Survival Mechanisms in B Lymphoid Malignancies and Associated Therapies

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Abstract

The apoptotic pathway plays critical roles in regulating lymphocyte survival throughout B cell development, maturation and differentiation. The whole process involves clonal expansion. In the normal B lymphocyte population the majority of cells and their progenitors derived from each stage die from induction of apoptosis under specific mechanisms of control. Failure to do so can result in malignant transformation. This project focuses on apoptotic pathways and associated survival mechanisms in neoplasms of lymphoid provenance with an emphasis on B cell malignancies. The role of galectin-3, a molecule implicated in signal transduction and apoptotic pathways, has been investigated in both primary CLL cells and cell lines of human B lineage, using GCS-100, a novel galectin-3 antagonist. The potential interaction between galectin-3 and Bcl-2 and its contribution to cell death have been explored in depth. The role of NADPH oxidase and ROS in mitochondria has also been examined in the context of apoptosis. PK11195, a small pro-apoptotic molecule, has been studied in relation to mitochondria-mediated apoptosis. The above investigations could contribute to a rationale for potential novel strategies in the treatment of B cell malignancies.

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Contents

Chapters

List of figures

List of tables

Abbreviations

7-AAD	7-Aminoactinomycin D
Ab	antibody
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
AIF	apoptosis-inducing factor
Ala	alanine
AML	Acute myeloid leukaemia
ANOVA	analysis of variance
ANT	adenine nucleotide translocator
Apaf-1	apoptosis protease associated factor 1
APS	ammonium persulfate
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
Bak	Bcl-2 antagonist killer
Bax	Bcl-2 associated X protein
BCR	B cell receptor
BD	Becton Dickinson
BH	Bcl-2 homology
Bid	Bcl-2 interacting domain
BL	Burkitt lymphoma
BLNK	B cell linker protein
BMEC	bone-marrow-derived endothelial cell
bp	base pair
BSA	bovine serum albumin
CAD	caspase-activated DNAase
CARD	caspase recruitment domain
CBD	carbohydrate-binding domain
CD40L	CD40 ligand
CIAP	calf intestinal alkaline phosphatase
CLL	B-chronic lymphocytic leukaemia
CLP	common lymphocyte progenitor
CMV	cytomegalovirus
CO_2	carbon dioxide
CRD	cysteine-rich domain

CSH	Cold Spring Harbour
C-terminal	carboxyl-terminal
CXCR	chemokine, CXC motif, receptor
DAG	Diacylglycerol
DAPI	4,6-diamino-2-phenylindole
dATP	Deoxyadenosine triphosphate
DD	death domain
? ? m	mitochondrial inner membrane potential
DHE	dihydroethidium
DIABLO	direct IAP-binding protein with low PI
DiOC6(3)	3,3'-dihexyloxacarbocyanine iodide
DISC	death-inducing signalling complex
DLBCL	diffuse large B-cell lymphoma
DME	Dulbecco’s Modified Eagle’s medium
DMSO	dimethyl sulphoxide
DNA	deoxy-ribonucleoc acid
DR	death receptor
DUOX	dual domain oxidase
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
Endo G	Endonuclease G
ER	endoplasmic reticulum
EtOH	ethanol
FADD	Fas-associated protein with a death domain
Fc	fragment crystallizable
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FKHR	Forkhead (Drosophila) homolog 1 (rhabdomyosarcoma)
FLICE	FADD-like interleukin-1-converting enzyme
FLIP	FLICE-inhibitory proteins
GST	glutathione S-transferase
HBS	HEPES buffer saline
HBSS	Hanks balanced salt solution
HCl	hydrochloric acid
HEK293	human embryonic kidney 293
hr	hour
HSC	haematopoietic stem cell

HSP	heat shock protein
HUVEC	human umbilical vein endothelial cell
IAP	inhibitor of apoptosis
ICAD	inhibitor of caspase-activated DNAase
IFN	interferon
Ig	immunoglobulin
IgH	Ig heavy chain
I?B	inhibitor of ?B
IKK	I?B kinase
IL	interleukin
IP	immunoprecipitation
IP3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
kb	a thousand base pair
kD	kilodalton
L	Liter
LB	Luria Bertani
LOD	lactate oxidase
M	molar
MAPK	mitogen activated protein kinase
MC540	merocyanine 540
Mcl-1	myeloid cell leukaemia 1
mdm	murine double minute
Met	methionine
MFI	mean of fluorescence intensity
min	minute
MM	multiple myeloma
MnTBAP	manganese(III)-tetrakis(4-benzoic acid)porphyrin
mPBR	mitochondrial peripheral benzodiazepine receptor
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MW	molecular weight
MtOH	methanol
mTOR	mammalian target of rapamycin
NADPH	nicotinamide adenine dinucleotide phosphate
NaOAc	Sodium acetate
NEMO	NF-?B essential modulator
NF-AT	Nuclear factor of activated T-cells

NF-?B	Nuclear Factor ?B
NHL	non-Hodgkin’s lymphoma
Ni-NTA	nickel-nitrilotriacetic acid
NLC	nurse-like cell
Nonidet	non-ionic detergent
Nox	NAD(P)H oxidase
NSCLC	non-small cell lung cancer
N-terminal	amine-terminal
PARP	poly(ADP-ribose) polymerase
PBR	Peripheral benzodiazepine receptor
PBS	phosphate buffer saline
PBST	PBS Tween 20
PC	positive control
PCR	Polymerase chain reaction
PDK	phosphoinositide-dependent kinase
PE	phycoerythrin
PEI	polyethyleneimine
PEL	primary effusion lymphoma
PFA	paraformaldehyde
PH	pleckstrin homology
Phox	phagocyte oxidase
PIPES	1,4-Piperazinediethanesulfonic acid
pI	pH of the isoelectric point
PI	Isoelectric point
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol bisphosphate
PIP_3	Phosphatidylinositol trisphosphate
PK11195	[1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinoline-carboxamide]
PKC	Protein Kinase C
PLC?2	Phospholipase C ?2
PT	permeability transition
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PVDF	Polyvinylidene fluoride
r	radius

REL	reticuloendotheliosis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	revolution per minute
R.T.	room temperature
SDF	stromal-derived factor
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophresis
Ser	serine
SHIP	SH2-domain-containing inositol phosphatase
SMAC/DIABLO	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI
STAT	signal transducers and activators of transcription protein
tBid	truncated Bid
Tcl-1	T-cell leukaemia/lymphoma 1
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	transmembrane domain
TNF	Tumour necrosis factor
TRADD	TNFRSF1A-associated via death domain
TRAIL	TNF-related apoptosis-inducing ligand
TSPO	Translocator protein
UV	ultraviolet
VDAC	voltage-dependent anion channel
WB	washing buffer
ZAP-70	zeta-associated protein-70

Publications

New Immunological Principles: GCS-100 a novel Galectin-3 antagonist and Caspase-9 activating therapy for indolent B-Cell malignancies, **B Su**, M Corbo, L Maharaj, S Joel, Y Chang, B Carver, CD Fegan, RL Auer, FE Cotter, *Annals of Oncology*, 2005, Vol. 16, Suppl. 5, ppv70.

GCS-100, a Galectin 3 antagonist, is a novel Caspase-9 apoptosis activating agent for the treatment of indolent B-cell malignancies, **B Su**, M Corbo, Y Chang, J Schindler, B Carver, CD Fegan, AC Newland, RL Auer, FE Cotter, *British Journal of Haematology*, 2005, Vol. 129, Suppl. 1, pp5.

GCS-100, a Galectin 3 antagonist, is a novel Caspase-9 apoptosis activating agent for the treatment of indolent B-Cell malignancies, **B Su**, M Corbo, Y Chang, J Schindler, B Carver, CD Fegan, AC Newland, RL Auer, FE Cotter, *Blood*, 2004, Vol. 104 (11), 3286.

Chapter 1 Introduction

1.1 Malignancy and apoptosis

Malignant growth is characterised by an increased number of cells often in a relatively immature state. This process is brought about by deregulated gene expression, the fundamental process by which normal tissue cells become neoplastic. By contrast to the hypothesis that a 'direct hit' somatic mutation leads to the initiation of carcinogenesis, Bignold proposes a non-mutational mechanism of genetic instability which involves inhibition of proof-reading enzymes by carcinogens in DNA synthesis during local stem cell division as the first step in tumour formation (Bignold, Coghlan *et al*. 2006; Bignold 2003). Systematic 'signature' alterations of gene expression may be found for specific tumours. The loss of normal tissue homeostasis, which is a hallmark of malignancy, can fundamentally be caused by one or two processes acting alone or in concert, namely, deregulation of proliferation genes or disruption of apoptosis. Either or both result in increased cell numbers. Disrupted apoptosis is widely recognised as the main defect in indolent malignancies, e.g. certain types of chronic leukaemia (Caligaris-Cappio, Cignetti *et al*. 2002).

Malignant cells typically develop a survival advantage, in which the apoptotic cascade is blocked, thereby generating resistance to apoptosis-inducing therapies. A plethora of genetic and epigenetic modifications contribute to this outcome including translocated chromosomes, activated oncogenes or

mutated tumour-suppressor genes, leading to abnormal expression of transcription factors, receptors and signalling components. Externally, cells receive supportive signals from the extracellular environment which further serve as encouragement to proliferation and survival, all of which may contribute to oncogenic transformation.

B-cell malignancies comprise a group of neoplasms that involve maturational arrest and/or immortalization at specific points of B-cell development. They are classified in terms of the developmental stage from which the malignant clone appears to derive. Accumulation of malignant B cells can occur in the bone marrow or periphery as a consequence of either a lack of apoptotic cell death or rapid division of a particular type of B-lineage cell.

The apoptotic pathway plays critical roles in regulating lymphocyte survival throughout B-cell development, maturation and differentiation. Strict selection and clonal expansion are tightly regulated by homeostatic control. In normal lymphocytes the majority of lymphoid progenitors and lymphocytes derived from each stage die from induction of apoptosis. Failure to do so can result in B-cell malignancies. This thesis focuses on the modification of survival factors observed in B-cell malignancies with a view to identifying routes to therapeutic intervention based on an understanding of the survival process.

1.2 Apoptotic pathways

Apoptosis is a Greek word meaning the falling of leaves from trees or petals from flowers in autumn. It is a complicated phenomenon. Over 40 years have passed since the process was first described. Kerr describes the discovery of the ‘shrinkage necrosis’ of liver cells which occurs after ligation of the portal vein branches in rats, a non-degenerative process of cell death that differs from typical necrosis in histological appearance (Kerr 2002). He noted that only isolated cells were affected and no inflammation accompanied cell death in 1962. However, the term “apoptosis” to connote this phenomenon was not widely accepted until the 1970s on publication of a landmark paper (Kerr, Wyllie *et al*. 1972). Since then, research in apoptosis has grown rapidly. Studying alterations in apoptotic pathways in cancer cells has provided more detailed mechanisms for cancer survival and immortalization, leading to new strategies for pharmacological intervention.

Apoptosis is a vital, programmed, highly regulated and evolutionarily conserved process, which is common to physiological and pathological phenomena. A cell that dies by apoptosis ‘commits suicide’ in an ordered and controlled way. The cell goes through a series of events in which it is finally broken down by enzymes internally, to render small apoptotic particles that are removed by phagocytosis. Diverse pro-apoptotic stimuli activate signal-specific pathways via two conventional apoptotic pathways: the ‘extrinsic’

ATP-independent caspase-8 pathway and the 'intrinsic' ATP-dependent mithochondria/caspase-9 pathway (Ferrari, Stepczynska *et al*. 1998). However, as there is crosstalk between these two pathways at several points and many alternative pathways have been discovered, it is an oversimplification to frame apoptosis within this scenario alone (Figure 1.1). In addition, as apoptosis is such a complicated phenomenon, detailed review of the whole apoptotic pathway is beyond the scope of this thesis. Therefore the overview below is just a brief description of the relevant pathways, *i.e.* from receipt of the pro-apoptotic signal to caspase-3 activation.

