 cooperatively inhibits procaspase-3 activation by the caspase-9 apoptosome. *J Biol Chem*, 285, 30061-8.
- BURSCH, W. 2001. The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ*, 8, 569-81.
- BUYTAERT, E., CALLEWAERT, G., VANDENHEEDE, J. R. & AGOSTINIS, P. 2006. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. *Autophagy*, 2, 238-40.
- CANCER-RESEARCH-IRELAND 2010. Cancer Research Ireland - <http://www.cancer.ie/research/why.php>.
- CERESOLI, G. L., ZUCALI, P. A., GIANONCELLI, L., LORENZI, E. & SANTORO, A. 2009. Second-line treatment for malignant pleural mesothelioma. *Cancer Treat Rev*.
- CHAI, J., DU, C., WU, J. W., KYIN, S., WANG, X. & SHI, Y. 2000. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature*, 406, 855-62.
- CHAI, J., SHIOZAKI, E., SRINIVASULA, S. M., WU, Q., DATTA, P., ALNEMRI, E. S. & SHI, Y. 2001. Structural basis of caspase-7 inhibition by XIAP. *Cell*, 104, 769-80.
- CHANCE, B. 1948. The enzyme-substrate compounds of catalase and peroxides. *Nature*, 161, 914-7.

- CHECINSKA, A., HOOGELAND, B. S., RODRIGUEZ, J. A., GIACCONE, G. & KRUYT, F. A. 2007. Role of XIAP in inhibiting cisplatin-induced caspase activation in non-small cell lung cancer cells: a small molecule Smac mimic sensitizes for chemotherapy-induced apoptosis by enhancing caspase-3 activation. *Exp Cell Res*, 313, 1215-24.
- CHEN, J., NIKOLOVSKA-COLESKA, Z., WANG, G., QIU, S. & WANG, S. 2006. Design, synthesis, and characterization of new embelin derivatives as potent inhibitors of X-linked inhibitor of apoptosis protein. *Bioorg Med Chem Lett*, 16, 5805-8.
- CHEN, Z., ZHANG, H., LU, W. & HUANG, P. 2009. Role of mitochondria-associated hexokinase II in cancer cell death induced by 3-bromopyruvate. *Biochim Biophys Acta*, 1787, 553-60.
- CHEUNG, H. H., PLENCHETTE, S., KERN, C. J., MAHONEY, D. J. & KORNELUK, R. G. 2008. The RING domain of cIAP1 mediates the degradation of RING-bearing inhibitor of apoptosis proteins by distinct pathways. *Mol Biol Cell*, 19, 2729-40.
- CHIARA, F., CASTELLARO, D., MARIN, O., PETRONILLI, V., BRUSILOV, W. S., JUHASZOVA, M., SOLLOTT, S. J., FORTE, M., BERNARDI, P. & RASOLA, A. 2008. Hexokinase II detachment from mitochondria triggers apoptosis through the permeability transition pore independent of voltage-dependent anion channels. *PLoS One*, 3, e1852.
- CHOI, Y. E., BUTTERWORTH, M., MALLADI, S., DUCKETT, C. S., COHEN, G. M. & BRATTON, S. B. 2009. The E3 ubiquitin ligase cIAP1 binds and ubiquitinates caspase-3 and -7 via unique mechanisms at distinct steps in their processing. *J Biol Chem*, 284, 12772-82.
- CHOU, J. J., LI, H., SALVESEN, G. S., YUAN, J. & WAGNER, G. 1999. Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell*, 96, 615-24.
- CHOUCROUN, P., GILLET, D., DORANGE, G., SAWICKI, B. & DEWITTE, J. D. 2001. Comet assay and early apoptosis. *Mutat Res*, 478, 89-96.
- CHOWDHURY, S., CHAFEEV, M., LIU, S., SUN, J., RAINA, V., CHUI, R., YOUNG, W., KWAN, R., FU, J. & CADIEUX, J. A. 2011. Discovery of XEN907, a spirooxindole blocker of NaV1.7 for the treatment of pain. *Bioorg Med Chem Lett*, 21, 3676-81.
- CLOONAN, S. M., KEATING, J. J., BUTLER, S. G., KNOX, A. J., JORGENSEN, A. M., PETERS, G. H., RAI, D., CORRIGAN, D., LLOYD, D. G., WILLIAMS, D. C. & MEEGAN, M. J. 2009. Synthesis and serotonin transporter activity of sulphur-substituted alpha-alkyl phenethylamines as a new class of anticancer agents. *Eur J Med Chem*, 44, 4862-88.
- CLOONAN, S. M. & WILLIAMS, D. C. 2010. The antidepressants maprotiline and fluoxetine induce Type II autophagic cell death in drug-resistant Burkitt's lymphoma. *Int J Cancer*.
- CONKLIN, K. A. 2004. Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness. *Integr Cancer Ther*, 3, 294-300.
- CONZE, D. B., ALBERT, L., FERRICK, D. A., GOEDDEL, D. V., YEH, W. C., MAK, T. & ASHWELL, J. D. 2005. Posttranscriptional downregulation of c-IAP2 by the ubiquitin protein ligase c-IAP1 in vivo. *Mol Cell Biol*, 25, 3348-56.
- CORSON, J. M. 2004. Pathology of mesothelioma. *Thorac Surg Clin*, 14, 447-60.
- CORY, S. & ADAMS, J. M. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*, 2, 647-56.

- COSSU, F., MILANI, M., MASTRANGELO, E., VACHETTE, P., SERVIDA, F., LECIS, D., CANEVARI, G., DELIA, D., DRAGO, C., RIZZO, V., MANZONI, L., SENEI, P., SCOLASTICO, C. & BOLOGNESI, M. 2009. Structural basis for bivalent Smac-mimetics recognition in the IAP protein family. *J Mol Biol*, 392, 630-44.
- CREAGH, E. M. & MARTIN, S. J. 2003. Cell stress-associated caspase activation: intrinsically complex? *Sci STKE*, 2003, pe11.
- CRISOSTOMO, F. R., FENG, Y., ZHU, X., WELSH, K., AN, J., REED, J. C. & HUANG, Z. 2009. Design and synthesis of a simplified inhibitor for XIAP-BIR3 domain. *Bioorg Med Chem Lett*, 19, 6413-8.
- DALLINGER, D. & KAPPE, C. O. 2005. Creating chemical diversity space by scaffold decoration of dihydropyrimidines. *Pure and Applied Chemistry*, 77, 155-161.
- DAVOODI, J., GHAHREMANI, M. H., ES-HAGHI, A., MOHAMMAD-GHOLI, A. & MACKENZIE, A. 2010. Neuronal apoptosis inhibitory protein, NAIP, is an inhibitor of procaspase-9. *Int J Biochem Cell Biol*.
- DEBAERE, D., VANDER POORTEN, V., NUYTS, S., HAUBEN, E., SCHOENAERS, J., SCHOFFSKI, P. & CLEMENT, P. M. 2011. Cyclophosphamide, doxorubicin, and cisplatin in advanced salivary gland cancer. *B-ENT*, 7, 1-6.
- DETER, R. L., BAUDHUIN, P. & DE DUVE, C. 1967. Participation of lysosomes in cellular autophagy induced in rat liver by glucagon. *J Cell Biol*, 35, C11-6.
- DEVERAUX, Q. L., LEO, E., STENNICKE, H. R., WELSH, K., SALVESEN, G. S. & REED, J. C. 1999. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J*, 18, 5242-51.
- DEVERAUX, Q. L., ROY, N., STENNICKE, H. R., VAN ARSDALE, T., ZHOU, Q., SRINIVASULA, S. M., ALNEMRI, E. S., SALVESEN, G. S. & REED, J. C. 1998. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J*, 17, 2215-23.
- DEVERAUX, Q. L., TAKAHASHI, R., SALVESEN, G. S. & REED, J. C. 1997. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature*, 388, 300-4.
- DING, W. X., NI, H. M. & YIN, X. M. 2007. Absence of Bax switched MG132-induced apoptosis to non-apoptotic cell death that could be suppressed by transcriptional or translational inhibition. *Apoptosis*, 12, 2233-44.
- DOHI, T., OKADA, K., XIA, F., WILFORD, C. E., SAMUEL, T., WELSH, K., MARUSAWA, H., ZOU, H., ARMSTRONG, R., MATSUZAWA, S., SALVESEN, G. S., REED, J. C. & ALTIERI, D. C. 2004. An IAP-IAP complex inhibits apoptosis. *J Biol Chem*, 279, 34087-90.
- DU, C., FANG, M., LI, Y., LI, L. & WANG, X. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell*, 102, 33-42.
- DUCKETT, C. S., NAVA, V. E., GEDRICH, R. W., CLEM, R. J., VAN DONGEN, J. L., GILFILLAN, M. C., SHIELS, H., HARDWICK, J. M. & THOMPSON, C. B. 1996. A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *EMBO J*, 15, 2685-94.
- DURIEZ, P. J. & SHAH, G. M. 1997. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem Cell Biol*, 75, 337-49.
- DVORAKOVA, K., PAYNE, C. M., TOME, M. E., BRIEHL, M. M., VASQUEZ, M. A., WALTIRE, C. N., COON, A. & DORR, R. T. 2002. Molecular and cellular characterization of imexon-resistant RPMI8226/I myeloma cells. *Mol Cancer Ther*, 1, 185-95.

- EA, C. K., DENG, L., XIA, Z. P., PINEDA, G. & CHEN, Z. J. 2006. Activation of IKK by TNF α requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol Cell*, 22, 245-57.
- EASTMAN, A. 1990. Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells*, 2, 275-80.
- ECKELMAN, B. P. & SALVESEN, G. S. 2006. The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *J Biol Chem*, 281, 3254-60.
- ECKELMAN, B. P., SALVESEN, G. S. & SCOTT, F. L. 2006. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep*, 7, 988-94.
- EDINGER, A. L. & THOMPSON, C. B. 2004. Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol*, 16, 663-9.
- EDWARDS, J. G., COX, G., ANDI, A., JONES, J. L., WALKER, R. A., WALLER, D. A. & O'BYRNE, K. J. 2001. Angiogenesis is an independent prognostic factor in malignant mesothelioma. *Br J Cancer*, 85, 863-8.
- ELDRIDGE, A. G. & O'BRIEN, T. 2010. Therapeutic strategies within the ubiquitin proteasome system. *Cell Death Differ*, 17, 4-13.
- ELMORE, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol*, 35, 495-516.
- ENDO, K., KOHNOE, S., WATANABE, A., TASHIRO, H., SAKATA, H., MORITA, M., KAKEJI, Y. & MAEHARA, Y. 2009. Clinical significance of Smac/DIABLO expression in colorectal cancer. *Oncol Rep*, 21, 351-5.
- ERKIZAN, H. V., KONG, Y., MERCHANT, M., SCHLOTTMANN, S., BARBER-ROTENBERG, J. S., YUAN, L., ABAAN, O. D., CHOU, T. H., DAKSHANAMURTHY, S., BROWN, M. L., UREN, A. & TORETSKY, J. A. 2009. A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing's sarcoma. *Nat Med*, 15, 750-6.
- ERKIZAN, H. V., UVERSKY, V. N. & TORETSKY, J. A. 2010. Oncogenic partnerships: EWS-FLI1 protein interactions initiate key pathways of Ewing's sarcoma. *Clin Cancer Res*, 16, 4077-83.
- FADOK, V. A., VOELKER, D. R., CAMPBELL, P. A., COHEN, J. J., BRATTON, D. L. & HENSON, P. M. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*, 148, 2207-16.
- FANG, Y., ZHOU, X., LIN, M., YING, M., LUO, P., ZHU, D., LOU, J., YANG, B. & HE, Q. 2011. Inhibition of all-trans-retinoic acid-induced proteasome activation potentiates the differentiating effect of retinoid in acute myeloid leukemia cells. *Mol Carcinog*, 50, 24-35.
- FARBER, S. & DIAMOND, L. K. 1948. Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid. *N Engl J Med*, 238, 787-93.
- FISCHER, S., WIESNET, M., RENZ, D. & SCHAPER, W. 2005. H₂O₂ induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. *Eur J Cell Biol*, 84, 687-97.
- FISCHER, S. J., BENSON, L. M., FAUQ, A., NAYLOR, S. & WINDEBANK, A. J. 2008. Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity. *Neurotoxicology*, 29, 444-52.
- FORMAN, B. M., TONTONOV, P., CHEN, J., BRUN, R. P., SPIEGELMAN, B. M. & EVANS, R. M. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell*, 83, 803-12.

- FU, J., JIN, Y. & AREND, L. J. 2003. Smac3, a novel Smac/DIABLO splicing variant, attenuates the stability and apoptosis-inhibiting activity of X-linked inhibitor of apoptosis protein. *J Biol Chem*, 278, 52660-72.
- FU, Z., GUO, J., JING, L., LI, R., ZHANG, T. & PENG, S. 2010. Enhanced toxicity and ROS generation by doxorubicin in primary cultures of cardiomyocytes from neonatal metallothionein-I/II null mice. *Toxicol In Vitro*, 24, 1584-91.
- FULDA, S., WICK, W., WELLER, M. & DEBATIN, K. M. 2002. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. *Nat Med*, 8, 808-15.
- GALBAN, S. & DUCKETT, C. S. 2010. XIAP as a ubiquitin ligase in cellular signaling. *Cell Death Differ*, 17, 54-60.
- GALLUZZI, L. & KROEMER, G. 2008. Necroptosis: a specialized pathway of programmed necrosis. *Cell*, 135, 1161-3.
- GAO, Z., SARSOOR, E. H., KALEN, A. L., LI, L., KUMAR, M. G. & GOSWAMI, P. C. 2008. Late ROS accumulation and radiosensitivity in SOD1-overexpressing human glioma cells. *Free Radic Biol Med*, 45, 1501-9.
- GIRNUN, G. D., NASERI, E., VAFAI, S. B., QU, L., SZWAYA, J. D., BRONSON, R., ALBERTA, J. A. & SPIEGELMAN, B. M. 2007. Synergy between PPARGgamma ligands and platinum-based drugs in cancer. *Cancer Cell*, 11, 395-406.
- GLOVER, C. J., HITE, K., DELOSH, R., SCUDIERO, D. A., FIVASH, M. J., SMITH, L. R., FISHER, R. J., WU, J. W., SHI, Y., KIPP, R. A., MCLENDON, G. L., SAUSVILLE, E. A. & SHOEMAKER, R. H. 2003. A high-throughput screen for identification of molecular mimics of Smac/DIABLO utilizing a fluorescence polarization assay. *Anal Biochem*, 320, 157-69.
- GOLDBERG, A. L. 2003. Protein degradation and protection against misfolded or damaged proteins. *Nature*, 426, 895-9.
- GOLDIN, N., ARZOINE, L., HEYFETS, A., ISRAELSON, A., ZASLAVSKY, Z., BRAVMAN, T., BRONNER, V., NOTCOVICH, A., SHOSHAN-BARMATZ, V. & FLESCHER, E. 2008. Methyl jasmonate binds to and detaches mitochondria-bound hexokinase. *Oncogene*, 27, 4636-43.
- GONG, J., CHEN, N., ZHOU, Q., YANG, B., WANG, Y. & WANG, X. 2005. Melanoma inhibitor of apoptosis protein is expressed differentially in melanoma and melanocytic naevus, but similarly in primary and metastatic melanomas. *J Clin Pathol*, 58, 1081-5.
- GONZALEZ-POLO, R. A., BOYA, P., PAULEAU, A. L., JALIL, A., LAROCLETTE, N., SOUQUERE, S., ESKELINEN, E. L., PIERRON, G., SAFTIG, P. & KROEMER, G. 2005. The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death. *J Cell Sci*, 118, 3091-102.
- GOODMAN, L. S., WINTROBE, M. M., DAMESHEK, W., GOODMAN, M. J., GILMAN, A. & MCLENNAN, M. T. 1984. Landmark article Sept. 21, 1946: Nitrogen mustard therapy. Use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris(beta-chloroethyl)amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. By Louis S. Goodman, Maxwell M. Wintrobe, William Dameshek, Morton J. Goodman, Alfred Gilman and Margaret T. McLennan. *JAMA*, 251, 2255-61.
- GORDON, G. J., APPASANI, K., PARCELLS, J. P., MUKHOPADHYAY, N. K., JAKLITSCH, M. T., RICHARDS, W. G., SUGARBAKER, D. J. & BUENO, R. 2002. Inhibitor of apoptosis protein-1 promotes tumor cell survival in mesothelioma. *Carcinogenesis*, 23, 1017-24.
- GORDON, G. J., MANI, M., MAULIK, G., MUKHOPADHYAY, L., YEAP, B. Y., KINDLER, H. L., SALGIA, R., SUGARBAKER, D. J. & BUENO, R. 2008. Preclinical studies of the proteasome inhibitor bortezomib in malignant pleural mesothelioma. *Cancer Chemother Pharmacol*, 61, 549-58.

- GORDON, G. J., MANI, M., MUKHOPADHYAY, L., DONG, L., EDENFIELD, H. R., GLICKMAN, J. N., YEAP, B. Y., SUGARBAKER, D. J. & BUENO, R. 2007a. Expression patterns of inhibitor of apoptosis proteins in malignant pleural mesothelioma. *J Pathol*, 211, 447-54.
- GORDON, G. J., MANI, M., MUKHOPADHYAY, L., DONG, L., YEAP, B. Y., SUGARBAKER, D. J. & BUENO, R. 2007b. Inhibitor of apoptosis proteins are regulated by tumour necrosis factor- α in malignant pleural mesothelioma. *J Pathol*, 211, 439-46.
- GOZUACIK, D. & KIMCHI, A. 2004. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene*, 23, 2891-906.
- GRASBERGER, B. L., LU, T., SCHUBERT, C., PARKS, D. J., CARVER, T. E., KOBLISH, H. K., CUMMINGS, M. D., LAFRANCE, L. V., MILKIEWICZ, K. L., CALVO, R. R., MAGUIRE, D., LATTANZE, J., FRANKS, C. F., ZHAO, S., RAMACHANDREN, K., BYLEBYL, G. R., ZHANG, M., MANTHEY, C. L., PETRELLA, E. C., PANTOLIANO, M. W., DECKMAN, I. C., SPURLINO, J. C., MARONEY, A. C., TOMCZUK, B. E., MOLLOY, C. J. & BONE, R. F. 2005. Discovery and cocrystal structure of benzodiazepinedione HDM2 antagonists that activate p53 in cells. *J Med Chem*, 48, 909-12.
- GREGORY, C. D., ROWE, M. & RICKINSON, A. B. 1990. Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. *J Gen Virol*, 71 (Pt 7), 1481-95.
- GREGORY, E. M., KOWALSKI, J. B. & HOLDEMAN, L. V. 1977. Production and some properties of catalase and superoxide dismutase from the anaerobe *Bacteroides distasonis*. *J Bacteriol*, 129, 1298-302.
- GRIMM, S. & BAEUERLE, P. A. 1993. The inducible transcription factor NF- κ B: structure-function relationship of its protein subunits. *Biochem J*, 290 (Pt 2), 297-308.
- GUEDAT, P. & COLLAND, F. 2007. Patented small molecule inhibitors in the ubiquitin proteasome system. *BMC Biochem*, 8 Suppl 1, S14.
- GUO, X. N. & DING, J. 2003. [Gleevec: an novel inhibitor of bcr-abl tyrosine kinase]. *Sheng Li Ke Xue Jin Zhan*, 34, 183-6.
- GUTIERREZ, M. I., CHERNEY, B., HUSSAIN, A., MOSTOWSKI, H., TOSATO, G., MAGRATH, I. & BHATIA, K. 1999. Bax is frequently compromised in Burkitt's lymphomas with irreversible resistance to Fas-induced apoptosis. *Cancer Res*, 59, 696-703.
- HACKER, G. 2000. The morphology of apoptosis. *Cell Tissue Res*, 301, 5-17.
- HAFERKAMP, A., BEDKE, J., VETTER, C., PRITSCH, M., WAGENER, N., BUSE, S., CRNKOVIC-MERTENS, I., HOPPE-SEYLER, K., MACHER-GOEPFINGER, S., HOPPE-SEYLER, F., AUTSCHBACH, F. & HOHENFELLNER, M. 2008. High nuclear Livin expression is a favourable prognostic indicator in renal cell carcinoma. *BJU Int*, 102, 1700-6.
- HAMAGUCHI, N., HAMADA, H., MIYOSHI, S., IRIFUNE, K., ITO, R., MIYAZAKI, T. & HIGAKI, J. 2010. In vitro and in vivo therapeutic efficacy of the PPAR- γ agonist troglitazone in combination with cisplatin against malignant pleural mesothelioma cell growth. *Cancer Sci*, 101, 1955-64.
- HAMBLIN, T. J., DAVIS, Z., GARDINER, A., OSCIER, D. G. & STEVENSON, F. K. 1999. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*, 94, 1848-54.
- HANES, C. S. 1932. Studies on plant amylases: The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochem J*, 26, 1406-21.
- HARDCASTLE, I. R., AHMED, S. U., ATKINS, H., CALVERT, A. H., CURTIN, N. J., FARNIE, G., GOLDING, B. T., GRIFFIN, R. J., GUYENNE, S., HUTTON, C., KALLBLAD, P., KEMP, S. J., KITCHING, M. S., NEWELL, D. R., NORBEDO, S., NORTHERN, J. S., REID, R. J., SARAVANAN, K., WILLEMS, H. M. & LUNEC, J. 2005.