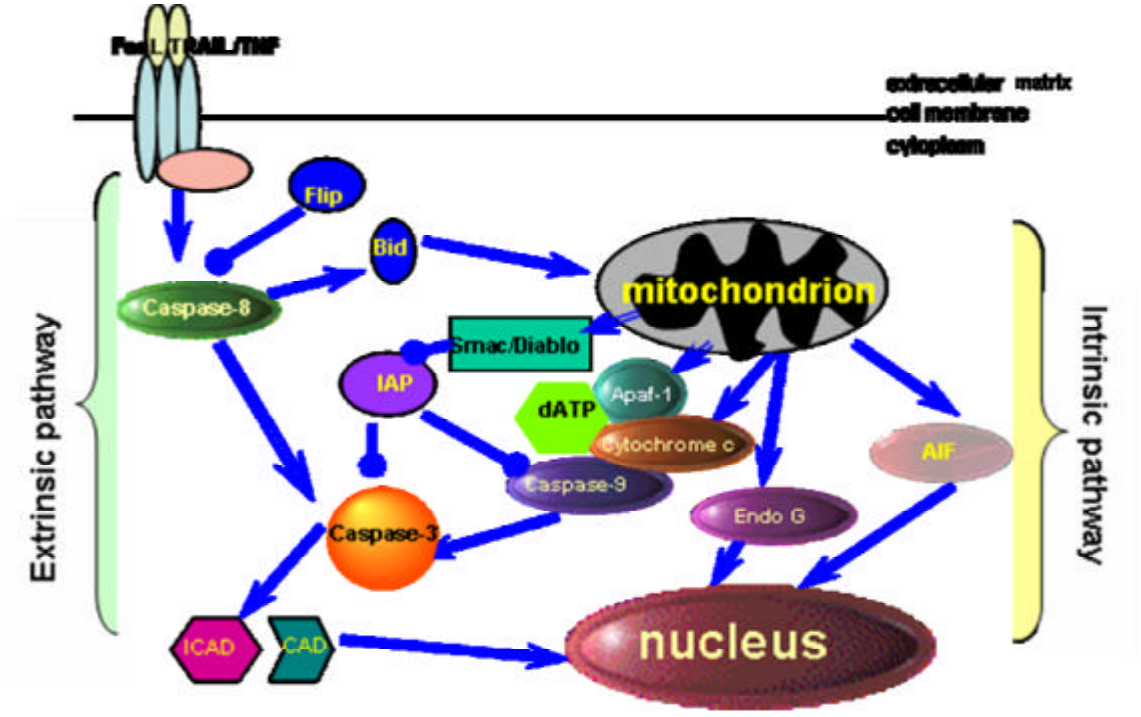


Figure 1.1 A diagrammatic representation of apoptotic pathways. Different stimuli activate signal-specific apoptotic pathways, including the death receptor-caspase-8 (extrinsic) and the mitochondria-caspase-9 (intrinsic) pathways (see 1.2.1 & 1.2.2), leading to the

activation of caspase-3 and subsequent DNA fragmentation. Arrows indicate activation, points indicate inhibition.

1.2.1 TNF superfamily receptors – caspase 8 pathway

Apoptosis can be induced through cell surface receptors that belong to the tumour necrosis factor (TNF) superfamily, *e.g*. Fas/CD95/Apo-1, TNF-related apoptosis-inducing ligand (TRAIL), death receptors (DR) and TNF receptors. As most of them share a homologous cytoplasmic sequence termed the *death domain*, they are sometimes designated as *death receptors*. This pathway mostly involves binding of cognate ligands/antibodies, receptor clustering, formation of death-inducing signalling complex (DISC) and subsequent activation of caspase-8 (Ferrari, Stepczynska *et al*. 1998). The negative regulator of this process is the FADD-like interleukin-1-converting enzyme (FLICE) inhibitory protein (FLIP) (Siegmund, Mauri *et al*. 2001) that resembles caspase-8, although it has no enzyme activity.

Procaspase-8 exists in two isoforms (55/53 kD). Upon activation, it forms two intermediate products of 43/41 kD that are further cleaved into the 18/10 kD heterodimer (Ferrari, Stepczynska *et al.* 1998). Activated caspase-8 can relay the upstream signal in two ways: by cleavage of caspase-3 or by cleavage of Bid, a Bcl-2 family protein. The former is independent of mitochondria,

whereas the latter forms a link between the two conventional apoptotic pathways (Fehlberg, Gregel *et al.* 2003), namely intrinsic (caspase-9) and extrinsic (caspase-8) (Figure 1.1). Truncated Bid (tBid) translocates onto mitochondria upon cleavage, induces oligomerization of Bax and Bak (Wei, Zong *et al*. 2001) and mediates mitochondrial change and the subsequent apoptotic events (Luo, Budihardjo *et al*. 1998).

1.2.2 Mitochondrial pathway

Mitochondria play a pivotal role in apoptosis by acting as a sensor and an integrator to receive and amplify signals from diverse upstream signalling pathways. They also initiate downstream execution steps. The mitochondrion has a double-membrane structure, with an outer membrane permeable to solutes <1.5 kD (Degterev, Boyce *et al*. 2001; Narita, Shimizu *et al*. 1998), and an essentially impermeable inner membrane. Apoptogenic factors, e.g. cytochrome c, apoptosis inducing factor (AIF), endonuclease G (Endo G), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI (SMAC/DIABLO) and pro-caspases, are larger than 1.5 kD, and safely sequestered in the mitochondrial intermembrane space under normal conditions. The mitochondrial inner membrane potential (DeltaPsi(m)) is a voltage and pH gradient created by H^+

pumping during electron transfer, and is crucial for mitochondrial functions (Fennell and Cotter 2000).

Whether mitochondria control cell death in a dominant (sensing damage and initiating death signals) or passive (receiving death signals and passing them on) way is still a question of debate (Cristea and Degli Esposti 2004). However, the majority of evidence points towards a dominant process (Jacobson and McCarthy 2002).

1.2.2.a Permeability transition (PT) and DeltaPsi(m) dissipation

The mitochondrial permeability transition (PT) is a critical early event in the apoptotic cascade. It is characterized as a Ca^{2+}-dependent increase of the inner membrane permeability to molecules <1.5 kD (Beutner, Ruck *et al*. 1998; Zamzami, Marchetti *et al*. 1996). Direct induction of PT is an effective trigger of apoptosis (Marchetti, Hirsch *et al*. 1996). PT is regulated by a multiprotein complex located at the contact site between the inner and outer mitochondrial membrane, termed the PT pore complex. It comprises the mitochondrial peripheral benzodiazepine receptor (mPBR), the voltage-dependent anion channel (VDAC or porin) (Fennell and Cotter 2000; Hirsch, Decaudin *et al.* 1998), the adenine nucleotide translocator (ANT) (Fennell,

Corbo *et al.* 2001; McEnery, Snowman *et al*. 1992), cyclophilin D and two enzymes, hexo-kinase and creatine kinase (Figure 1.2).

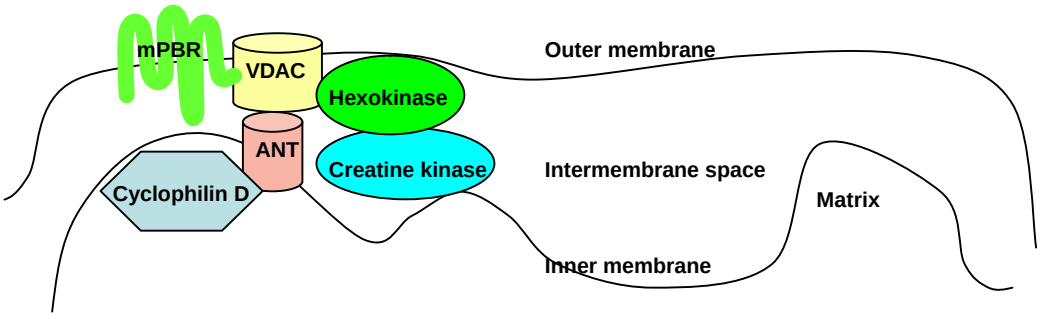


Figure 1.2 A diagramatic representation of the mitochondrial membrane, showing the putative structure of the PT pore complex. The 18 kD mitochondrial peripheral benzo-diazepine receptor (PBR) localizes to the outer mitochondrial membrane in close association with the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT). Cyclophilin D is on the inner membrane or matrix-facing side (Zamzami, Larochette *et al*. 2005).

The relationship between PT, DeltaPsi(m) dissipation and apoptosis, although not completely clear, is thought to follow the pattern of opening of PT pores, causing PT and then DeltaPsi(m) dissipation. The latter leads to an immediate shutdown of mitochondrial function, resulting in uncoupling of the respiratory chain and failure of ATP synthesis, as well as swelling of the mitochondrial matrix. Rupture of the outer membrane and release of apoptogenic factors then occur (Tang, Gao *et al*. 2005). Thus, agents that target components of the PT pore complex can interfere with the apoptotic

pathway. Bongkrekic acid, a specific ligand for ANT (Marchetti, Hirsch *et al*. 1996), and cyclosporin A, an inhibitor of cyclophilin D (Zamzami, Marchetti *et al*. 1996; Zoratti and Szabo 1995), are both characteristic PT inhibitors (Narita, Shimizu *et al*. 1998) with the ability to prevent DeltaPsi(m) dissipation (Hirsch, Marzo *et al*. 1997; Zamzami, Marchetti *et al*. 1996) and apoptosis.

As well as PT pore ligands, the gating of PT pore is regulated by the redox state of some critical vicinal thiols (Fennell and Cotter 2000), in particular, the thiol groups on ANT (Halestrap, McStay *et al*. 2002). Thiol-reactive agents that act on ANT can induce PT pore opening (Costantini, Belzacq *et al*. 2000).

Recent studies show that DeltaPsi(m) dissipation can be independent of PT. As one of the main functions of mitochondria is energy production, ATP synthesized in mitochondria and ADP generated in the cytosol need to be exchanged to maintain the energy chain. This exchange can be achieved by ANT (Martinou 1999), acting alone or together with VDAC, and any disturbance in this function may lead to failure of energy production and apoptosis (Murphy, Imahashi *et al*. 2005). Bcl-2 family proteins regulate the gating of VDAC and ANT, and the closure of VDAC is induced by Bax-like proteins (Lucken-Ardjomande and Martinou 2005). Closure of VDAC results in ATP/ADP exchange failure, cytoplasmic acidification, mitochondrial

hyperpolarization and DeltaPsi(m) collapse (Gottlob, Majewski *et al*. 2001). Thus, DeltaPsi(m) dissipation is not necessarily downstream of PT.

1.2.2.b NADPH oxidase and ROS

Reactive oxygen species (ROS) include superoxide anions, hydrogen peroxide, hypochlorite ions, hydroxyl radicals and peroxynitrite (Brandes and Janiszewski 2005; Richter, Gogvadze *et al*. 1995). They are unavoidable byproducts of cell respiration as well as an important weapon for killing microorganisms during the respiratory burst in phagocytes. They have also been implicated in several signal transduction pathways. ROS are produced initially by oxygen reduction using NADPH as an electron donor (Babior 1999). The products, superoxide anions, are precursors of other ROS molecules. This reaction is catalysed by NADPH oxidase, the key enzyme in ROS generation.

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2O_2^- + NADP^+ + H^+$$

NADPH oxidase is a family of enzymes that were originally discovered in phagocytes, e.g. neutrophils and macrophages. They play important roles in host defence. These enzymes are divided into phagocytic and non-phagocytic types. They are structurally and functionally homologous, but have

different biological distribution. Phagocytic NADPH oxidase comprises three groups of components (De Deken, Wang *et al*. 2000).

i) gp91^{phox} and p22^{phox} transmembrane heterodimer with four NADPH-binding sites, one FAD and two haems, all together known as *cytochrome b558*, located at the organelle membrane (Banfi, Molnar *et al*. 2001).
ii) p40^{phox}, p47^{phox} and p67^{phox} forming a cytosolic trimer complex.
iii) Two G-proteins: membrane-associated Rap-1A and cytosolic Rac-2.

Upon activation, p47^{phox} is phosphorylated and the trimer complex, together with Rac-2, migrate through the cytosol towards the membrane-bound cytochrome b558 and Rap-1A to assemble active NADPH oxidase. This enables the catalysis of oxygen reduction, the electron transfer reaction, generation of superoxide anions and other ROS molecules (Babior 1999).

The major biological process leading to ROS generation is electron transport at the mitochondrial membrane. Mitochondria actively and continuously generate ROS during cell respiration (Chandra, Samali *et al*. 2000). These molecules are biologically toxic and may damage vital macromolecules such as nucleic acids, proteins, carbohydrates and lipids. The principal defences against this are the natural antioxidant systems of the cell. Among these systems, the glutathione redox cycle is the most rapid and abundant weapon against ROS. In mitochondria, H_2O_2 is converted to H_2O, at the expense of

GSH oxidation to its disulfide type, GSSG, by the catalysis of glutathione peroxidase (Fennell, Corbo *et al*. 2001).

NADPH oxidase is involved in apoptosis as ROS have been implicated in apoptotic pathways. The redox state of the mitochondrial NADPH pool is found to be associated with PT (Zoratti and Szabo 1995). Intracellular ROS generation has been detected and monitored as an index of apoptotic events. A biphasic increase of intracellular ROS has been observed following apoptosis induction by mPBR ligands, *i.e.* an early sharp peak followed by a secondary, more sustained increase (Blatt, Bednarski *et al*. 2002). The late increase is accompanied by DeltaPsi(m) collapse and the release of cytochrome c, indicating that ROS are implicated in the mitochondrial apoptotic pathway. Other evidence suggests that ROS may act as mediators of growth factor signal transduction (Finkel 2000). In addition, activation of NADPH oxidase can be induced by many pro-apoptotic drugs, e.g. capsaicin (Lee, Kang *et al*. 2004), salicylates (Chung, Bae *et al*. 2003) and tamoxifen (Lee, Kang *et al*. 2000), indicating that ROS production can be a common mechanism of apoptosis. NADPH oxidase has also been activated by H_2O_2 in non-phagocytic cell types, suggesting the existence of a positive feedback loop in ROS production (Li, Miller *et al*. 2001).

Following the discovery of a variety of non-phagocytic NADPH oxidases with different tissue distribution, it has become clear that ROS generation is an

ubiquitous, rather than phagocyte-specific, physiological phenomenon. As all these enzymes have the homologous catalytic subunit, $gp91^{phox}$, and share the conserved core structure, *i.e.* six transmembrane domains and a long cytoplasmic C terminus (Banfi, Tirone *et al*. 2004), they are termed the Nox family, including Nox and dual domain oxidases (DUOX). Nox-5 is a member of the Nox family, and is expressed in lymphoid tissues and testis. Nox-5 has an additional N-terminal extension with Ca^{2+}-binding EF-hand domains (Krause 2004) and therefore is activated and catalyses generation of superoxide anions in response to elevated cytosolic Ca^{2+} (Banfi, Molnar *et al.* 2001). During phagocytosis, NADPH oxidase assembles the cytosolic and membrane-bound subunits necessary for its activation. It appears that the regulatory and catalytic modules of Nox-5 are combined within one protein. The N-terminal part of Nox-5 acts as an intracellular [Ca^{2+}] sensor and undergoes conformational changes upon Ca^{2+}-binding. This results in activation of Nox-5 and subsequent catalysis of ROS generation (Banfi, Tirone *et al*. 2004).

1.2.2.c Apoptogenic factor release

It is understood that DeltaPsi(m) dissipation leads to mitochondrial dysfunction, resulting in uncoupling of the respiratory chain. ATP synthesis fails and all the other ion pumps shut down resulting in swelling of

mitochondrial matrix, rupture of the outer mitochondrial membrane and release of apoptogenic factors, *e.g*. cytochrome c and apoptosis-inducing factor (AIF). These factors either activate caspases and nucleases directly or neutralise cytosolic inhibitors of the apoptotic process.

However, these observations cannot explain the fact that in some apoptotic cells mitochondria do not manifest any major ultrastructural abnormalities (Martinou 1999). Furthermore, it has been reported that apoptogenic factors, *e.g*. cytochrome c, can be released before DeltaPsi(m) collapse (Yang, Liu *et al*. 1997), or be unaccompanied by DeltaPsi(m) changes (Kluck, Bossy-Wetzel *et al*. 1997). More recently the focus has moved to outer mitochondrial membrane permeabilization, a process that can be independent of DeltaPsi(m) collapse. The new proposal is that the outer membrane permeability transition is more important and happens earlier than the inner membrane potential changes, as the latter is a late event that also occurs in necrotic cell death (Cristea and Degli Esposti 2004). One possible mechanism is that pro-apoptotic Bcl-2 proteins, *e.g*. Bax or Bak, form large conductance pores, either alone or in combination with VDAC, at the outer mitochondrial membrane and mediate the release of apoptogenic factors. This can be blocked by Bcl-2 via its interaction with either Bax/Bak or VDAC (Halestrap, McStay *et al*. 2002; Lucken-Ardjomande and Martinou 2005; Murphy, Imahashi *et al*. 2005; Wang 2001).

Cytochrome c is a key component of the electron transport chain (Murphy, Imahashi *et al*. 2005) and normally resides in the intermembrane space of mitochondria. Release into the cytoplasm occurs during apoptosis. Cytosolic cytochrome c interacts with apoptotic protease-activating factor 1 (Apaf-1) and deoxyadenosine triphosphate (dATP), leading to conformational change in Apaf-1 and the exposure of its caspase recruitment domain (CARD) (Ferrari, Stepczynska *et al.* 1998). Caspase-9, having its own CARD at the N-terminal, binds to it and becomes autoproteolytically cleaved and activated. Caspase-9 in turn cleaves and activates caspase-3, the main executioner of apoptosis.

Apoptosis-inducing factor (AIF) is a flavoprotein that has oxidoreductase activity, an important function in cell respiration when sequestered in the mitochondrial intermembrane space. Once released to the cytosol, AIF translocates to the nucleus and activates endonucleases, inducing large scale DNA fragmentation (Reed 2001). The effect of AIF is independent of caspase activation (Tsujimoto 2002; Wang 2001), although it can also activate caspase-3 (Kroemer, Dallaporta *et al.* 1998). AIF can bind to DNA and RNA (Vahsen, Cande *et al*. 2005), but does not have DNase activity (Kroemer, Dallaporta *et al*. 1998).

Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI (SMAC/DIABLO) is also released from

the mitochondrial intermembrane space to the cytosol where it binds inhibitors of apoptosis (IAPs), a group of cytosolic apoptosis inhibitors that can inactivate caspases. Inhibition of IAPs by SMAC/DIABLO promotes apoptosis (Wang 2001).

Endonuclease G (Endo G) is another apoptogenic factor released in the cytosol during apoptosis. Like AIF, it causes DNA fragmentation (apoptosis) independent of caspases. Unlike AIF, it acts directly as a nuclease (Wang 2001).

1.2.3 Final common pathway

The two pathways, death receptor–caspase-8 (extrinsic) and mitochondria–caspase-9 (intrinsic) pathways, are not independent of each other. There are cells that mainly follow the death receptor-caspase-8 pathway in response to certain stimuli which leads to cell death with minimal involvement of mitochondria. They are termed Type I cells. By contrast, cells that need the mitochondrial amplification loop to respond to death signals are termed Type II cells (Scorrano, Oakes *et al.* 2003). Both types converge at the caspase-3 point to induce the final stage of apoptosis (Figure 1.3).

Apart from AIF and Endo G, *etc*, the apoptotic pathways converge on a group of caspases that perform the final stage of apoptosis and cause the typical apoptotic morphological changes, *e.g*. cell shrinkage, membrane blebbing, exposure of phosphatidylserine, chromatin condensation, DNA fragmentation and apoptotic body formation. Caspases are synthesized as inactive pro-enzymes and become activated upon autoproteolysis or cleavage by other caspases at aspartate residues (Ferrari, Stepczynska *et al.* 1998). There are more than 280 types of known caspase substrates (Fischer, Janicke *et al.* 2003). Caspase-3 is considered to be the key executioner of apoptosis due to its ability to cleave a wide range of vital intracellular substrates including the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), actin-regulatory protein gelsolin, structural protein fodrin, actin and nuclear lamin (Hajra and Liu 2004; Janicke, Ng *et al*. 1998), and the DNA-dependent protein kinase catalytic subunit (Porter and Janicke 1999). Caspase-3 also cleaves the inhibitory binding partner of DNA fragmentation factor (DFF), DFF45/ICAD, to release the active caspase-activated DNase (CAD) and DFF40/CAD endonuclease (Springer, Azbill *et al.* 1999). Furthermore, Bcl-2 family proteins, *e.g*. Bcl-2, Bcl-xl and Mcl-1 (Weng, Li *et al.* 2005) are also targets for caspase-3 (Fujita, Nagahashi *et al*. 1998; Fujita and Tsuruo 1998). Cleavage of Bcl-2 converts its anti-apoptotic effect to pro-apoptotic and amplifies upstream mitochondrial signals by facilitating the release of cytochrome c, forming a positive signal loop and hastening the apoptotic process (Porter and Janicke 1999). As caspase-3 can be activated via

mitochondria-independent signals, Bcl-2 cleavage may activate the mitochondrial pathway downstream of caspase-3 activation and form another link between these two apoptotic pathways besides Bid. Caspase-3 activity is also required for the activation of caspase-8, as knock-out of the caspase-3 gene causes inhibition of caspase-8 activity (Aouad, Cohen *et al*. 2004). The effect of caspase-3 on Bcl-2 proteins and caspase-8 suggests the existence and importance of the mitochondrial amplification loop in apoptosis, *i.e*. pro-apoptotic signals after mitochondria-caspase-9-caspase-3 can further feed back and amplify the process (Figure 1.3).

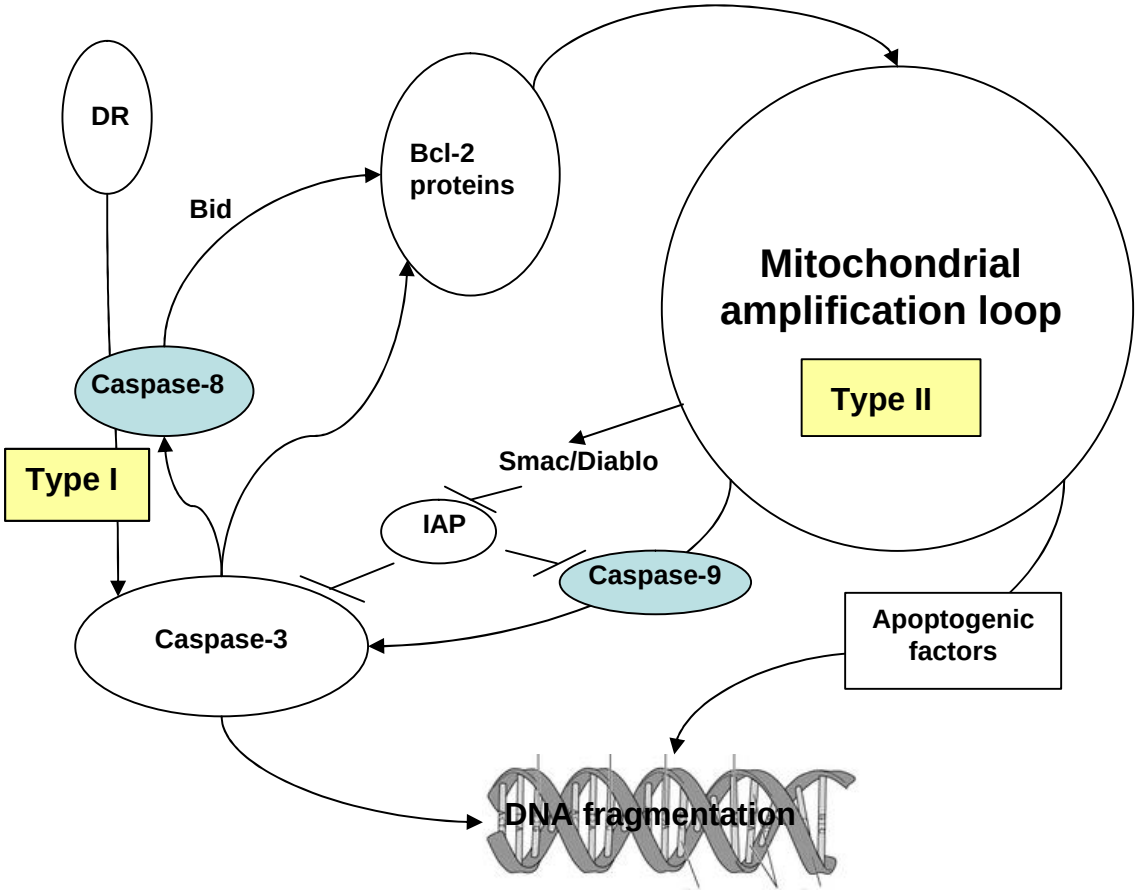


Figure 1.3 A diagramatic representation of the crosstalk between apoptotic pathways and the mitochondrial amplification loop. Caspase-3, activated by caspase-8 and/or caspase-9, can directly or indirectly act on Bcl-2 proteins and thus form links between the two pathways (see 1.2.3).