Isoindolinone-based inhibitors of the MDM2-p53 protein-protein interaction. *Bioorg Med Chem Lett*, 15, 1515-20.

HARDCASTLE, I. R., AHMED, S. U., ATKINS, H., FARNIE, G., GOLDING, B. T., GRIFFIN, R. J., GUYENNE, S., HUTTON, C., KALLBLAD, P., KEMP, S. J., KITCHING, M. S., NEWELL, D. R., NORBEDO, S., NORTHEN, J. S., REID, R. J., SARAVANAN, K., WILLEMS, H. M. & LUNEC, J. 2006. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction based on an isoindolinone scaffold. *J Med Chem*, 49, 6209-21.

HARDCASTLE, I. R., LIU, J., VALEUR, E., WATSON, A., AHMED, S. U., BLACKBURN, T. J., BENNACEUR, K., CLEGG, W., DRUMMOND, C., ENDICOTT, J. A., GOLDING, B. T., GRIFFIN, R. J., GRUBER, J., HAGGERTY, K., HARRINGTON, R. W., HUTTON, C., KEMP, S., LU, X., MCDONNELL, J. M., NEWELL, D. R., NOBLE, M. E., PAYNE, S. L., REVILL, C. H., RIEDINGER, C., XU, Q. & LUNEC, J. 2011. Isoindolinone inhibitors of the murine double minute 2 (MDM2)-p53 protein-protein interaction: structure-activity studies leading to improved potency. *J Med Chem*, 54, 1233-43.

HEGEMANN, L., TOSO, S. M., LAHIJANI, K. I., WEBSTER, G. F. & UITTO, J. 1993. Direct interaction of antifungal azole-derivatives with calmodulin: a possible mechanism for their therapeutic activity. *J Invest Dermatol*, 100, 343-6.

HENDRICKX, M. 2009. Naturally occurring asbestos in eastern Australia: a review of geological occurrence, disturbance and mesothelioma risk. 909-926.

HENKEL, T., MACHLEIDT, T., ALKALAY, I., KRONKE, M., BEN-NERIAH, Y. & BAEUERLE, P. A. 1993. Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature*, 365, 182-5.

HESTERBERG, T. W., BUTTERICK, C. J., OSHIMURA, M., BRODY, A. R. & BARRETT, J. C. 1986. Role of phagocytosis in Syrian hamster cell transformation and cytogenetic effects induced by asbestos and short and long glass fibers. *Cancer Res*, 46, 5795-802.

HO, Y. S., WU, C. H., CHOU, H. M., WANG, Y. J., TSENG, H., CHEN, C. H., CHEN, L. C., LEE, C. H. & LIN, S. Y. 2005. Molecular mechanisms of econazole-induced toxicity on human colon cancer cells: G0/G1 cell cycle arrest and caspase 8-independent apoptotic signaling pathways. *Food Chem Toxicol*, 43, 1483-95.

HODGSON, J. T., MCELVENNY, D. M., DARNTON, A. J., PRICE, M. J. & PETO, J. 2005. The expected burden of mesothelioma mortality in Great Britain from 2002 to 2050. *Br J Cancer*, 92, 587-93.

HOVES, S., TRAPANI, J. A. & VOSKOBOINIK, I. 2010. The battlefield of perforin/granzyme cell death pathways. *J Leukoc Biol*, 87, 237-43.

HU, Y., CHERTON-HORVAT, G., DRAGOWSKA, V., BAIRD, S., KORNELUK, R. G., DURKIN, J. P., MAYER, L. D. & LACASSE, E. C. 2003. Antisense oligonucleotides targeting XIAP induce apoptosis and enhance chemotherapeutic activity against human lung cancer cells in vitro and in vivo. *Clin Cancer Res*, 9, 2826-36.

HUANG, X., MARONA-LEWICKA, D. & NICHOLS, D. E. 1992. p-methylthioamphetamine is a potent new non-neurotoxic serotonin-releasing agent. *Eur J Pharmacol*, 229, 31-8.

HUANG, Y., PARK, Y. C., RICH, R. L., SEGAL, D., MYSZKA, D. G. & WU, H. 2001. Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. *Cell*, 104, 781-90.

HUANG, Y., RICH, R. L., MYSZKA, D. G. & WU, H. 2003. Requirement of both the second and third BIR domains for the relief of X-linked inhibitor of apoptosis protein (XIAP)-mediated caspase inhibition by Smac. *J Biol Chem*, 278, 49517-22.

- HURTADO-GUZMAN, C., FIERRO, A., ITURRIAGA-VASQUEZ, P., SEPULVEDA-BOZA, S., CASSELS, B. K. & REYES-PARADA, M. 2003. Monoamine oxidase inhibitory properties of optical isomers and N-substituted derivatives of 4-methylthioamphetamine. *J Enzyme Inhib Med Chem*, 18, 339-47.
- ISLAM, A., KAGEYAMA, H., TAKADA, N., KAWAMOTO, T., TAKAYASU, H., ISOGAI, E., OHIRA, M., HASHIZUME, K., KOBAYASHI, H., KANEKO, Y. & NAKAGAWARA, A. 2000. High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. *Oncogene*, 19, 617-23.
- JAIN, D., PATEL, N., SHELTON, M., BASU, A., ROQUE, R. & SIEDE, W. 2010. Enhancement of cisplatin sensitivity by NSC109268 in budding yeast and human cancer cells is associated with inhibition of S-phase progression. *Cancer Chemother Pharmacol*, 66, 945-52.
- JAKOBSEN, J. N. & SORENSEN, J. B. 2011. Review on clinical trials of targeted treatments in malignant mesothelioma. *Cancer Chemother Pharmacol*, 68, 1-15.
- JAN, C. R., HO, C. M., WU, S. N. & TSENG, C. J. 1999. Multiple effects of econazole on calcium signaling: depletion of thapsigargin-sensitive calcium store, activation of extracellular calcium influx, and inhibition of capacitative calcium entry. *Biochim Biophys Acta*, 1448, 533-42.
- JANNI, W. & HEPP, P. 2010. Adjuvant aromatase inhibitor therapy: outcomes and safety. *Cancer Treat Rev*, 36, 249-61.
- JANSON, V., JOHANSSON, A. & GRANKVIST, K. 2010. Resistance to caspase-8 and -9 fragments in a malignant pleural mesothelioma cell line with acquired cisplatin-resistance. *Cell Death and Dis*, 1, e78.
- JIN, Z. & EL-DEIRY, W. S. 2005. Overview of cell death signaling pathways. *Cancer Biol Ther*, 4, 139-63.
- JOE, A. K., LIU, H., SUZUI, M., VURAL, M. E., XIAO, D. & WEINSTEIN, I. B. 2002. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin Cancer Res*, 8, 893-903.
- JONES, H. W., JR. 1997. Record of the first physician to see Henrietta Lacks at the Johns Hopkins Hospital: history of the beginning of the HeLa cell line. *Am J Obstet Gynecol*, 176, S227-8.
- JOURDAIN, A. & MARTINOU, J. C. 2009. Mitochondrial outer-membrane permeabilization and remodelling in apoptosis. *Int J Biochem Cell Biol*, 41, 1884-9.
- JOY, B., SIVADASAN, R., ABRAHAM, T. E., JOHN, M., SOBHAN, P. K., SEERVI, M. & T, R. S. 2010. Lysosomal destabilization and cathepsin B contributes for cytochrome c release and caspase activation in embelin-induced apoptosis. *Mol Carcinog*, 49, 324-36.
- KABEYA, Y., MIZUSHIMA, N., UENO, T., YAMAMOTO, A., KIRISAKO, T., NODA, T., KOMINAMI, E., OHSUMI, Y. & YOSHIMORI, T. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*, 19, 5720-8.
- KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, 26, 239-57.
- KHEDKAR, S. A., MALDE, A. K., COUTINHO, E. C. & SRIVASTAVA, S. 2007. Pharmacophore Modeling in drug discovery and development: An overview. *Medicinal Chemistry*, 3, 187-197.
- KIM, A. S., W 2002. TNF-alpha-mediated apoptosis in chondrocytes sensitized by MG132 or actinomycin D. *Biochem Biophys Res Commun*, 295, 937-44.
- KLEINBERG, L., LIE, A. K., FLORENES, V. A., NESLAND, J. M. & DAVIDSON, B. 2007. Expression of inhibitor-of-apoptosis protein family members in malignant mesothelioma. *Hum Pathol*, 38, 986-94.