1.3 Resistance to apoptosis in tumour cells

Resistance to apoptosis is a crucial survival mechanism in tumour cells achieved by either downregulation of pro-apoptotic or upregulation of anti-apoptotic signals. The latter include elevated expression of negative

regulators of apoptosis, including Bcl-2, galectin-3 and Akt, the primary topics of this investigation.

1.3.1 Bcl-2 family proteins

Bcl-2 (B-cell leukaemia/lymphoma gene 2), a critical survival protein, was first discovered following the cloning of the t(14:18) chromosomal translocation found in 85% of follicular B-cell lymphoma (Tsujimoto 2002). This places the immunoglobulin gene enhancer on chromosome 14 alongside the complete protein coding region of Bcl-2 on chromosome 18. Thus, the enhancer switches on Bcl-2 expression in B-cells, leading to development of lymphoma (Kitada, Pedersen *et al*. 2002). Regulation of Bcl-2 family proteins can be obtained by increased synthesis, increased degradation and phosphorylation. Following the discovery of other Bcl-2 members, it becomes clear that this is a large family of proteins that regulate cell death and cause drug resistance.

1.3.1.a Structure of Bcl-2 proteins

Two main structural homologies have been found in Bcl-2 proteins (Figure 1.4).

i) *Bcl-2 homology (BH) domains*. These include BH1, BH2, BH3 and BH4 domains. Both BH1 and BH2 domains are critical for the anti-apoptotic function and dimerisation (Yin, Oltvai *et al.* 1994), in particular the *NWGR* motif in the BH1 domain. BH4 is conserved among anti-apoptotic but not pro-apoptotic members with the exception of Bcl-xs and Mcl-1 (Day, Chen *et al.* 2005; Kroemer 1997).
ii) *Transmembrane domain (TM)*. This is the C-terminal hydrophobic tail that is responsible for insertion into intracellular membrane structures, *e.g*. mitochondrial, nuclear and endoplasmic reticulum membranes (Hanada, Aime-Sempe *et al*. 1995).

Bcl-2 family proteins are mainly classified into three sub-families (Figure 1.4), based on their structures as well as functions.

i) Bcl-2-like sub-family, *e.g.* Bcl-2, Bcl-xl, Bcl-w, *etc*. They share BH1, BH2, BH3, BH4 and TM, and are anti-apoptotic.
ii) Bax-like sub-family, *e.g*. Bax, Bak, *etc*. They are pro-apoptotic and share BH1, BH2, BH3 and TM, but not necessarily BH4.

iii) BH3-only sub-family. They all share BH3 and are pro-apoptotic. Apart from BH3, Bik and Bim possess TM, whereas Bid, Bad and Puma do not (Cory, Huang *et al*. 2003; Hutcheson, Scatizzi *et al*. 2005; Kroemer 1997).

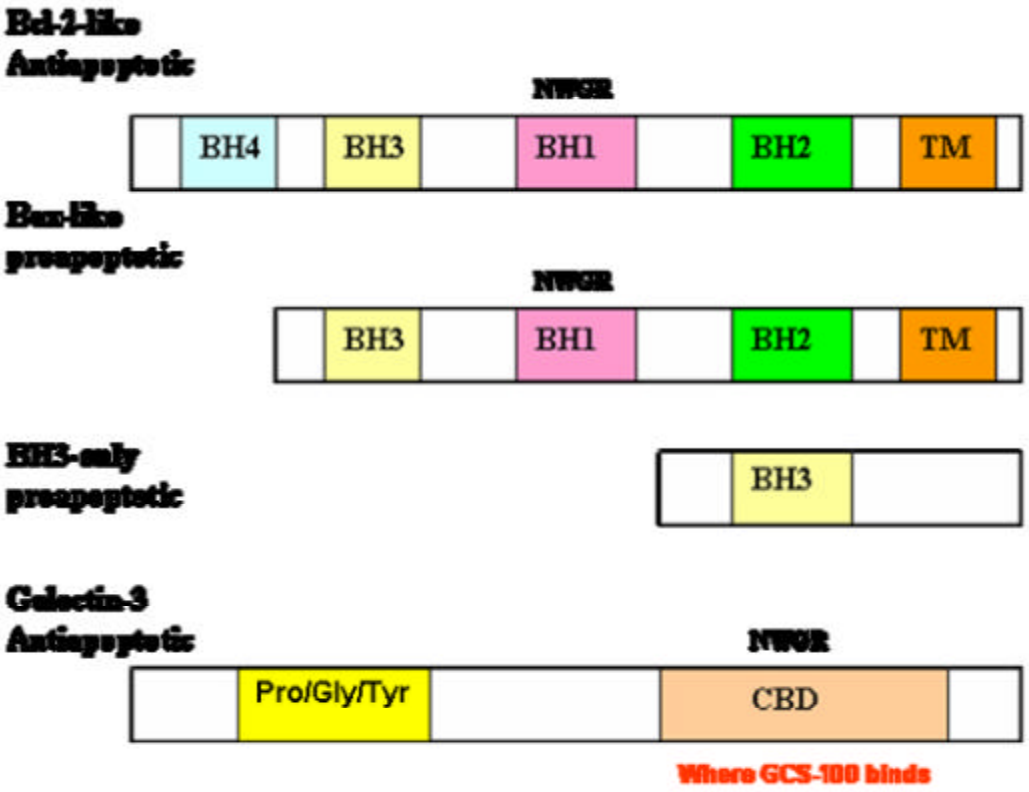


Figure 1.4 A diagramatic comparison of the structure of Bcl-2 proteins and galectin-3. The BH1 domain shared by Bcl-2-like and Bax-like proteins contains the NWGR motif which is also found in the CBD domain of galectin-3 (see 1.3.2 for detailed description). BH, Bcl-2 homology domain; CBD, carbohydrate-binding domain; TM, transmembrane domain.

There are exceptions to these broad groupings. Bcl-xs is the alternative splicing product of bcl-xl gene. It is pro-apoptotic and has only BH3, BH4 and TM (Lindenboim, Yuan *et al*. 2000). Mcl-1 lacks the N-terminal BH4 domain (Day, Chen *et al*. 2005) but is anti-apoptotic.

The BH3 domain of pro-apoptotic members, *e.g*. Bax, Bak, and Bid, is normally inactive and folded into the molecule. To become active and to heteroligomerize with other Bcl-2 proteins, the N-terminal part around the BH3 domain needs to be exposed by either conformational change, *e.g*. Bax and Bak (Griffiths, Dubrez *et al.* 1999), or N-terminal truncation by caspase-8 in the case of Bid (Tsujimoto 2002).

Bax is mainly phosphorylated and inactive in healthy cells, forming monomers or heterodimers with anti-apoptotic Bcl-2 members, *e.g.* Bcl-xl, Bcl-2 and Mcl-1. Akt is thought to be involved in direct phosphorylation of Bax and thus keeps Bax inactive (Gardai, Hildeman *et al.* 2004). Bax is dephosphorylated in response to upstream pro-apoptotic signals and undergoes conformational changes, *i.e*. exposure of the N-terminal part and release of the C-terminal tail, to become activated (Lucken-Ardjomande and Martinou 2005). Activated Bax loses its ability to heteroligomerize and translocates from cytosol to mitochondria (Wolter, Hsu *et al.* 1997) to exert its function.

1.3.1.b Function of Bcl-2 proteins

Bcl-2 family proteins predominantly control the fate, *i.e*. life or death, of a cell. Their pro-apoptotic and anti-apoptotic members act independently, synergistically, counter-actively or via interaction with other family molecules. In transgenic animal models, Bax promotes apoptosis in Bcl-2-null mice, whereas Bcl-2 represses apoptosis in Bax-null mice, suggesting that Bcl-2 and Bax can act independently (Knudson and Korsmeyer 1997). However, the intricate mechanisms for their function are largely unknown. It has been hypothesized that Bcl-2 proteins form pores on lipid membrane and interfere with ion exchange across intracellular membrane, thus stabilising the PT pore and preventing DeltaPsi(m) collapse with subsequent release of apoptogenic factors.

i) Pore-forming

The discovery of the structural similarities between some Bcl-2 members, bacterial colicins and diphteria toxin leads to the investigation of their pore-forming function (Lucken-Ardjomande and Martinou 2005). Bcl-2 (Schendel, Xie *et al*. 1997), Bcl-x (Minn, Velez *et al*. 1997), Bax (Antonsson, Conti *et al*. 1997) and Bid (Tsujimoto and Shimizu 2000) can form pore structures in a synthetic lipid membrane. These pore structures have different properties.

The pore-forming activity of Bax is inhibited by Bcl-2 at physiological pH (Antonsson, Conti *et al*. 1997).

It appears that Bax/Bak as well as other Bcl-2 members can translocate to the outer mitochondrial membrane and form pore structures, directly mediating the release of apoptogenic factors (see 1.2.2.c). However, this theory has been challenged due to the finding that Bax/Bak alone cannot induce cytochrome c release from artificial liposomes or isolated mitochondria, and the existence of VDAC is necessary for this process (Shimizu, Narita *et al*. 1999).

ii) *Interference with mitochondrial ion exchange, PT, DeltaPsi(m) and apoptogenic factor release*

Bcl-2 resides at the outer mitochondrial membrane (also other organelle membranes) and regulates the ion exchange across the membrane (Hajra and Liu 2004). It increases the mitochondrial matrix volume and its ability to take up cytosolic Ca^{2+} (Murphy, Imahashi *et al*. 2005). Thus Bcl-2 plays a crucial role in the control of intracellular Ca^{2+} homeostasis (Burlacu 2003).

Recombinant Bax induces PT and cytochrome c release from isolated mitochondria, possibly by direct interaction with the PT pore, which can be

inhibited by exogenous Bcl-xl, Bcl-2 and cyclosporin A (Gardai, Hildeman *et al*. 2004; Jurgensmeier, Xie *et al*. 1998; Narita, Shimizu *et al*. 1998).

Although the pathway is not fully elucidated for events downstream of Bcl-2 inhibition of PT, *e.g*. the release of cytochrome c (Kluck, Bossy-Wetzel *et al*. 1997) and AIF (Susin, Zamzami *et al*. 1996), it is thought that Bcl-2 (and Bcl-xl) interacts with the PT pore complex and stabilises it (Marzo, Brenner *et al. 1998)*. As stated in 1.2.2.a, it is hypothesized that the closure of VDAC, induced by pro-apoptotic signals, plays important roles in apoptosis. Bcl-2 interacts with VDAC to keep it in the open state (Murphy, Imahashi *et al.* 2005), whereas Bax-like proteins promote its closure (Lucken-Ardjomande and Martinou 2005).

The role of anti- and pro-apoptotic proteins on VDAC is, however, contradictory. Bcl-xl, the anti-apoptotic protein, also closes VDAC (essentially the opposite effect to Bax). Possibly the role of these molecules on ANT or the ANT state at the time of VDAC closure is the important factor (see Figure 1.2). Greater clarification will be required to determine the full effect of these molecules on cytochrome c release.

iii) Interactions between Bcl-2 members

The ratio between the anti-apoptotic and pro-apoptotic Bcl-2 family members is crucial for the susceptibility of a cell to apoptosis. This “death-life rheostat” is mediated, at least in part, by competitive oligomerization between antagonists and agonists of apoptosis. One of the classical examples is the Bcl-2/Bax heteroligomers (Zhang, Lapolla *et al*. 2004). When Bcl-2 is in excess, Bcl-2 homoligomers dominate and cells are protected from apoptosis. When Bax is in excess, Bax homoligomers dominate and cells are susceptible to apoptosis. The balance between activated Bax-like proteins (forming oligomers at the outer mitochondrial membrane) and inhibitory Bcl-2-like proteins (stabilising mitochondrial membrane) decides the release of apoptogenic factors such as cytochrome c (Zhang, Lapolla *et al*. 2004).