- KLIEWER, S. A., LEHMANN, J. M., MILBURN, M. V. & WILLSON, T. M. 1999. The PPARs and PXR: nuclear xenobiotic receptors that define novel hormone signaling pathways. *Recent Prog Horm Res*, 54, 345-67; discussion 367-8.
- KNOX, A. J., PRICE, T., PAWLAK, M., GOLFIS, G., FLOOD, C. T., FAYNE, D., WILLIAMS, D. C., MEEGAN, M. J. & LLOYD, D. G. 2009. Integration of ligand and structure-based virtual screening for the identification of the first dual targeting agent for heat shock protein 90 (Hsp90) and tubulin. *J Med Chem*, 52, 2177-80.
- KO, Y. H., SMITH, B. L., WANG, Y., POMPER, M. G., RINI, D. A., TORBENSON, M. S., HULLIHEN, J. & PEDERSEN, P. L. 2004. Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. *Biochem Biophys Res Commun*, 324, 269-75.
- KONOPLEVA, M., ZHAO, S., XIE, Z., SEGALL, H., YOUNES, A., CLAXTON, D. F., ESTROV, Z., KORNBLAU, S. M. & ANDREEFF, M. 1999. Apoptosis. Molecules and mechanisms. *Adv Exp Med Biol*, 457, 217-36.
- KOSTOVA, Z., TSAI, Y. C. & WEISSMAN, A. M. 2007. Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. *Semin Cell Dev Biol*, 18, 770-9.
- KROEMER, G., MARINO, G. & LEVINE, B. 2010. Autophagy and the integrated stress response. *Mol Cell*, 40, 280-93.
- KROLL, M., MARGOTTIN, F., KOHL, A., RENARD, P., DURAND, H., CONCORDET, J. P., BACHELERIE, F., ARENZANA-SEISDEDOS, F. & BENAROUS, R. 1999. Inducible degradation of I κ B α by the proteasome requires interaction with the F-box protein h-betaTrCP. *J Biol Chem*, 274, 7941-5.
- KUBINYI, H. 1990. Quantitative structure-activity relationships (QSAR) and molecular modelling in cancer research. *J Cancer Res Clin Oncol*, 116, 529-37.
- KUMARSWAMY, R. & CHANDNA, S. 2009. Putative partners in Bax mediated cytochrome-c release: ANT, CypD, VDAC or none of them? *Mitochondrion*, 9, 1-8.
- KUSSIE, P. H., GORINA, S., MARECHAL, V., ELENBAAS, B., MOREAU, J., LEVINE, A. J. & PAVLETICH, N. P. 1996. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science*, 274, 948-53.
- LACASSE, E. C., MAHONEY, D. J., CHEUNG, H. H., PLENCHETTE, S., BAIRD, S. & KORNELUK, R. G. 2008. IAP-targeted therapies for cancer. *Oncogene*, 27, 6252-75.
- LAMMERING, G., DE RUYSSCHER, D., VAN BAARDWIJK, A., BAUMERT, B. G., BORGER, J., LUTGENS, L., VAN DEN ENDE, P., OLLERS, M. & LAMBIN, P. 2010. The use of FDG-PET to target tumors by radiotherapy. *Strahlenther Onkol*, 186, 471-81.
- LANDOWSKI, C. P., BOLANZ, K. A., SUZUKI, Y. & HEDIGER, M. A. 2011. Chemical inhibitors of the calcium entry channel TRPV6. *Pharm Res*, 28, 322-30.
- LEBOVITZ, H. E. & BANERJI, M. A. 2001. Insulin resistance and its treatment by thiazolidinediones. *Recent Prog Horm Res*, 56, 265-94.
- LEMAIRE, I. & OUELLET, S. 1996. Distinctive profile of alveolar macrophage-derived cytokine release induced by fibrogenic and nonfibrogenic mineral dusts. *J Toxicol Environ Health*, 47, 465-78.
- LEONG, S., COHEN, R. B., GUSTAFSON, D. L., LANGER, C. J., CAMIDGE, D. R., PADAVIC, K., GORE, L., SMITH, M., CHOW, L. Q., VON MEHREN, M., O'BRYANT, C., HARIHARAN, S., DIAB, S., FOX, N. L., MICELI, R. & ECKHARDT, S. G. 2009. Mapatumumab, an antibody targeting TRAIL-R1, in combination with paclitaxel and carboplatin in patients with advanced solid malignancies: results of a phase I and pharmacokinetic study. *J Clin Oncol*, 27, 4413-21.

- LEWIS, M. E., LEVINE, R. M., YORK, J. T. & GRUBBS, W. T. 2009. A Quick and Accurate Oxygen-Based Pressure-Sensor Assay for Catalase Activity. *Journal of Chemical Education*, 86, 1227-1230.
- LI, L., THOMAS, R. M., SUZUKI, H., DE BRABANDER, J. K., WANG, X. & HARRAN, P. G. 2004. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. *Science*, 305, 1471-4.
- LIN, S. C., HUANG, Y., LO, Y. C., LU, M. & WU, H. 2007. Crystal structure of the BIR1 domain of XIAP in two crystal forms. *J Mol Biol*, 372, 847-54.
- LINDEN, M., GELLERFORS, P. & NELSON, B. D. 1982. Pore protein and the hexokinase-binding protein from the outer membrane of rat liver mitochondria are identical. *FEBS Lett*, 141, 189-92.
- LIU, G. Y. & STORZ, P. 2010. Reactive oxygen species in cancer. *Free Radic Res*, 44, 479-96.
- LIU, H., LI, Y. & RAISCH, K. P. 2010a. Clotrimazole induces a late G1 cell cycle arrest and sensitizes glioblastoma cells to radiation in vitro. *Anticancer Drugs*, 21, 841-9.
- LIU, Y., DAI, B., FU, L., JIA, J. & MEI, C. 2010b. Rosiglitazone inhibits cell proliferation by inducing G1 cell cycle arrest and apoptosis in ADPKD cyst-lining epithelia cells. *Basic Clin Pharmacol Toxicol*, 106, 523-30.
- LIU, Z., SUN, C., OLEJNICZAK, E. T., MEADOWS, R. P., BETZ, S. F., OOST, T., HERRMANN, J., WU, J. C. & FESIK, S. W. 2000. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature*, 408, 1004-8.
- LOMONOSOVA, E., RYERSE, J. & CHINNADURAI, G. 2009. BAX/BAK-independent mitoptosis during cell death induced by proteasome inhibition? *Mol Cancer Res*, 7, 1268-84.
- LUCKEN-ARDJOMANDE, S., MONTESSUIT, S. & MARTINO, J. C. 2008. Bax activation and stress-induced apoptosis delayed by the accumulation of cholesterol in mitochondrial membranes. *Cell Death Differ*, 15, 484-93.
- MACE, P. D., SHIRLEY, S. & DAY, C. L. 2010. Assembling the building blocks: structure and function of inhibitor of apoptosis proteins. *Cell Death Differ*, 17, 46-53.
- MACHIDA, K., OHTA, Y. & OSADA, H. 2006. Suppression of apoptosis by cyclophilin D via stabilization of hexokinase II mitochondrial binding in cancer cells. *J Biol Chem*, 281, 14314-20.
- MAHALINGAM, D., SZEGEZDI, E., KEANE, M., JONG, S. & SAMALI, A. 2009. TRAIL receptor signalling and modulation: Are we on the right TRAIL? *Cancer Treat Rev*, 35, 280-8.
- MAJEWSKI, N., NOGUEIRA, V., BHASKAR, P., COY, P. E., SKEEN, J. E., GOTTLOB, K., CHANDEL, N. S., THOMPSON, C. B., ROBEY, R. B. & HAY, N. 2004a. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol Cell*, 16, 819-30.
- MAJEWSKI, N., NOGUEIRA, V., ROBEY, R. B. & HAY, N. 2004b. Akt inhibits apoptosis downstream of BID cleavage via a glucose-dependent mechanism involving mitochondrial hexokinases. *Mol Cell Biol*, 24, 730-40.
- MAKI, C. G., HUIBREGTSE, J. M. & HOWLEY, P. M. 1996. In vivo ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Res*, 56, 2649-54.
- MALIA, T. J. & WAGNER, G. 2007. NMR structural investigation of the mitochondrial outer membrane protein VDAC and its interaction with antiapoptotic Bcl-xL. *Biochemistry*, 46, 514-25.
- MANN, S. S. & HAMMARBACK, J. A. 1994. Molecular characterization of light chain 3. A microtubule binding subunit of MAP1A and MAP1B. *J Biol Chem*, 269, 11492-7.

- MANNING, L. S., WHITAKER, D., MURCH, A. R., GARLEPP, M. J., DAVIS, M. R., MUSK, A. W. & ROBINSON, B. W. 1991. Establishment and characterization of five human malignant mesothelioma cell lines derived from pleural effusions. *Int J Cancer*, 47, 285-90.
- MARCONDES, M. C., SOLA-PENNA, M. & ZANCAN, P. 2010. Clotrimazole potentiates the inhibitory effects of ATP on the key glycolytic enzyme 6-phosphofructo-1-kinase. *Arch Biochem Biophys*, 497, 62-7.
- MASOOD, A., SHER, T., PAULUS, A., MILLER, K. C., CHITTA, K. S. & CHANAN-KHAN, A. 2011. Targeted treatment for chronic lymphocytic leukemia. *Onco Targets Ther*, 4, 169-83.
- MATSUI, H., SAKANASHI, Y., OYAMA, T. M., OYAMA, Y., YOKOTA, S., ISHIDA, S., OKANO, Y., OYAMA, T. B. & NISHIMURA, Y. 2008. Imidazole antifungals, but not triazole antifungals, increase membrane Zn²⁺ permeability in rat thymocytes Possible contribution to their cytotoxicity. *Toxicology*, 248, 142-50.
- MCMANUS, D. C., LEFEBVRE, C. A., CHERTON-HORVAT, G., ST-JEAN, M., KANDIMALLA, E. R., AGRAWAL, S., MORRIS, S. J., DURKIN, J. P. & LACASSE, E. C. 2004. Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. *Oncogene*, 23, 8105-17.
- MEIRA, D. D., MARINHO-CARVALHO, M. M., TEIXEIRA, C. A., VEIGA, V. F., DA POIAN, A. T., HOLANDINO, C., DE FREITAS, M. S. & SOLA-PENNA, M. 2005. Clotrimazole decreases human breast cancer cells viability through alterations in cytoskeleton-associated glycolytic enzymes. *Mol Genet Metab*, 84, 354-62.
- MERINO, J. J. & CORDERO-CAMPANA, M. I. 1998. [Molecular bases of the programmed cell death process: implications of tumor suppressor protein p53 and other proteins in the control of cell cycle. Mechanisms of apoptotic action. Review]. *Invest Clin*, 39, 323-58.
- MICHALIK, L., DESVERGNE, B. & WAHLI, W. 2004. Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nat Rev Cancer*, 4, 61-70.
- MICHEAU, O. & TSCHOPP, J. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell*, 114, 181-90.
- MITA, A. C., MITA, M. M., NAWROCKI, S. T. & GILES, F. J. 2008. Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. *Clin Cancer Res*, 14, 5000-5.
- MIZUSHIMA, N., LEVINE, B., CUERVO, A. M. & KLIONSKY, D. J. 2008. Autophagy fights disease through cellular self-digestion. *Nature*, 451, 1069-75.
- MIZUSHIMA, N. & YOSHIMORI, T. 2007. How to interpret LC3 immunoblotting. *Autophagy*, 3, 542-5.
- MIZUTANI, Y., NAKANISHI, H., YAMAMOTO, K., LI, Y. N., MATSUBARA, H., MIKAMI, K., OKIHARA, K., KAWAUCHI, A., BONAVIDA, B. & MIKI, T. 2005. Downregulation of Smac/DIABLO expression in renal cell carcinoma and its prognostic significance. *J Clin Oncol*, 23, 448-54.
- MODY, M., DHARKER, N., BLOOMSTON, M., WANG, P. S., CHOU, F. S., GLICKMAN, T. S., MCCAFFREY, T., YANG, Z., PUMFERY, A., LEE, D., RINGEL, M. D. & PINZONE, J. J. 2007. Rosiglitazone sensitizes MDA-MB-231 breast cancer cells to anti-tumour effects of tumour necrosis factor-alpha, CH11 and CYC202. *Endocr Relat Cancer*, 14, 305-15.
- MORENO-SANCHEZ, R., RODRIGUEZ-ENRIQUEZ, S., MARIN-HERNANDEZ, A. & SAAVEDRA, E. 2007. Energy metabolism in tumor cells. *FEBS J*, 274, 1393-418.
- MOSSMAN, B. T. & CHURG, A. 1998. Mechanisms in the pathogenesis of asbestosis and silicosis. *Am J Respir Crit Care Med*, 157, 1666-80.