One of the recent models of how Bcl-2 proteins regulate apoptosis proposes that pro-apoptotic signals activate BH3-only proteins, *e.g.* Bid, which translocate to mitochondria and either activate Bax-like proteins directly or interact with anti-apoptotic Bcl-2 proteins, *e.g*. Bcl-xl or Bcl-2. The latter action frees Bax-like proteins from heteroligomerizing with Bcl-2/Bcl-xl and leads to homoligomerization of Bax/Bak and permeabilization of the outer mitochondrial membrane (Hajra and Liu 2004; Li, Zhu *et al.* 1998; Wei, Zong *et al*. 2001; Zhang, Lapolla *et al*. 2004). This is not necessarily accompanied by DeltaPsi(m) disruption (Katoh, Tomimori *et al.* 2004).

1.3.2 Galectin-3

Lectins, or glycoproteins, are carbohydrate-binding proteins, excluding enzymes and antibodies, which have a C-terminal carbohydrate-binding domain (Nangia-Makker, Conklin *et al*. 2002) and an affinity for specific oligosaccharides (Krzeslak and Lipinska 2004). The most widely expressed family of lectins are *galectins* (galactoside-binding lectins) with a specific affinity for beta-galactosides. *Galectin-3* is a member of the galectin family.

1.3.2.a Structure of galectin-3

The N-terminal part of galectin-3 has a repetitive sequence of 100-150 amino acids rich in proline, glycine and/or tyrosine (Figure 1.4) (Krzeslak and Lipinska 2004), and the length of the repeats is species-dependent (Barboni, Bawumia *et al*. 2000; Gong, Honjo *et al*. 1999; Herrmann, Turck *et al.* 1993; Morris, Ahmad *et al*. 2004). It contains collagenase-cleavage sites (Hsu, Zuberi *et al*. 1992) and serves as a substrate for matrix metalloproteinases (Yoshii, Fukumori *et al*. 2002).

The C-terminal part of galectin-3 has a globular structure and contains the carbohydrate-binding domain (Gong, Honjo *et al*. 1999). The highly-

conserved *NWGR* motif, also shared with Bcl-2 family proteins as part of the BH1 domain, is found within the carbohydrate-binding domain (Figure 1.4) (Akahani, Nangia-Makker *et al.* 1997). This motif is crucial for its anti-apoptotic effect, as substitution of glycine within the motif by alanine abrogates its anti-apoptotic effect against genistein (Lin, Moon *et al*. 2000). This is reminiscent of the abolition of the anti-apoptotic effect of Bcl-2 by substitution of glycine, the same amino acid within the same motif (Yin, Oltvai *et al.* 1994).

Several research groups have been trying to distinguish the function of N-terminal and C-terminal parts using truncated galectin-3. It was found that galectin-3-C, with its N-terminal part truncated, has similar affinity for lactose and other types of saccharide to intact galectin-3 (Hsu, Zuberi *et al*. 1992). Transfection of galectin-3-positive PEL cells with the N-terminal truncated galectin-3 resulted in increased sensitivity to anti-Fas-induced apoptosis (Hoyer, Pang *et al*. 2004).

Galectin-3 shows the ability to form homodimers (Barboni, Bawumia *et al*. 2000; Kuklinski and Probstmeier 1998; Yang, Hill *et al.* 1998). Electron microscopy shows that, at a certain concentration, recombinant galectin-3 exists mostly in a Y-shaped dimer form (Ochieng, Platt *et al*. 1993).

Galectin-3 is post-translationally modified by Met-1 cleavage and Ala-2 acetylation (Herrmann, Turck *et al*. 1993). The first twelve amino acids contain phosphorylation sites, *e.g*. Ser6 and Ser12, and are involved in cellular compartmentalization (Gong, Honjo *et al*. 1999). The Ser6 site can be phosphorylated by casein kinase ? (Huflejt, Turck *et al.* 1993), which is believed to be an 'on/off' switch for some of its important functions, *e.g*. carbohydrate-binding capability (Mazurek, Conklin *et al*. 2000; Nangia-Makker, Conklin *et al*. 2002), exportation from the nucleus to the cytoplasm (Takenaka, Fukumori *et al*. 2004) and anti-apoptotic effect (Yoshii, Fukumori *et al.* 2002). Galectin-3 can also be phosphorylated by glycogen synthase kinase-3ß, similar to beta-catenin, and is implicated in the Wnt/beta-catenin signalling pathway (Shimura, Takenaka *et al.* 2005).

1.3.2.b Intracellular and extracellular distribution and trafficking

Galectin-3 is synthesized on free ribosomes and mainly found in the cytoplasm in its phosphorylated form as a result of its N-acetylation, a marker for cytoplasmic proteins (Gong, Honjo *et al*. 1999; Krzeslak and Lipinska 2004; Mehul and Hughes 1997). Galectin-3 is also found on the cell surface, in the nucleus and extracellular matrix, depending on cell type, cell cycle and external stimulus. Phosphorylated galectin-3 is found in both cytosol and nucleus, whereas its non-phosphorylated form exists exclusively in the

nucleus (Dagher, Wang *et al*. 1995; Gong, Honjo *et al*. 1999). Galectin-3 in proliferating 3T3 mouse fibroblasts is predominantly localized in the nucleus, whereas in quiescent cells it is primarily found in the cytoplasm (Moutsatsos, Wade *et al.* 1987).

It has been reported that galectin-3 translocates to the perinuclear mitochondrial membrane following pro-apoptotic stimulation (Yu, Finley *et al.* 2002). Galectin-3 is also secreted in vesicles into the extracellular matrix (Zhu and Ochieng 2001) via a non-classical export pathway independent of the ER and Golgi system, as it lacks the signal sequence for translocation to the ER (Hughes 1999; Krzeslak and Lipinska 2004; Mehul and Hughes 1997; Menon and Hughes 1999). The trafficking of galectin-3 from free ribosomes to the plasma membrane is thought to be the rate-limiting step in its secretion (Mehul and Hughes 1997). The first twelve amino acids are crucial for the secretion as truncated galectin-3 without this N-terminal part cannot be secreted (Gong, Honjo *et al*. 1999).

1.3.2.c Function of galectin-3

Galectin-3 is a multi-functional protein involved in a wide range of cell behaviour, but this study has focused on its apoptosis-related functions.

Galectin-3 has affinities for a variety of molecules of varied sizes and functions. Galectin-3 has an affinity for beta-galactoside-containing sugar molecules or glycoconjugates, and acts as a receptor for ligands with sugar moieties, *e.g.* asialofetuin, lactose and N-acetyllactosamine (Castronovo, Van Den Brule *et al*. 1996; Krzeslak and Lipinska 2004). Initially galectin-3 was termed *IgE-binding protein*, suggesting its affinity for IgE (Hsu, Zuberi *et al*. 1992). Some complex carbohydrates, *e.g*. modified citrus pectin, can bind galectin-3 and interfere with its functions (Nangia-Makker, Conklin *et al*. 2002). Galectin-3 binds to extracellular matrix molecules such as laminin (Castronovo, Van Den Brule *et al*. 1996; Ochieng, Furtak *et al*. 2004) and fibronectin (Ortega, Behonick *et al.* 2005). Galectin-3 also binds single-stranded DNA and RNA with higher affinity than for carbohydrate (Wang, Inohara *et al*. 1995).

Galectin-3 has an anti-apoptotic function. Galectin-3 overexpression shows protection in Jurkat T cells against anti-Fas- and stauroporine-induced apoptosis (Yang, Hsu *et al*. 1996) and in breast cancer cell lines against genistein- and nitric-oxide-induced apoptosis (Lin, Moon *et al*. 2000). Galectin-3 transfected breast cancer cells show resistance to ROS generation and mitochondrial membrane potential collapse induced by TNF-alpha and menadione (Matarrese, Tinari *et al*. 2000). High levels of galectin-3 expression have been found in B cell malignancies such as diffuse large B-cell lymphoma (DLBCL), primary effusion lymphoma (PEL) and multiple

myeloma (MM) (Hoyer, Pang *et al.* 2004). Transfection of galectin-3 in galectin-3-negative Burkitt lymphoma (BL) cells results in increased resistance to anti-Fas-induced apoptosis (Hoyer, Pang *et al.* 2004). In normal B-cell development, galectin-3 expression is low in germinal centre B cells and plasma B cells, but high in long-lived B cells, *e.g*. memory B cells (Hoyer, Pang *et al*. 2004), suggesting a protective advantage associated with galectin-3 levels in B cells. Galectin-3 serum levels are significantly elevated in patients with breast, gastrointestinal, lung and ovarian cancer, as well as melanoma and non-Hodgkin's lymphoma (NHL) (Iurisci, Tinari *et al*. 2000).

How galectin-3 exerts its anti-apoptotic effect is unclear, but its role in the PI3K/Akt pathway may explain some of its action. Galectin-3 promotes strong activation of PI3K/Akt pathway in bladder carcinoma cells (Oka, Nakahara *et al.* 2005), and the expression of galectin-3 appears to be regulated by the NF-(kappa)B pathway (Dumic, Lauc *et al*. 2000; Nakahara, Oka *et al*. 2005). Galectin-3 is implicated in the canonical Wnt signalling pathway. Activation of Wnt signalling results in dephosphorylation of beta-catenin and protection from its subsequent ubiquitination and degradation. beta-catenin enters the nucleus where it binds transcription factors and regulates Wnt-target genes such as *c-myc* and *cyclin D1*. This process appears to be galectin-3-dependent, and structurally beta-catenin/galectin-3/transcription-factors together form one complex in the nucleus (Shimura, Takenaka *et al*. 2004).

However, galectin-3 can be pro-apoptotic. Many haematopoietic tumour cells do not express high levels of galectin-3. Galectin-3 expression is down-regulated in prostate cancer (Pacis, Pilat *et al*. 2000). Downregulation of galectin-3 has also been observed in relation to progression and invasiveness of breast cancer (Castronovo, Van Den Brule *et al*. 1996). There has been increasing evidence that galectin-3 plays different or even opposite roles intracellularly and extracellularly. Exogenous galectin-3 induces T cell line apoptosis via cell surface CD29/CD7 and the mitochondrial apoptotic pathways (Fukumori, Takenaka *et al*. 2003). This implies a role of galectin-3 in immune escape (Nakahara, Oka *et al.* 2005). It is known that many types of tumour cells export galectin-3 extracellularly which correlates with the high serum level of galectin-3 in breast cancer (Iurisci, Tinari *et al*. 2000). Galectin-3-null cells seem to be more sentitive to exogenous galectin-3 than galectin-3-expressing cells, and there may be a balance between its intracellular anti-apoptotic effect and extracellular pro-apoptotic effect (Nakahara, Oka *et al*. 2005). The effect of galectin-3 on TRAIL-induced apoptosis has been controversial, as in bladder carcinoma cells it renders resistance to TRAIL-induced apoptosis (Oka, Nakahara *et al*. 2005), whereas in breast cancer cells it sensitises them to TRAIL (Lee, Song *et al*. 2003).

1.3.3 Akt and NF-?B

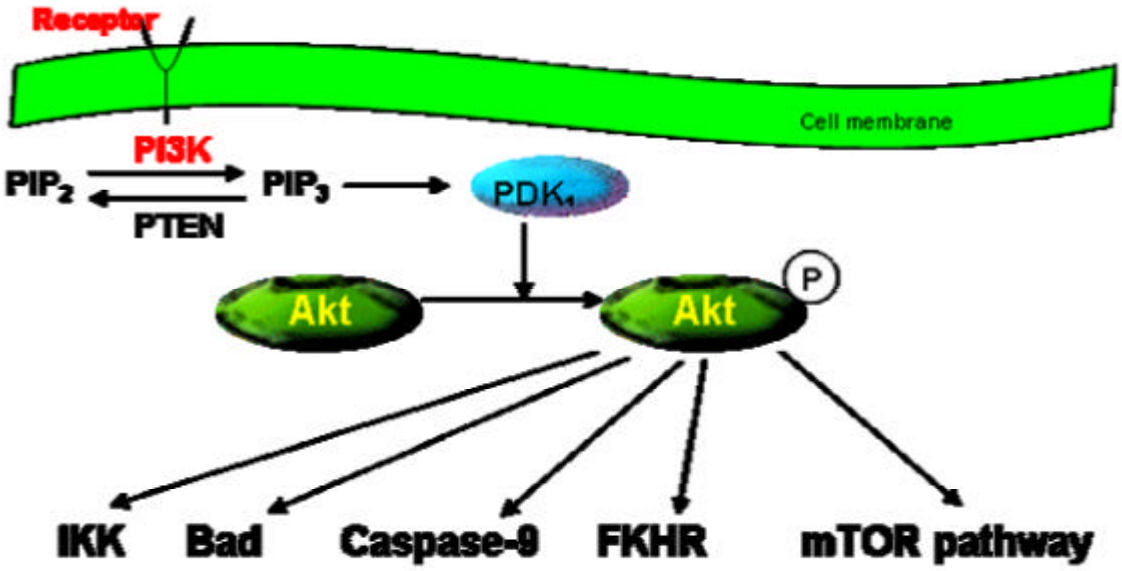


Figure 1.5 Simplified diagrammatic representation of the PI3K/Akt pathway. Specific receptors on the cell membrane receive signals and lead to the activation of PI3K, which phosphorylates PIP_2 and produces PIP_3. PIP_3 recruits and activates PDK1, which in turn phosphorylates and activates Akt. Akt is a protein kinase involved in a number of downstream signalling pathways, including IKK (NF-(kappa)B pathway), Bad (Bcl-2 family proteins), caspase-9 pathway, FKHR transcription factors and mTOR. IKK, I(kappa)B (inhibitor of NF-(kappa)B) kinase; FKHR, Forkhead (Drosophila) homolog 1 (rhabdomyosarcoma); PIP_2, phosphatidylinositol bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PI3K, phosphoinositide 3-kinase; PDK1, phosphoinositide-dependent kinase 1; mTOR, mammalian target of rapamycin.

The PI-3K/Akt pathway (Figure 1.5) is one of the most important signalling pathways that regulate cell survival. Akt, also known as protein kinase B, is a serine/threonine protein kinase and another major determinant of cell survival (Datta, Dudek *et al.* 1997). Constitutive activation and hyperactivity of Akt have been found in many human tumour types (Kitada, Pedersen *et al*. 2002; Nicholson and Anderson 2002). Overexpression of activated Akt in primary non-small cell lung cancer (NSCLC) cells is correlated with poor prognosis (David, Jett *et al*. 2004). Elevated levels of Akt activation can also be an independent biomarker for biomedical recurrence of prostate cancer (Ayala, Thompson *et al*. 2004). Inhibition of Akt activity induces apoptosis in human cell lines that overexpress its activated form (Yang, Dan *et al*. 2004).

Growth factors signal through the PI-3K/Akt pathway to promote cell survival. PI3K is activated via tyrosine kinase and G-protein-coupled receptors, generating phospholipids PIP3, secondary messengers for downstream signals. Akt binds PIP3 via its pleckstrin homology (PH) domain and translocates from cytoplasm to the inner face of the plasma membrane (Datta, Brunet *et al.* 1999; Oka, Nakahara *et al*. 2005) to become phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) and thus activated (Kennedy, Kandel *et al*. 1999; Nicholson and Anderson 2002). PTEN and SHIP are lipid phosphatases that dephosphorylate PIP3 at different sites and act as negative regulators of the PI3K/Akt pathway (Datta, Brunet *et al.* 1999; Nicholson and Anderson 2002). Mutations and defective

function of PTEN are found in NHL and MM (Kitada, Pedersen *et al*. 2002). Enhanced PI3K signalling or impaired PTEN activity leads to enhanced activation of Akt and promotes cell survival (Jones, Saibil *et al*. 2005).

Activated Akt dissociates from the membrane and regulates a variety of cell signalling pathways by phosphorylation. It can also enter the nucleus where it phosphorylates nuclear proteins (Lawlor and Alessi 2001). At least five major pathways have been suggested through which Akt may prevent apoptosis.

i) One of the first and best known substrates of Akt is Bad, the pro-apoptotic member of Bcl-2 family. Bad has no mitochondria-targeting sequence and translocates to mitochondria in a phosphorylation-dependent manner by heteroligomerization with other Bcl-2 proteins, *e.g*. Bcl-xl (Kennedy, Kandel *et al*. 1999). Dephosphorylated Bad promotes cell death possibly via forming heterodimers with Bcl-xl to release Bax and generate Bax homodimers. Akt can directly phosphorylate and inactivate Bad (Franke and Cantley 1997; Hajra and Liu 2004; Karin and Lin 2002). Phosphorylated Bad is sequestered by 14-3-3, the cytosolic phospho-serine-binding protein, from Bcl-xl and thus promotes cell survival (Datta, Dudek *et al.* 1997).
ii) Akt directly phosphorylates and inactivates caspase-9, and thus prevents apoptosis downstream of the release of cytochrome c from mitochondria (Datta, Brunet *et al*. 1999).

iii) Akt can act independently of Bad, upstream of cytochrome c release and caspase activation (Kennedy, Kandel *et al*. 1999), *e.g*. via preventing Bid cleavage (Oka, Nakahara *et al*. 2005). In the presence of glucose, Akt also prevents the closure of VDAC associated with hexokinase, another possible mitochondrial effector of Akt-mediated cell survival (Gottlob, Majewski *et al*. 2001). Akt also phosphorylates Bax and suppresses its translocation to mitochondria during apoptosis (Gardai, Hildeman *et al*. 2004; Tsuruta, Masuyama *et al.* 2002).

iv) Akt phosphorylates transcription factors that regulate apoptosis, *e.g.* phosphorylation of Forkhead family transcription factors by Akt sequesters them in the cytoplasm from entering the nucleus and therefore prevents the transcription of pro-apoptotic genes, *e.g*. *FasL* (Datta, Brunet *et al*. 1999).

v) I(kappa)B kinase (IKK), containing two catalytic subunits IKK-alpha and IKK-beta (NEMO), and a regulatory subunit IKK-gamma (Karin and Lin 2002), is another important substrate of Akt (Nicholson and Anderson 2002). Akt stimulates the signalling pathway that leads to upregulation of NF-(kappa)B activity (Madrid, Wang *et al.* 2000) and it has been suggested that IKK is a direct downstream target of Akt. Phosphorylation of I(kappa)B-alpha is detected following Akt activation. Akt and IKK-alpha constitutively co-immunoprecipitate (Ozes, Mayo *et al*. 1999; Romashkova and Makarov 1999).

NF-(kappa)B comprises a group of dimer transcription factors composed of Rel family subunits, Rel-A (p65), Rel-B, c-Rel, NF-(kappa)B1-p105 and NF-(kappa)B2-p100. The latter two are synthesized as big precursors and have an I(kappa)B-like C-terminus. Degradation of the I(kappa)B-like C-terminus in response to upstream signal forms the activated p50 and p52 (Karin and Lin 2002). Two NF-?B activation pathways have been described.

i) *The canonical pathway*

NF-(kappa)B dimers, *e.g*. Rel-A (p65)/p50, are sequestered in the cytoplasm by inhibitors of NF-(kappa)B (I(kappa)B). IKK-beta becomes phosphorylated and activated by upstream kinases, possibly Akt, and phosphorylates I(kappa)B. Phosphorylated I(kappa)B is subjected to ubiquitination and degradation via proteasome and releases NF-(kappa)B dimers that translocate to the nucleus where they activate the transcription of NF-(kappa)B-target genes (Datta, Brunet *et al*. 1999).

ii) *IKK-alpha pathway*

Rel-B and NF-(kappa)B2-p100 form dimers in the cytoplasm. Upon stimulation, the I(kappa)B-like C-terminus of NF-(kappa)B2-p100 becomes phosphorylated by IKK-alpha and degraded, leaving the Rel-B/p52 to actvate transcription of target genes (Karin and Lin 2002).

Tumour cells that express constitutively active NF-(kappa)B are highly resistant to drug or radiation treatment, and inhibition of NF-(kappa)B activity increases their sensitivity to such treatment (Karin, Cao *et al.* 2002). Many tumours of lymphoid origin show constitutively activated NF-(kappa)B (Karin and Lin 2002). Aberrant NF-(kappa)B activation has also been found in association with defective apoptosis in breast cancer cells (Biswas, Shi *et al*. 2004). In a similar manner to Bcl-2 proteins, NF-(kappa)B is another major regulator of cell death. Unlike Bcl-2, NF-(kappa)B has direct DNA-binding activity and binds promoters of a variety of genes involved in host defence, inflammatory responses and anti-apoptosis, regulating their transcription (Kitada, Pedersen *et al*. 2002). The anti-apoptotic proteins under NF-(kappa)B regulation include Bcl-2 proteins, IAPs, FLIP, *etc* (Karin and Lin 2002; Kitada, Pedersen *et al*. 2002).

1.3.4 p53 mutation and apoptosis

The *TP53* gene was discovered as a tumour supressor gene, mutations of which are found in more than 50% of human cancers (Soussi and Beroud 2001). It regulates gene expression and cellular response after stress by inducing a series of signals leading to DNA repair, cell cycle arrest or apoptosis (Soussi and Lozano 2005). Recent studies reveal that it also plays roles in regulating longevity and ageing, glycolytic pathways and overall

fitness of an organism (Vousden and Lane 2007). More than 1500 p53 mutants have been described in various types of human tumours and these correlate with anti-apoptotic activity and drug resistance (Soussi, Kato *et al*. 2005). p53 translocates to mitochondria in apoptotic tumour cells and causes permeablization of the outer membrane of mitochondria and release of cytochrome c, by forming complex with Bcl-xl and Bcl-2 proteins (Mihara, Erster *et al*. 2003).

1.4 Normal B cell development

A detailed understanding of the molecular basis of normal B-cell development helps elucidate defects in B cell malignancies. The life cycle of B cells includes development in the bone marrow followed by maturation and differentiation in the periphery (Table 1.1). Sequential stages of B-cell development are defined by expression of particular molecular markers, including the rearrangement status and expression of the immunoglobulin genes. In adult life B cell development occurs predominantly in the bone marrow. *Haematopoietic stem cells* (HSC), the pluripotent primitive $CD34^+$, $CD38^-$ cells in the bone marrow, have self-renewal capacity and differentiate into committed precursors of various haematopoietic lineages. These include the *common lymphocyte progenitor* (CLP) cells that further differentiate into T, B or natural killer cells (Auer 2003).

Table 1.1 B cell development

Pluripotent stem cells	Both heavy and light Ig genes are germline. No surface Ig expression. $CD34^+$
Pro-B	Light chain genes remain germline, heavy chain genes rearrange. Cell surface expression of Pro-BCR. $CD43^+$
Pre-B	Light chain genes rearrange. Cell surface expression of pre-BCR.
Immature B	Heavy and light Ig genes are both rearranged. IgM is expressed on cell surface. Cell surface expression of BCR.
Mature B	Cell surface expression of BCR, IgD

B cells at the earliest stage are $CD43^+$ *pro-B* cells. They have limited self-renewal capacity and are further divided into *early pro-B* and *late pro-B* cells. Rearrangement of immunoglobulin heavy chain occurs at this stage, with D_H to J_H joining at early pro-B and V_H to D_HJ_H joining at late pro-B stage. Productive rearrangement leads to the expression of mu heavy chain in the cytoplasm and the next stage, *pre-B* cells. At early pre-B stage, mu heavy chain starts to be expressed on the cell surface, together with a surrogate light chain, to form the pre-B receptor. These cells proliferate and differentiate into $CD43^-$ late pre-B cells when V_L to J_L rearrangement of light chain begins.

Finally intact IgM molecules are expressed on the surface of *immature B* cells (Rudin and Thompson 1998).

Immature B cells undergo a series of negative selections, depending on the specificity of their B-cell receptors (BCR). Self-reactive B cells are eliminated or rendered tolerant. The latter also die, but take longer than clonally aborted cells (Burrows and Kearney 1993). Those survivors of the selection enter the periphery and become *naïve mature B cells*. They express IgD as a second surface Immunoglobulin (Janeway, Travers *et al*. 2001) and are subject to T-cell-dependent or -independent activation. These cells die within a few days in the periphery unless they encounter their cognate antigen and become either anergic or activated (Shaffer, Rosenwald *et al*. 2002). The dramatic effects of B cell activation include the formation of germinal centres, IgH isotype switching, antibody affinity maturation, *plasma cell* differentiation, and the generation of *memory B cells*.