- MOZAFFAR, S., UEDA, M., KITATSUJI, K., SHIMIZU, S., OSUMI, M. & TANAKA, A. 1986. Properties of catalase purified from a methanol-grown yeast, *Kloeckera* sp. 2201. *Eur J Biochem*, 155, 527-31.
- MUTSAERS, S. E. 2004. The mesothelial cell. *Int J Biochem Cell Biol*, 36, 9-16.
- NADAL, M. & GOLD, S. E. 2012. Assessment of autophagosome formation by transmission electron microscopy. *Methods Mol Biol*, 835, 481-9.
- NAHLE, Z. 2004. PPAR trilogy from metabolism to cancer. *Curr Opin Clin Nutr Metab Care*, 7, 397-402.
- NAKAGAWA, Y., ABE, S., KURATA, M., HASEGAWA, M., YAMAMOTO, K., INOUE, M., TAKEMURA, T., SUZUKI, K. & KITAGAWA, M. 2006. IAP family protein expression correlates with poor outcome of multiple myeloma patients in association with chemotherapy-induced overexpression of multidrug resistance genes. *Am J Hematol*, 81, 824-31.
- NAKAHARA, T., TAKEUCHI, M., KINOYAMA, I., MINEMATSU, T., SHIRASUNA, K., MATSUHISA, A., KITA, A., TOMINAGA, F., YAMANAKA, K., KUDOH, M. & SASAMATA, M. 2007. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. *Cancer Res*, 67, 8014-21.
- NIKOLOVSKA-COLESKA, Z., WANG, R., FANG, X., PAN, H., TOMITA, Y., LI, P., ROLLER, P. P., KRAJEWSKI, K., SAITO, N. G., STUCKEY, J. A. & WANG, S. 2004a. Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. *Anal Biochem*, 332, 261-73.
- NIKOLOVSKA-COLESKA, Z., XU, L., HU, Z., TOMITA, Y., LI, P., ROLLER, P. P., WANG, R., FANG, X., GUO, R., ZHANG, M., LIPPMAN, M. E., YANG, D. & WANG, S. 2004b. Discovery of embelin as a cell-permeable, small-molecular weight inhibitor of XIAP through structure-based computational screening of a traditional herbal medicine three-dimensional structure database. *J Med Chem*, 47, 2430-40.
- O'DWYER, M. E. & DRUKER, B. J. 2000. STI571: an inhibitor of the BCR-ABL tyrosine kinase for the treatment of chronic myelogenous leukaemia. *Lancet Oncol*, 1, 207-11.
- O'SULLIVAN-COYNE, G., O'SULLIVAN, G. C., O'DONOVAN, T. R., PIWOCKA, K. & MCKENNA, S. L. 2009. Curcumin induces apoptosis-independent death in oesophageal cancer cells. *Br J Cancer*, 101, 1585-95.
- OIKAWA, T., UNNO, Y., MATSUNO, K., SAWADA, J., OGO, N., TANAKA, K. & ASAI, A. 2010. Identification of a small-molecule inhibitor of the interaction between Survivin and Smac/DIABLO. *Biochem Biophys Res Commun*, 393, 253-8.
- OOST, T. K., SUN, C., ARMSTRONG, R. C., AL-ASSAAD, A. S., BETZ, S. F., DECKWERTH, T. L., DING, H., ELMORE, S. W., MEADOWS, R. P., OLEJNICZAK, E. T., OLEKSIJEW, A., OLTERSDORF, T., ROSENBERG, S. H., SHOEMAKER, A. R., TOMASELLI, K. J., ZOU, H. & FESIK, S. W. 2004. Discovery of potent antagonists of the antiapoptotic protein XIAP for the treatment of cancer. *J Med Chem*, 47, 4417-26.
- ORLOWSKI, R. Z., STINCHCOMBE, T. E., MITCHELL, B. S., SHEA, T. C., BALDWIN, A. S., STAHL, S., ADAMS, J., ESSELTINE, D. L., ELLIOTT, P. J., PIEN, C. S., GUERCIOLINI, R., ANDERSON, J. K., DEPCIK-SMITH, N. D., BHAGAT, R., LEHMAN, M. J., NOVICK, S. C., O'CONNOR, O. A. & SOIGNET, S. L. 2002. Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies. *J Clin Oncol*, 20, 4420-7.
- OTT, M., GOGVADZE, V., ORRENIUS, S. & ZHIVOTOVSKY, B. 2007. Mitochondria, oxidative stress and cell death. *Apoptosis*, 12, 913-22.
- OWA, T., YOSHINO, H., YOSHIMATSU, K. & NAGASU, T. 2001. Cell cycle regulation in the G1 phase: a promising target for the development of new chemotherapeutic anticancer agents. *Curr Med Chem*, 8, 1487-503.

- OYAMA, T. M., OYAMA, T. B., OYAMA, K., SAKANASHI, Y., MORIMOTO, M., MATSUI, H. & OYAMA, Y. 2007. Clotrimazole, an antifungal drug possessing diverse actions, increases membrane permeation of cadmium in rat thymocytes. *Toxicol In Vitro*, 21, 1505-12.
- PALCHAUDHURI, R., NESTERENKO, V. & HERGENROTHER, P. J. 2008. The complex role of the triphenylmethyl motif in anticancer compounds. *J Am Chem Soc*, 130, 10274-81.
- PARK, C., JIN, C. Y., KIM, G. Y., CHEONG, J., JUNG, J. H., YOO, Y. H. & CHOI, Y. H. 2010. A methyl jasmonate derivative, J-7, induces apoptosis in human hepatocarcinoma Hep3B cells in vitro. *Toxicol In Vitro*, 24, 1920-6.
- PARTANEN, R., KOSKINEN, H. & HEMMINKI, K. 1995. Tumour necrosis factor-alpha (TNF-alpha) in patients who have asbestosis and develop cancer. *Occup Environ Med*, 52, 316-9.
- PASTORINO, J. G. & HOEK, J. B. 2008. Regulation of hexokinase binding to VDAC. *J Bioenerg Biomembr*, 40, 171-82.
- PASTORINO, J. G., SHULGA, N. & HOEK, J. B. 2002. Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. *J Biol Chem*, 277, 7610-8.
- PENG, L., JIANG, H. & BRADELY, C. 2002. Detection of B lymphoma cells undergoing apoptosis by Annexin-V assay. *Chin Med Sci J*, 17, 17-21.
- PENNATI, M., FOLINI, M. & ZAFFARONI, N. 2007. Targeting survivin in cancer therapy: fulfilled promises and open questions. *Carcinogenesis*, 28, 1133-9.
- PENSO, J. & BEITNER, R. 1998. Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells. *Eur J Pharmacol*, 342, 113-7.
- PERCY, M. E. 1984. Catalase: an old enzyme with a new role? *Can J Biochem Cell Biol*, 62, 1006-14.
- PETERSEN, S. L., PEYTON, M., MINNA, J. D. & WANG, X. 2010. Overcoming cancer cell resistance to Smac mimetic induced apoptosis by modulating cIAP-2 expression. *Proc Natl Acad Sci U S A*, 107, 11936-41.
- PETERSEN, S. L., WANG, L., YALCIN-CHIN, A., LI, L., PEYTON, M., MINNA, J., HARRAN, P. & WANG, X. 2007. Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. *Cancer Cell*, 12, 445-56.
- PLESCIA, J., SALZ, W., XIA, F., PENNATI, M., ZAFFARONI, N., DAIDONE, M. G., MELI, M., DOHI, T., FORTUGNO, P., NEFEDOVA, Y., GABRILOVICH, D. I., COLOMBO, G. & ALTIERI, D. C. 2005. Rational design of shepherdin, a novel anticancer agent. *Cancer Cell*, 7, 457-68.
- PORTER, J. R., FRITZ, C. C. & DEPEW, K. M. 2010. Discovery and development of Hsp90 inhibitors: a promising pathway for cancer therapy. *Curr Opin Chem Biol*.
- PROBST, B. L., LIU, L., RAMESH, V., LI, L., SUN, H., MINNA, J. D. & WANG, L. 2010. Smac mimetics increase cancer cell response to chemotherapeutics in a TNF-alpha-dependent manner. *Cell Death Differ*, 17, 1645-54.
- RAHIM, S., BEAUCHAMP, E. M., KONG, Y., BROWN, M. L., TORETSKY, J. A. & UREN, A. 2011. YK-4-279 inhibits ERG and ETV1 mediated prostate cancer cell invasion. *PLoS One*, 6, e19343.
- RAVAGNAN, L., ROUMIER, T. & KROEMER, G. 2002. Mitochondria, the killer organelles and their weapons. *J Cell Physiol*, 192, 131-7.