Only a small fraction of the newly generated B precursors survive the entire development process and enter the periphery. In order to protect themselves from all the pro-apoptotic signals and accomplish the whole developmental and maturational process, B cells need to receive survival signals or express pro-survival proteins once they are selected as the desired clone. These include:

i) *CD40/CD40L*: CD40, a 48 kD glycoprotein expressed on mature B cells, interacts with CD40L (CD154) on activated $CD4^+$ T cells and delivers costimulation and survival signals to B cells (Furman, Asgary *et al*. 2000; Klaus, Choi *et al.* 1997). CD40 ligation is critical for T-cell-dependent B-cell activation and evidence shows that it acts via activation of the NF-(kappa)B pathway (Furman, Asgary *et al*. 2000).
ii) *Surface immunoglobulins*: early pre-B cells express pre-B receptors and immature B cells express surface IgM. They are markers of successful rearrangement of immunoglobulin heavy and light chain, respectively, and prevent B cells from undergoing apoptosis. In mature B cells, surface immunoglobulins are the key part of the B-cell receptor (BCR) and determine antigen specificity, B cell survival and activation (Rudin and Thompson 1998).

Apoptosis is a critical mechanism to eliminate self-reactive cells, attenuate immune responses and maintain the crucial state of dynamic homeostasis in the lymphoid immune system. It happens at several stages:

i) Early pre-B cells that lack surrogate immunoglobulin, mostly due to non-productive rearrangement of the heavy chain gene, are deleted by induction of apoptosis.
ii) Late pre-B cells that fail to express functional surface immunoglobulin die from apoptosis.

iii) Immature B cells that express self-reactive immunoglobulin undergo apoptosis within the bone marrow.

iv) Peripheral naïve B cells are also highly susceptible to apoptosis induction unless stimulated by their cognate antigen.

v) Apart from the differentiation of a minute fraction of the activated B cells into memory B cells, the majority of selected, activated and amplified B cell clones undergo apoptosis on termination of an immune response.

1.5 B cell receptor (BCR) signalling

B cell receptor signalling is required for both B cell development and survival, as well as the pivotal role that B cell plays in the immune system. The signal transduction through BCR is a complex labyrinth of interconnecting pathways and there follows below only a brief description of the main pathways relevant to this thesis.

As with most receptor-based signal transduction, BCR signalling begins with receptor aggregation initiated by recognition of antigens and activation of an array of effector molecules, and ends with phenotypic and/or genotypic modifications which determine the cell response to the stimulus. The receptor comprises a surface immunoglobulin, non-covalently associated with a disulfide-linked CD79a and CD79b heterodimer. The cytoplasmic part of the

CD79a/b contains an important motif, the immunoreceptor tyrosine-based activation motif (ITAM). At least two major events occur rapidly after BCR ligation, ITAM phosphorylation by Src-family kinases, and generation of PIP3 by PI3K (Gold 2002; Kurosaki 2002).

i) Phosphorylation of the ITAM of CD79a/b leads to the recruitment and activation of Syk, the cytosolic tyrosine kinase (Rowley 1995), which in turn phosphorylates B cell linker protein (BLNK), the adaptor molecule. Phospholipase C(gamma)2 (PLC(gamma)2) is recruited via its SH2 domain to BLNK and becomes phosphorylated by Syk.

ii) PI3K catalyses the generation of PIP3, which interacts with PH-domain containing proteins, including Akt, PLC(gamma)2 as well as Btk, which subsequently phosphorylates PLC(gamma)2, like Syk.

The two events converge on the phosphorylation and activation of PLC(gamma)2, which cleaves membrane-associated PIP2 into inositol trisphosphate (IP3) and diacylglycerol (DAG), the phospholipid-derived second messengers. IP3 frees the intracellular Ca^{2+}, whereas DAG mediates activation of protein kinase C (PKC) and subsequent activation of mitogen activated protein kinase (MAPK) family. Both lead to the activation of transcription factors, *e.g.* NF-(kappa)B and nuclear factor of activated T-cells (NF-AT), which modify gene expression and determine the cell response to

stimuli. BCR stimulation does not always result in B cell activation and proliferation, and how a B cell responds to a signal depends not only on the maturational stage and its extracellular environment, but also on properties of the relevant antigen and the availability of T cell help (Dal Porto, Gauld *et al*. 2004). BCR signalling is also modulated by co-receptors and other accessory molecules, including CD19 and CD45, the positive regulators, and CD22, Fc(gamma)RIIB, the negative regulators (Dal Porto, Gauld *et al*. 2004; Monroe 2006).

1.6 Chronic lymphocytic leukaemia (CLL)

Chronic lymphocytic leukaemia (CLL) involves the abnormal clonal expansion of the $CD5^+$ /$CD19^+$/$CD23^+$ B cells that have a prolonged life span (Furman, Asgary *et al.* 2000; von Bergwelt-Baildon, Maecker *et al*. 2004). These non-proliferating cells are arrested at the G_0/early G_1 phase of the cell cycle and gradually accumulate in lymphoid tissues, bone marrow and peripheral blood (Granziero, Ghia *et al*. 2001; Kolb, Kern *et al*. 2003). The prolonged survival mechanisms in CLL include defective apoptosis and cell cycle arrest that result from both intrinsic and extrinsic factors.

1.6.1 Genetic abnormalities and CLL biomarkers

Chromosomal abnormalities and genomic aberrations are frequently seen in CLL (Byrd, Stilgenbauer *et al*. 2004; Kokhaei, Palma *et al*. 2005; Shanafelt, Geyer *et al*. 2004) and may determine response to therapies and cell survival. Although none of these defects are fully unifying (Chiorazzi, Rai *et al*. 2005), reported mutations and altered gene expression patterns include *TP53*, *ATM*, *Tcl-1* and *mdm2* (Bentley and Pepper 2000; Kipps 2003; Munk Pedersen and Reed 2004). Extended cell survival favours genetic instability and accumulation of gene mutations, as well as new and more 'damaging' genetic abnormalities that coincide with disease progression.

The mutation status of the variable region of the immunoglobulin heavy chain (V_H) gene is an important characteristic of CLL, as it divides the disease into two prognostic subtypes: V_H-unmutated and V_H-mutated CLL (Herishanu and Polliack 2005; Kokhaei, Palma *et al*. 2005; Shaffer, Rosenwald *et al*. 2002;). The V_H-unmutated CLL is associated with poor prognosis, relative to the V_H-mutated subtype (Damle, Wasil *et al*. 1999; Hamblin, Davis *et al*. 1999). Zeta-associated protein-70 (ZAP-70), a cytoplasmic tyrosine kinase involved in T cell receptor signalling, is preferentially expressed in V_H-unmutated CLL cells (Crespo, Bosch *et al*. 2003; Kokhaei, Palma *et al.* 2005; Wiestner 2005). CD38 is another surrogate marker associated with V_H-unmutated CLL, although it is less reliable and expression of CD38 may change through the

course of disease (Kharfan-Dabaja, Chavez *et al.* 2008; Kokhaei, Palma *et al*. 2005; Shanafelt, Geyer *et al*. 2004).

1.6.2 Aberrant apoptotic pathway

CLL cells have a survival advantage *in vivo* in association with defective apoptosis. This is reflected in the following mechanisms:

a. CLL cells have consistent overexpression of Bcl-2 and Mcl-1 (Bentley and Pepper 2000; Granziero, Ghia *et al*. 2001). Bcl-2 gene is hypomethylated in over 95% of CLL cells, resulting in elevated expression of this anti-apoptotic protein (Blaise, Masdehors *et al*. 2001).
b. Signal transducers and activators of transcription protein 1 and 3 (STAT1 and STAT3) are constitutively phosphorylated and activated in CLL cells. STATs have been implicated in upregulation of Bcl-xl and Mcl-1 and downregulation of caspases (Munk Pedersen and Reed 2004).
c. The PI3K/Akt and NF-(kappa)B signalling pathways are constitutively active and play important roles in CLL cell survival (Cuni, Perez-Aciego *et al*. 2004). In normal B cells, T-cell leukaemia/lymphoma 1 (Tcl-1), a 14 kD protein, is expressed at early B cell maturation stages and downregulated possibly following antigen encounter and transition through the germinal

centre. Tcl-1 overexpression is detected in CLL and reportedly binds and activates Akt (Kipps 2003; Munk Pedersen and Reed 2004).

d. CLL cells express defective apoptotic receptors on their surface, *e.g.* Fas and TRAIL. The activities of Fas and TRAIL are impaired, contributing to resistance to spontaneous and drug-induced apoptosis (Kolb, Kern *et al*. 2003). CLL cells express and secrete Fas ligand and induce apoptosis in Fas^+ T cells, a possible mechanism for immune escape (Jewell 2002).
e. The ubiquitin system involved in apoptosis of normal lymphocytes is deregulated in CLL cells (Blaise, Masdehors *et al*. 2001).

1.6.3 BCR signalling in CLL cells

In CLL, low expression of surface immunoglobulin has been reported (Kokhaei, Palma *et al.* 2005; Shaffer, Rosenwald *et al.* 2002). CD79b is downregulated (Chiorazzi, Rai *et al*. 2005) and a truncated form has been detected with the extracellular domain absent, resulting from alternative splicing of the CD79b gene (Munk Pedersen and Reed 2004). These properties may contribute to the unresponsiveness and survival mechanisms in CLL.

BCR signalling leads to either cell death or progression into the cell cycle. This can be upregulated or downregulated in CLL, depending partly on the

surface density of IgM and IgD as well as the levels of CD38 and ZAP70 (Herishanu and Polliack 2005). Medium-cultured CLL cells show reduced responsiveness to IgM stimulation (Wiestner 2005). Crosslinking of surface IgM leads to apoptosis or cell survival, whereas crosslinking of surface IgD consistently protects CLL cells from apoptosis (Chiorazzi, Rai *et al.* 2005). As IgM and IgD can transduce opposite signals, the final results of BCR signalling may depend on the balance between these two types of receptors (Zupo, Massara *et al.* 2000).

1.7 Modified citrus pectin – GCS-100

Carbohydrates are molecules that contain only hydrogen, oxygen and carbon. They may have simple structures, *e.g*. five- and six-carbon sugars, or complex polymer chains, *i.e*. polysaccharides. Carbohydrates play finely-tuned roles in regulation of cell function. Even minor changes in their structures may have a major impact on cell signalling. They interact and form conjugates with biologically active molecules such as carbohydrate-binding proteins to interfere with their functions.

Citrus pectin, a type of polysaccharide obtained from citrus fruit, is highly branched and rich in galactoside residues. In its modified form after hydrolysis, it acts as a specific inhibitor of galectin-3, which binds the beta-

galactoside residues through its carbohydrate binding domain (Inohara and Raz 1994; Nangia-Makker, Hogan *et al*. 2002). As galectin-3 is a multi-functional protein involved in immune response, cell cycle, apoptosis, cell-cell and cell-matrix interaction, tumour metastasis and angiogenesis (Inohara and Raz 1994), potentially modified citrus pectin may interfere with a wide spectrum of cell functions. Experimentally it can inhibit cell adhesion to laminin and, in at least partial consequence of this, tumour metastasis (Inohara and Raz 1994; Pienta, Naik *et al*. 1995).

GCS-100, formerly designated as *GBC-590*, is a type of modified citrus pectin derived by acid/base hydrolysis of citrus pectin isolated from the skin and pulp of citrus fruits (GlycoGenesys, Boston). Structurally it is rich in galactoside residues and has a high-affinity for galectin-3, which is thought to be instrumental in its biological effects. GCS-100 appears to interfere with tumour metastasis and inhibit angiogenesis, and has been in Phase II clinical trial for pancreatic, prostate and colorectal cancer (Cotter 2004; Daliani, Pratt *et al*. 1999; Grous, Redfern *et al*. 2006; Gunzburg and Salmons 2001; Springgate, Cartwright *et al*. 2001; Staddon, Bonnem *et al.* 1999; Stuart, Kindler *et al.* 2001). Recently it has been reported that GCS-100 induces apoptosis in multiple myeloma cells without affecting normal lymphocytes (Chauhan, Li *et al*. 2005).

The potential anti-cancer role of modified pectin has recently excited considerable interest as a dietary component of fruit and vegetables, and as an agent used in food processing. The specific binding between the bioactive fragments from pectin and human galectin-3 has also been demonstrated (Gunning, Bongaerts *et al*. 2009).