- REDDY, R. C., SRIRANGAM, A., REDDY, K., CHEN, J., GANGIREDDY, S., KALEMKERIAN, G. P., STANDIFORD, T. J. & KESHAMOUNI, V. G. 2008. Chemotherapeutic drugs induce PPAR-gamma expression and show sequence-specific synergy with PPAR-gamma ligands in inhibition of non-small cell lung cancer. *Neoplasia*, 10, 597-603.
- RICCABONI, M., BIANCHI, I. & PETRILLO, P. 2010. Spleen tyrosine kinases: biology, therapeutic targets and drugs. *Drug Discov Today*, 15, 517-30.
- RICCARDI, C. & NICOLETTI, I. 2006. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc*, 1, 1458-61.
- RICE, D. C., STEVENS, C. W., CORREA, A. M., VAPORCIYAN, A. A., TSAO, A., FORSTER, K. M., WALSH, G. L., SWISHER, S. G., HOFSTETTER, W. L., MEHRAN, R. J., ROTH, J. A., LIAO, Z. & SMYTHE, W. R. 2007. Outcomes after extrapleural pneumonectomy and intensity-modulated radiation therapy for malignant pleural mesothelioma. *Ann Thorac Surg*, 84, 1685-92; discussion 1692-3.
- RIEDL, S. J., RENATUS, M., SCHWARZENBACHER, R., ZHOU, Q., SUN, C., FESIK, S. W., LIDDINGTON, R. C. & SALVESEN, G. S. 2001. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*, 104, 791-800.
- RIEDL, S. J. & SALVESEN, G. S. 2007. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol*, 8, 405-13.
- ROBEY, R. B. & HAY, N. 2006. Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. *Oncogene*, 25, 4683-96.
- ROSEN, E. D. & SPIEGELMAN, B. M. 2001. PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. *J Biol Chem*, 276, 37731-4.
- ROTHER, M., PAN, M. G., HENZEL, W. J., AYRES, T. M. & GOEDEL, D. V. 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell*, 83, 1243-52.
- SAMUEL, T., WELSH, K., LOBER, T., TOGO, S. H., ZAPATA, J. M. & REED, J. C. 2006. Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. *J Biol Chem*, 281, 1080-90.
- SANCHIS-SEGURA, C., MIQUEL, M., CORREA, M. & ARAGON, C. M. 1999. The catalase inhibitor sodium azide reduces ethanol-induced locomotor activity. *Alcohol*, 19, 37-42.
- SARTORE-BIANCHI, A., GASPARRI, F., GALVANI, A., NICI, L., DARNOWSKI, J. W., BARBONE, D., FENNELL, D. A., GAUDINO, G., PORTA, C. & MUTTI, L. 2007. Bortezomib inhibits nuclear factor-kappaB dependent survival and has potent in vivo activity in mesothelioma. *Clin Cancer Res*, 13, 5942-51.
- SCHIMMER, A. D., DALILI, S., BATEY, R. A. & RIEDL, S. J. 2006. Targeting XIAP for the treatment of malignancy. *Cell Death Differ*, 13, 179-88.
- SCHNEIDER, P. & TSCHOPP, J. 2000. Apoptosis induced by death receptors. *Pharm Acta Helv*, 74, 281-6.
- SCHRADER, M. & FAHIMI, H. D. 2008. The peroxisome: still a mysterious organelle. *Histochem Cell Biol*, 129, 421-40.
- SCHRADER, M., REUBER, B. E., MORRELL, J. C., JIMENEZ-SANCHEZ, G., OBIE, C., STROH, T. A., VALLE, D., SCHROER, T. A. & GOULD, S. J. 1998. Expression of PEX11beta mediates peroxisome proliferation in the absence of extracellular stimuli. *J Biol Chem*, 273, 29607-14.
- SCHWARTZ, A. L. & CIECHANOVER, A. 1999. The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annu Rev Med*, 50, 57-74.

- SCOTT, F. L., DENAULT, J. B., RIEDL, S. J., SHIN, H., RENATUS, M. & SALVESEN, G. S. 2005. XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *EMBO J*, 24, 645-55.
- SCOTT, K. A., ARNOTT, C. H., ROBINSON, S. C., MOORE, R. J., THOMPSON, R. G., MARSHALL, J. F. & BALKWILL, F. R. 2004. TNF-alpha regulates epithelial expression of MMP-9 and integrin alphavbeta6 during tumour promotion. A role for TNF-alpha in keratinocyte migration? *Oncogene*, 23, 6954-66.
- SEARGENT, J. M., YATES, E. A. & GILL, J. H. 2004. GW9662, a potent antagonist of PPARgamma, inhibits growth of breast tumour cells and promotes the anticancer effects of the PPARgamma agonist rosiglitazone, independently of PPARgamma activation. *Br J Pharmacol*, 143, 933-7.
- SEGLIN, P. O. & GORDON, P. B. 1982. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci U S A*, 79, 1889-92.
- SEN, C. K. & PACKER, L. 1996. Antioxidant and redox regulation of gene transcription. *FASEB J*, 10, 709-20.
- SENECI, P., BIANCHI, A., BATTAGLIA, C., BELVISI, L., BOLOGNESI, M., CAPRINI, A., COSSU, F., FRANCO, E., MATTEO, M., DELIA, D., DRAGO, C., KHALED, A., LECIS, D., MANZONI, L., MARIZZONI, M., MASTRANGELO, E., MILANI, M., MOTTO, I., MORONI, E., POTENZA, D., RIZZO, V., SERVIDA, F., TURLIZZI, E., VARRONE, M., VASILE, F. & SCOLASTICO, C. 2009. Rational design, synthesis and characterization of potent, non-peptidic Smac mimics/XIAP inhibitors as proapoptotic agents for cancer therapy. *Bioorg Med Chem*, 17, 5834-56.
- SENSINTAFFAR, J., SCOTT, F. L., PEACH, R. & HAGER, J. H. 2010. XIAP is not required for human tumor cell survival in the absence of an exogenous death signal. *BMC Cancer*, 10, 11.
- SHACKA, J. J., KLOCKE, B. J., SHIBATA, M., UCHIYAMA, Y., DATTA, G., SCHMIDT, R. E. & ROTH, K. A. 2006. Bafilomycin A1 inhibits chloroquine-induced death of cerebellar granule neurons. *Mol Pharmacol*, 69, 1125-36.
- SHARMA, B., DEO, S. K., BACHAS, L. G. & DAUNERT, S. 2005. Competitive binding assay using fluorescence resonance energy transfer for the identification of calmodulin antagonists. *Bioconjug Chem*, 16, 1257-63.
- SHI, Y. 2004. Caspase activation: revisiting the induced proximity model. *Cell*, 117, 855-8.
- SHIMIZU, M., TAKESHITA, A., TSUKAMOTO, T., GONZALEZ, F. J. & OSUMI, T. 2004. Tissue-selective, bidirectional regulation of PEX11 alpha and perilipin genes through a common peroxisome proliferator response element. *Mol Cell Biol*, 24, 1313-23.
- SHIMIZU, S., NARITA, M. & TSUJIMOTO, Y. 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature*, 399, 483-7.
- SHIOZAKI, E. N., CHAI, J., RIGOTTI, D. J., RIEDL, S. J., LI, P., SRINIVASULA, S. M., ALNEMRI, E. S., FAIRMAN, R. & SHI, Y. 2003. Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell*, 11, 519-27.
- SHIOZAKI, E. N. & SHI, Y. 2004. Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. *Trends Biochem Sci*, 29, 486-94.
- SHOSHAN-BARMATZ, V., KEINAN, N., ABU-HAMAD, S., TYOMKIN, D. & ARAM, L. 2010. Apoptosis is regulated by the VDAC1 N-terminal region and by VDAC oligomerization: release of cytochrome c, AIF and Smac/Diablo. *Biochim Biophys Acta*, 1797, 1281-91.

- SHU, H. B., TAKEUCHI, M. & GOEDEL, D. V. 1996. The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. *Proc Natl Acad Sci U S A*, 93, 13973-8.
- SHULGA, N., WILSON-SMITH, R. & PASTORINO, J. G. 2009. Hexokinase II detachment from the mitochondria potentiates cisplatin induced cytotoxicity through a caspase-2 dependent mechanism. *Cell Cycle*, 8, 3355-64.
- SIEGELIN, M. D., GAISER, T. & SIEGELIN, Y. 2009. The XIAP inhibitor Embelin enhances TRAIL-mediated apoptosis in malignant glioma cells by down-regulation of the short isoform of FLIP. *Neurochem Int*, 55, 423-30.
- SILKE, J. & BRINK, R. 2010. Regulation of TNFRSF and innate immune signalling complexes by TRAFs and cIAPs. *Cell Death Differ*, 17, 35-45.
- SINGH, N. P., MCCOY, M. T., TICE, R. R. & SCHNEIDER, E. L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*, 175, 184-91.
- SKAFF, D. A., KIM, C. S., TSAI, H. J., HONZATKO, R. B. & FROMM, H. J. 2005. Glucose 6-phosphate release of wild-type and mutant human brain hexokinases from mitochondria. *J Biol Chem*, 280, 38403-9.
- SKOUFIAS, D. A., MOLLINARI, C., LACROIX, F. B. & MARGOLIS, R. L. 2000. Human survivin is a kinetochore-associated passenger protein. *J Cell Biol*, 151, 1575-82.
- SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. & KLENK, D. C. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem*, 150, 76-85.
- SNAJDROVA, L., XU, A. & NARAYANAN, N. 1998. Clotrimazole, an antimycotic drug, inhibits the sarcoplasmic reticulum calcium pump and contractile function in heart muscle. *J Biol Chem*, 273, 28032-9.
- SOBOLOFF, J. & BERGER, S. A. 2002. Sustained ER Ca²⁺ depletion suppresses protein synthesis and induces activation-enhanced cell death in mast cells. *J Biol Chem*, 277, 13812-20.
- SRINIVASULA, S. M., HEGDE, R., SALEH, A., DATTA, P., SHIOZAKI, E., CHAI, J., LEE, R. A., ROBBINS, P. D., FERNANDES-ALNEMRI, T., SHI, Y. & ALNEMRI, E. S. 2001. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature*, 410, 112-6.
- SUGANUMA, M., OKABE, S., MARINO, M. W., SAKAI, A., SUEOKA, E. & FUJIKI, H. 1999. Essential role of tumor necrosis factor alpha (TNF-alpha) in tumor promotion as revealed by TNF-alpha-deficient mice. *Cancer Res*, 59, 4516-8.
- SUN, C., CAI, M., GUNASEKERA, A. H., MEADOWS, R. P., WANG, H., CHEN, J., ZHANG, H., WU, W., XU, N., NG, S. C. & FESIK, S. W. 1999. NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. *Nature*, 401, 818-22.
- SUN, C., CAI, M., MEADOWS, R. P., XU, N., GUNASEKERA, A. H., HERRMANN, J., WU, J. C. & FESIK, S. W. 2000. NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP. *J Biol Chem*, 275, 33777-81.
- SUN, H., NIKOLOVSKA-COLESKA, Z., YANG, C. Y., XU, L., TOMITA, Y., KRAJEWSKI, K., ROLLER, P. P. & WANG, S. 2004. Structure-based design, synthesis, and evaluation of conformationally constrained mimetics of the second mitochondria-derived activator of caspase that target the X-linked inhibitor of apoptosis protein/caspase-9 interaction site. *J Med Chem*, 47, 4147-50.

- SUN, Y., OTTOSSON, A., PERVAIZ, S. & FADEEL, B. 2007. Smac-mediated sensitization of human B-lymphoma cells to staurosporine- and lactacystin-triggered apoptosis is apoptosome-dependent. *Leukemia*, 21, 1035-43.
- SUNG, K. W., CHOI, J., HWANG, Y. K., LEE, S. J., KIM, H. J., KIM, J. Y., CHO, E. J., YOO, K. H. & KOO, H. H. 2009. Overexpression of X-linked inhibitor of apoptosis protein (XIAP) is an independent unfavorable prognostic factor in childhood de novo acute myeloid leukemia. *J Korean Med Sci*, 24, 605-13.
- SUZUKI, Y., NAKABAYASHI, Y. & TAKAHASHI, R. 2001. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc Natl Acad Sci U S A*, 98, 8662-7.
- TAKATSUKA, C., INOUE, Y., MATSUOKA, K. & MORIYASU, Y. 2004. 3-methyladenine inhibits autophagy in tobacco culture cells under sucrose starvation conditions. *Plant Cell Physiol*, 45, 265-74.
- TAMAI, M., YOKOO, C., MURATA, M., OGUMA, K., SOTA, K., SATO, E. & KANAOKA, Y. 1987. Efficient synthetic method for ethyl (+)-(2S,3S)-3-[(S)-3-methyl- 1-(3-methylbutylcarbamoyl)butylcarbamoyl]-2-oxiranecarboxylate (EST), a new inhibitor of cysteine proteinases. *Chem Pharm Bull (Tokyo)*, 35, 1098-104.
- TANAKA, S., CHOE, N., IWAGAKI, A., HEMENWAY, D. R. & KAGAN, E. 2000. Asbestos exposure induces MCP-1 secretion by pleural mesothelial cells. *Exp Lung Res*, 26, 241-55.
- TANIDA, I., MINEMATSU-KEGUCHI, N., UENO, T. & KOMINAMI, E. 2005. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy*, 1, 84-91.
- THEOCHARIS, S., MARGELI, A., VIELH, P. & KOURAKLIS, G. 2004. Peroxisome proliferator-activated receptor-gamma ligands as cell-cycle modulators. *Cancer Treat Rev*, 30, 545-54.
- TOLEDO, F. & WAHL, G. M. 2006. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer*, 6, 909-23.
- TOMEK, S., EMRI, S., KREJCY, K. & MANEGOLD, C. 2003. Chemotherapy for malignant pleural mesothelioma: past results and recent developments. *Br J Cancer*, 88, 167-74.
- TONG, W., WELSH, W. J., SHI, L. M., FANG, H. & PERKINS, R. 2003. Structure-activity relationship approaches and applications. *Environmental Toxicology and Chemistry*, 22, 1680-1695.
- TORETSKY, J. A., ERKIZAN, V., LEVENSON, A., ABAAN, O. D., PARVIN, J. D., CRIPE, T. P., RICE, A. M., LEE, S. B. & UREN, A. 2006. Oncoprotein EWS-FLI1 activity is enhanced by RNA helicase A. *Cancer Res*, 66, 5574-81.
- TOYOSHIMA, H. & HUNTER, T. 1994. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell*, 78, 67-74.
- TRAENCKNER, E. B., WILK, S. & BAEUERLE, P. A. 1994. A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. *EMBO J*, 13, 5433-41.
- TROSKEN, E. R., FISCHER, K., VOLKEL, W. & LUTZ, W. K. 2006. Inhibition of human CYP19 by azoles used as antifungal agents and aromatase inhibitors, using a new LC-MS/MS method for the analysis of estradiol product formation. *Toxicology*, 219, 33-40.
- TROSKEN, E. R., SCHOLZ, K., LUTZ, R. W., VOLKEL, W., ZARN, J. A. & LUTZ, W. K. 2004. Comparative assessment of the inhibition of recombinant human CYP19 (aromatase) by azoles used in agriculture and as drugs for humans. *Endocr Res*, 30, 387-94.

- TSANG, W. P., CHAU, S. P., KONG, S. K., FUNG, K. P. & KWOK, T. T. 2003. Reactive oxygen species mediate doxorubicin induced p53-independent apoptosis. *Life Sci*, 73, 2047-58.
- TUNG, Y. T., HSU, W. M., LEE, H., HUANG, W. P. & LIAO, Y. F. 2010. The evolutionarily conserved interaction between LC3 and p62 selectively mediates autophagy-dependent degradation of mutant huntingtin. *Cell Mol Neurobiol*, 30, 795-806.
- TURAN, V. K., MISHIN, V. M. & THOMAS, P. E. 2001. Clotrimazole is a selective and potent inhibitor of rat cytochrome P450 3A subfamily-related testosterone metabolism. *Drug Metab Dispos*, 29, 837-42.
- UMEZAWA, H., AOYAGI, T., MORISHIMA, H., MATSUZAKI, M. & HAMADA, M. 1970. Pepstatin, a new pepsin inhibitor produced by Actinomycetes. *J Antibiot (Tokyo)*, 23, 259-62.
- VAN ZANDWIJK, N. 1995. N-acetylcysteine (NAC) and glutathione (GSH): antioxidant and chemopreventive properties, with special reference to lung cancer. *J Cell Biochem Suppl*, 22, 24-32.
- VANDER HEIDEN, M. G., CANTLEY, L. C. & THOMPSON, C. B. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*, 324, 1029-33.
- VARFOLOMEEV, E., BLANKENSHIP, J. W., WAYSON, S. M., FEDOROVA, A. V., KAYAGAKI, N., GARG, P., ZOBEL, K., DYNEK, J. N., ELLIOTT, L. O., WALLWEBER, H. J., FLYGARE, J. A., FAIRBROTHER, W. J., DESHAYES, K., DIXIT, V. M. & VUCIC, D. 2007. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell*, 131, 669-81.
- VASSILEV, L. T., VU, B. T., GRAVES, B., CARVAJAL, D., PODLASKI, F., FILIPOVIC, Z., KONG, N., KAMMLOTT, U., LUKACS, C., KLEIN, C., FOTOUHI, N. & LIU, E. A. 2004. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*, 303, 844-8.
- VILLOUTREIX, B. O., LACONDE, G., LAGORCE, D., MARTINEAU, P., MITEVA, M. A. & DARIAVACH, P. 2011. Tyrosine kinase syk non-enzymatic inhibitors and potential anti-allergic drug-like compounds discovered by virtual and in vitro screening. *PLoS One*, 6, e21117.
- VINCE, J. E., WONG, W. W., KHAN, N., FELTHAM, R., CHAU, D., AHMED, A. U., BENETATOS, C. A., CHUNDURU, S. K., CONDON, S. M., MCKINLAY, M., BRINK, R., LEVERKUS, M., TERGAONKAR, V., SCHNEIDER, P., CALLUS, B. A., KOENTGEN, F., VAUX, D. L. & SILKE, J. 2007. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell*, 131, 682-93.
- VOGELZANG, N. J., PORTA, C. & MUTTI, L. 2005. New agents in the management of advanced mesothelioma. *Semin Oncol*, 32, 336-50.
- VOGELZANG, N. J., RUSTHOVEN, J. J., SYMANOWSKI, J., DENHAM, C., KAUKELE, E., RUFFIE, P., GATZEMEIER, U., BOYER, M., EMRI, S., MANEGOLD, C., NIYIKIZA, C. & PAOLETTI, P. 2003. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J Clin Oncol*, 21, 2636-44.
- VUCIC, D., DESHAYES, K., ACKERLY, H., PISABARRO, M. T., KADKHODAYAN, S., FAIRBROTHER, W. J. & DIXIT, V. M. 2002. SMAC negatively regulates the anti-apoptotic activity of melanoma inhibitor of apoptosis (ML-IAP). *J Biol Chem*, 277, 12275-9.
- VUCIC, D., FRANKLIN, M. C., WALLWEBER, H. J., DAS, K., ECKELMAN, B. P., SHIN, H., ELLIOTT, L. O., KADKHODAYAN, S., DESHAYES, K., SALVESEN, G. S. & FAIRBROTHER, W. J. 2005. Engineering ML-IAP to produce an extraordinarily potent caspase 9 inhibitor: implications for Smac-dependent anti-apoptotic activity of ML-IAP. *Biochem J*, 385, 11-20.
- VUCIC, D., STENNICKE, H. R., PISABARRO, M. T., SALVESEN, G. S. & DIXIT, V. M. 2000. ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. *Curr Biol*, 10, 1359-66.

- WANG, C. Y., MAYO, M. W., KORNELUK, R. G., GOEDEL, D. V. & BALDWIN, A. S., JR. 1998. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science*, 281, 1680-3.
- WANG, L., DU, F. & WANG, X. 2008. TNF-alpha induces two distinct caspase-8 activation pathways. *Cell*, 133, 693-703.
- WANG, X., XU, J., JU, S., NI, H., ZHU, J. & WANG, H. 2010. Livin gene plays a role in drug resistance of colon cancer cells. *Clin Biochem*, 43, 655-60.
- WARBURG, O., WIND, F. & NEGELEIN, E. 1927. THE METABOLISM OF TUMORS IN THE BODY. *J Gen Physiol*, 8, 519-30.
- WATSON, A. F., LIU, J., BENNACEUR, K., DRUMMOND, C. J., ENDICOTT, J. A., GOLDING, B. T., GRIFFIN, R. J., HAGGERTY, K., LU, X., MCDONNELL, J. M., NEWELL, D. R., NOBLE, M. E., REVILL, C. H., RIEDINGER, C., XU, Q., ZHAO, Y., LUNEC, J. & HARDCASTLE, I. R. 2011. MDM2-p53 protein-protein interaction inhibitors: a-ring substituted isoindolinones. *Bioorg Med Chem Lett*, 21, 5916-9.
- WERTZ, I. E. & DIXIT, V. M. 2010. Regulation of death receptor signaling by the ubiquitin system. *Cell Death Differ*, 17, 14-24.
- WHO-STATISTICS 2010. WHO statistics - <http://www.who.int/cancer/en/>.
- WIDLAK, P. & GARRARD, W. T. 2005. Discovery, regulation, and action of the major apoptotic nucleases DFF40/CAD and endonuclease G. *J Cell Biochem*, 94, 1078-87.
- WILSON, E. N., BRISTOL, M. L., DI, X., MALTESE, W. A., KOTERBA, K., BECKMAN, M. J. & GEWIRTZ, D. A. 2011. A switch between cytoprotective and cytotoxic autophagy in the radiosensitization of breast tumor cells by chloroquine and vitamin D. *Horm Cancer*, 2, 272-85.
- WILSON, J. E. 2003. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *J Exp Biol*, 206, 2049-57.
- WILSON, L. & JORDAN, M. A. 2004. New microtubule/tubulin-targeted anticancer drugs and novel chemotherapeutic strategies. *J Chemother*, 16 Suppl 4, 83-5.
- WONG, S. W., TIONG, K. H., KONG, W. Y., YUE, Y. C., CHUA, C. H., LIM, J. Y., LEE, C. Y., QUAH, S. I., FOW, C., CHUNG, C., SO, I., TAN, B. S., CHOO, H. L., ROSLI, R., CHEONG, S. K. & LEONG, C. O. 2011. Rapamycin synergizes cisplatin sensitivity in basal-like breast cancer cells through up-regulation of p73. *Breast Cancer Res Treat*, 128, 301-13.
- WRIGHT, M. E., HAN, D. K. & HOCKENBERY, D. M. 2000. Caspase-3 and inhibitor of apoptosis protein(s) interactions in *Saccharomyces cerevisiae* and mammalian cells. *FEBS Lett*, 481, 13-8.
- WU, C. H., JENG, J. H., WANG, Y. J., TSENG, C. J., LIANG, Y. C., CHEN, C. H., LEE, H. M., LIN, J. K., LIN, C. H., LIN, S. Y., LI, C. P. & HO, Y. S. 2002. Antitumor effects of miconazole on human colon carcinoma xenografts in nude mice through induction of apoptosis and G0/G1 cell cycle arrest. *Toxicol Appl Pharmacol*, 180, 22-35.
- WU, G., CHAI, J., SUBER, T. L., WU, J. W., DU, C., WANG, X. & SHI, Y. 2000. Structural basis of IAP recognition by Smac/DIABLO. *Nature*, 408, 1008-12.
- WU, S. N., LI, H. F., JAN, C. R. & SHEN, A. Y. 1999. Inhibition of Ca²⁺-activated K⁺ current by clotrimazole in rat anterior pituitary GH3 cells. *Neuropharmacology*, 38, 979-89.

- WU, T. Y., WAGNER, K. W., BURSULAYA, B., SCHULTZ, P. G. & DEVERAUX, Q. L. 2003. Development and characterization of nonpeptidic small molecule inhibitors of the XIAP/caspase-3 interaction. *Chem Biol*, 10, 759-67.
- WU, W. K., CHO, C. H., LEE, C. W., WU, K., FAN, D., YU, J. & SUNG, J. J. 2010a. Proteasome inhibition: a new therapeutic strategy to cancer treatment. *Cancer Lett*, 293, 15-22.
- WU, W. K., CHO, C. H., LEE, C. W., WU, Y. C., YU, L., LI, Z. J., WONG, C. C., LI, H. T., ZHANG, L., REN, S. X., CHE, C. T., WU, K., FAN, D., YU, J. & SUNG, J. J. 2010b. Macroautophagy and ERK phosphorylation counteract the antiproliferative effect of proteasome inhibitor in gastric cancer cells. *Autophagy*, 6, 228-38.
- WU, W. K., SAKAMOTO, K. M., MILANI, M., ALDANA-MASANKGAY, G., FAN, D., WU, K., LEE, C. W., CHO, C. H., YU, J. & SUNG, J. J. 2010c. Macroautophagy modulates cellular response to proteasome inhibitors in cancer therapy. *Drug Resist Updat*, 13, 87-92.
- WU, W. K., WU, Y. C., YU, L., LI, Z. J., SUNG, J. J. & CHO, C. H. 2008. Induction of autophagy by proteasome inhibitor is associated with proliferative arrest in colon cancer cells. *Biochem Biophys Res Commun*, 374, 258-63.
- XIE, C., REUSSE, A., DAI, J., ZAY, K., HARNETT, J. & CHURG, A. 2000. TNF-alpha increases tracheal epithelial asbestos and fiberglass binding via a NF-kappaB-dependent mechanism. *Am J Physiol Lung Cell Mol Physiol*, 279, L608-14.
- XU, L., YIN, S., BANERJEE, S., SARKAR, F. & REDDY, K. B. 2011. Enhanced anticancer effect of the combination of cisplatin and TRAIL in triple-negative breast tumor cells. *Mol Cancer Ther*, 10, 550-7.
- XUE, C., WU, J., LAN, F., LIU, W., YANG, X., ZENG, F. & XU, H. 2010. Nano titanium dioxide induces the generation of ROS and potential damage in HaCaT cells under UVA irradiation. *J Nanosci Nanotechnol*, 10, 8500-7.
- YAMAMOTO, A., TAGAWA, Y., YOSHIMORI, T., MORIYAMA, Y., MASAKI, R. & TASHIRO, Y. 1998. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct*, 23, 33-42.
- YAN, M., RAYAPURAM, N. & SUBRAMANI, S. 2005. The control of peroxisome number and size during division and proliferation. *Curr Opin Cell Biol*, 17, 376-83.
- YANG, C., KAUSHAL, V., SHAH, S. V. & KAUSHAL, G. P. 2008. Autophagy is associated with apoptosis in cisplatin injury to renal tubular epithelial cells. *Am J Physiol Renal Physiol*, 294, F777-87.
- YANG, H., BOCCHETTA, M., KROCZYNSKA, B., ELMISHAD, A. G., CHEN, Y., LIU, Z., BUBICI, C., MOSSMAN, B. T., PASS, H. I., TESTA, J. R., FRANZOSO, G. & CARBONE, M. 2006. TNF-alpha inhibits asbestos-induced cytotoxicity via a NF-kappaB-dependent pathway, a possible mechanism for asbestos-induced oncogenesis. *Proc Natl Acad Sci U S A*, 103, 10397-402.
- YANG, L., CAO, Z., YAN, H. & WOOD, W. C. 2003a. Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. *Cancer Res*, 63, 6815-24.
- YANG, L., MASHIMA, T., SATO, S., MOCHIZUKI, M., SAKAMOTO, H., YAMORI, T., OH-HARA, T. & TSURUO, T. 2003b. Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide. *Cancer Res*, 63, 831-7.
- YANG, Q. H. & DU, C. 2004. Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells. *J Biol Chem*, 279, 16963-70.

- YANG, Z. & KLIONSKY, D. J. 2010. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol*, 22, 124-31.
- YU, X., ACEHAN, D., MENETRET, J. F., BOOTH, C. R., LUDTKE, S. J., RIEDL, S. J., SHI, Y., WANG, X. & AKEY, C. W. 2005. A structure of the human apoptosome at 12.8 Å resolution provides insights into this cell death platform. *Structure*, 13, 1725-35.
- YUAN, B. Z., CHAPMAN, J. A. & REYNOLDS, S. H. 2008. Proteasome Inhibitor MG132 Induces Apoptosis and Inhibits Invasion of Human Malignant Pleural Mesothelioma Cells. *Transl Oncol*, 1, 129-40.
- ZAFFARONI, N., PENNATI, M., COLELLA, G., PEREGO, P., SUPINO, R., GATTI, L., PILOTTI, S., ZUNINO, F. & DAIDONE, M. G. 2002. Expression of the anti-apoptotic gene survivin correlates with taxol resistance in human ovarian cancer. *Cell Mol Life Sci*, 59, 1406-12.
- ZANCAN, P., ROSAS, A. O., MARCONDES, M. C., MARINHO-CARVALHO, M. M. & SOLA-PENNA, M. 2007. Clotrimazole inhibits and modulates heterologous association of the key glycolytic enzyme 6-phosphofructo-1-kinase. *Biochem Pharmacol*, 73, 1520-7.
- ZAYTSEVA, Y. Y., WALLIS, N. K., SOUTHARD, R. C. & KILGORE, M. W. 2011. The PPARgamma antagonist T0070907 suppresses breast cancer cell proliferation and motility via both PPARgamma-dependent and -independent mechanisms. *Anticancer Res*, 31, 813-23.
- ZHANG, L., YU, J., PARK, B. H., KINZLER, K. W. & VOGELSTEIN, B. 2000. Role of BAX in the apoptotic response to anticancer agents. *Science*, 290, 989-92.
- ZHANG, Y., SOBOLOFF, J., ZHU, Z. & BERGER, S. A. 2006. Inhibition of Ca²⁺ influx is required for mitochondrial reactive oxygen species-induced endoplasmic reticulum Ca²⁺ depletion and cell death in leukemia cells. *Mol Pharmacol*, 70, 1424-34.
- ZHAO, J. M., ZHU, Z. H., WU, Q. L., HOU, J. H., HUANG, X. P., ZHANG, P. Y. & RONG, T. H. 2006. [Expression of Smac in Stage I-II non-small cell lung cancer and its clinical significance]. *Ai Zheng*, 25, 631-4.
- ZHIVOTOVSKY, B. & ORRENIUS, S. 2009. The Warburg Effect returns to the cancer stage. *Semin Cancer Biol*, 19, 1-3.
- ZHIVOTOVSKY, B. & ORRENIUS, S. 2010. Cell death mechanisms: cross-talk and role in disease. *Exp Cell Res*, 316, 1374-83.
- ZHU, K., DUNNER, K., JR. & MCCONKEY, D. J. 2010. Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells. *Oncogene*, 29, 451-62.
- ZISTERER, D. M. & WILLIAMS, D. C. 1997. Calmidazolium and other imidazole compounds affect steroidogenesis in Y1 cells: lack of involvement of the peripheral-type benzodiazepine receptor. *J Steroid Biochem Mol Biol*, 60, 189-95.