1.8 PK11195

PK11195, or 1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinoline-carboxamide, is a ligand for the mitochondrial peripheral benzodiazepine receptor (mPBR) with high affinity. The mPBR, also designated as the 18 kD translocator protein (TSPO), is a transmembrane protein in mitochondria which forms part of the PT pore (see 1.2.2.a). PK11195 binds to mPBR at nanomolar concentrations (Fennell, Corbo *et al*. 2001). Higher doses of PK11195 induce apoptosis and have chemosensitisation effects in primary CLL, AML and MM cells (Banker, Cooper *et al.* 2002; Chauhan, Li *et al*. 2004; Santidrian, Cosialls *et al*. 2007; Walter, Pirga *et al*. 2005; Walter, Raden *et al*. 2004), and B-cell lymphoma cell lines (Muscarella, O'Brien *et al*. 2003). It has also shown pro-apoptotic effects in human glioma and insulinoma (Chelli, Salvetti *et al*. 2008; Park, Cho *et al.* 2005), and is currently in clinical development (Banker, Cooper *et al.* 2002).

The mechanism of the pro-apoptotic effect of PK11195 remains to be fully understood. It can induce PT pore opening, cytochrome c release as well as mitochondrial memebrane potential collapse (Li, Wang *et al*. 2007; Park, Cho *et al.* 2005). Anti-apoptotic activity due to Bcl-2 overexpression can also be overcome by PK11195 in human B lymphoma cells and other tumour cell lines (Hirsch, Decaudin *et al*. 1998; Muscarella, O'Brien *et al*. 2003). Based on the finding that the dose needed for its pro-apoptotic effects is much higher than that required for receptor engagement, and the fact that it induces apoptosis in mPBR-negative cells, PK11195-induced apoptosis must have an additional mPBR-independent mechanism (Fennell, Corbo *et al*. 2001; Walter, Pirga *et al*. 2005).

1.9 Purpose of investigation

The foregoing two types of drug candidates, GCS-100 and PK11195, currently of interest to the Department of Haematology at Queen Mary, University of London, of contrasting provenance and putative mechanisms of action, both with anti-cancer effects in haematopoietic malignancies and currently in clinical development (Banker, Cooper, *et al*. 2002; Chauhan, Li, *et al*. 2005), were evaluated for their capacity to induce apoptosis in established cell lines and primary neoplastic cells of B cell lineage, and the mechanisms of apoptosis-induction evaluated at the molecular level. For logistic reasons,

access to primary materials (*e.g*. primary CLL cells) was limited in this project. Therefore, for experimental purposes, the emphasis was on malignant cell lines of B cell origin for which no prior data on GCS-100 were available. In addition, cell lines of non-B-cell origin were included for comparison. The data presented have implications for the design of therapeutic strategies targeting apoptosis in haematological maliganancies.

Chapter 2 Materials and Methods

2.1 General reagents and stock solutions

Table 2.1 General reagents

Products	**Suppliers and Catalogue No.**
EDTA	Promega, V4231
EPOSIN (etoposide, VP-16)	Pharmachemie B.V.
Galectin-3 inhibitor	Organic Chemistry, Lund Uni.
GCS-100	GlycoGenesys, Batch.876284-HO1(unless otherwise indicated).
Glycine	BDH, 10119CU
HCl	BDH, 101254H
2-Mercaptoethanol	Sigma, M6250
MnTBAP	OxisResearch, 26532
Nonidet P40 Substitute	Fluka, 74385
PBS tablets	Fluka, 79382
PK11195	Sigma, C0424
2-Propanol (Isopropanol)	Sigma, I9516
Rotenone	Aldrich, 477737

Sodium chloride	BDH, 10241AP
Triton X-100	Sigma, T8787
Trizma base (Tris base)	Sigma, T6066
Spectrophotometer	Eppenforf, 6132. 000.008

Table 2.2 General stock solutions

Solution	Preparation
1 M Tris pH 7.5 (1 L)	• 121.14 g Tris base was dissolved in 900 ml H_2O. • The pH was adjusted to 7.5 with HCl. • The volume was adjusted to 1 L with H_2O.
5 M NaCl (1 L)	• 292.2 g NaCl was dissolved in 900 ml H_2O. • The volume was adjusted to 1 L with H_2O.
10% SDS (1 L)	• 100 g SDS was dissolved in 900 ml H_2O. A mask was used when weighing SDS. • The volume was adjusted to 1 L with H_2O.

2.2 Cell culture

2.2.1 Reagents

Table 2.3 Cell culture reagents

Products	Suppliers and Catalogue No.
Dulbecco's Modified Eagle's (DME) Medium	Sigma, D5546
FCS	PAA, A11-043
Ficoll-Paque Plus	Amersham Biosciences, 17-1440-03
L-glutamine (200 mM)	PAA, M11-004
Penicillin & streptomycin	PAA, P11-010
RPMI1640	PAA, E15-039
Trypsin-EDTA	PAA, L11-660

2.2.2 Cell lines

Table 2.4 Cell lines

Designation	Cell type	Origin	Features
DOHH2	Mature B cell	Follicular B-Non-Hodgkins Lymphoma	High expression of Bcl-2 due to t(14;18)
JURKAT	Immature T cell	Relapsed childhood T-acute lymphoblastic leukaemia	No galectin-3 expression, $CD95^{+}$,sensitive to various apoptotic

			signals
K562	Erythroid leukaemia cell	Myeloid leukaemia	Ph chromosome with bcr-abl b3-a2 fusion gene
RAMOS	B lymphoma cell	American Burkitt's Lymphoma	p53 mutation
RPMI8226	B-lineage cell	Myeloma; plasmacytoma	No heavy chain production
SUD4	Mature B cell	B lymphoma	p53 mutation
U266	B-lineage cell	Myeloma; plasmacytoma	Producing IL-6
HEK293	Epithelial	Human embryonic kidney	Adherent cells

2.2.3 Culture of suspension cell lines

All suspension cells were cultured in RPMI1640 medium supplemented with 10% FCS (inactivated at 56ºC for 0.5 hr), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin unless otherwise stated. Incubation was at 37ºC with 5% CO_2.

Cells were defrosted at 37ºC, immediately washed with 10 ml PBS, transferred to 20 ml medium (20% FCS) and incubated overnight. A further 20 ml medium (20% FCS) was added the following day. When cells became confluent, usually after 2 or 3 days, they were transferred to 40 ml fresh medium and kept in culture for 2 weeks. Medium was changed every 2-3 days during this period and culture volume expanded according to the degree

of confluence. The majority (leaving 5 ml in culture) of the confluent cell suspension was then resuspended in 3-5 ml (depending on the total cell number) of Freezing medium (50% FCS, 30% RPMI 1640 and 20% DMSO), aliquoted into 1 ml freezing vials and immediately stored in a pre-cooled (4ºC) Nalgen freezing box at -70ºC overnight before being transferred to a liquid nitrogen tank. The remaining 5ml cell suspension was subcultured by adding 35 ml fresh medium and maintained in culture as needed for 2 months before being discarded. Another vial of freezing stock was then defrosted in the same way as stated above.

2.2.4 Culture of adherent cell lines

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (inactivated at 56ºC for 0.5 hr), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin unless otherwise stated, in a cell-culture incubator at 37ºC with 5% CO_2.

Cells were defrosted at 37ºC, immediately washed with 10 ml PBS, re-suspended in 20 ml medium and seeded in a 75 cm^2 flask. When the confluence reached 80%, cells were detached with Trypsin-EDTA and re-seeded in two 150 cm^2 flasks. When both flasks became 90% confluent, cells were collected as much as possible, washed with PBS, resuspended in 10 ml

Freezing solution (10% DMSO + 90% FCS), aliquoted into 1 ml freezing vials and immediately stored in the pre-cooled Nalgen freezing box (4ºC) at -70ºC overnight before being transferred to the liquid nitrogen tank. 40ml fresh medium was added to each flask, and the remaining cells were subcultured and maintained in culture for 2 months before being discarded. Another vial of freezing stock was then defrosted in the same way as stated above.

2.2.5 Culture of primary CLL cells and normal lymphocytes

Peripheral whole blood samples were obtained from CLL patients at Birmingham Heartlands Hospital, or normal donors, following ethical approval. Lymphocytes were freshly isolated using Ficoll as described below.

i) Whole blood samples were diluted with an equal volume of PBS and mixed well.
ii) The Ficoll-Paque Plus bottle was inverted several times to ensure thorough mixing and 3.5 ml (2.4 cm of the height) Ficoll was added to a 15 ml centrifuge tube.
iii) 4.5 ml (3.0 cm of the height) diluted blood sample was carefully layered on top of Ficoll, and centrifuged at 400 g (1660 RPM, *r*=13 cm) for 40 minutes at 18ºC.

iv) The lymphocyte layer was transferred using a sterile Pasteur pipette to a clean centrifuge tube with 3 volumes of PBS. The lymphocytes were resuspended by gently inverting the tube several times and centrifuged at 100 g (830 RPM) for 10 minutes at 18ºC.

v) Supernatant was removed and the lymphocytes were resuspended in 8 ml PBS and centrifuged at 100 g (830 RPM) for 10 minutes at 18ºC.

vi) Lymphocytes were then resuspended in a suitable volume (depending on the size of cell pellets) of culture medium.

vii) Lymphocytes were counted and seeded in culture vessels, and treated as indicated.

2.2.6 Culture of PK11195-resistant cell line

Ramos cells were cultured as described in 2.2.3. At passage 3, cells were treated with 10 µM PK11195 and cultured until reaching confluency. Part of the cells were then frozen down for future use. Cells were then treated with 25 µM PK11195 and cultured until reaching confluency. Part of the cells were then frozen down for future use. Finally, cells were treated with 50 µM PK11195, and kept in culture for about 2 months before being discarded.

2.3 Flow cytometry

2.3.1 Fluorescent reagents

Table 2.5 Fluorescent reagents

Products	**Suppliers and Catalogue No.**
DHE	Sigma, D7008
DiOC6(3)	Molecular Probe, D273
MC540	Molecular Probe, M24571
Sphero Rainbow Calibration Particles	Pharmingen, 559123

Flow cytometry is a powerful and convenient method to detect apoptotic cells. By introducing a potentially fluorescent dye, which either binds to a specific protein or membrane or enters a specific cellular compartment, cells at certain stages of apoptosis can be detected and quantified. As specific probes detect different stages of apoptosis, a kinetic study was performed first to determine upon the time point for measurement.

i) *DiOC6(3)* stains the mitochondrial membrane at low concentrations. It has been specifically used to detect collapse of the mitochondrial trans-membrane potential (DeltaPsi(m)) and is regarded as a mitochondria-selective potentiometric probe (Blatt, Bednarski *et al*. 2002). Negative

DiOC6(3) staining correlates with apoptosis through the mitochonrial pathway, and this can be detected on the FL1 channel of the flow cytometer.

ii) *Merocyanine 540 (MC540)* is a polar dye which intercalates in the unpacked membrane and detects alterations in membrane symmetry. This often reflects a later stage of apoptosis in comparison with DiOC6(3). Positive MC540 staining correlates with apoptosis and this can be detected on the FL2 channel of the flow cytometer.

iii) *Dihydroethidium (DHE)* is a redox-sensitive fluorescent dye that reacts specifically with O_2^- (Blatt, Bednarski *et al*. 2002). It was used to detect intracellular ROS production and positive staining was detected on the FL2 channel of the flow cytometer.

2.3.2 Sample preparation and FACS analysis

a. Sample preparation

Calibration of the FAScan Machine (Becton Dickinson) was performed prior to detection using SPHERO^{TM} Rainbow Calibration particles (8 peaks, 3.0-3.4 µM). Titration of each fluorescent probe, or combination, was also performed to determine optimal concentration for cell types being analysed.

i) *DiOC6(3)/PI*: 100 mg DiOC6(3) was dissolved in 20 ml DMSO to make 5 mg/ml stock solution. 25 mg PI was dissolved in 5 ml PBS to make 5 mg/ml stock solution. 100 µl PI stock solution and 1 µl DiOC6(3) stock solution were added into 10 ml PBS to make the working solution, of which 40 µl/ml was used. The final concentration was 2 µg/ml PI + 20 ng/ml DiOC6(3).

ii) *MC540/7-AAD:* 25 mg MC540 was dissolved in 5 ml DMSO to make 5 mg/ml stock solution. 1:40 substock solution was made by adding 1 ml stock solution in 40 ml PBS. 40 µl/ml of substock solution was used to achieve the final concentration of 5 µg/ml. 1 mg *7-AAD* was dissolved in 5 ml PBS to make 200 µg/ml stock solution, and 5 µl/ml of stock was used to achieve the final concentration of 1 µg/ml.

iii) *DHE*: 10 mg DHE (MW. 315.4) was dissolved in 31.7 ml DMSO to make 1 mM stock solution. 5 µl/ml was used to achieve the final concentration of 5 µM.

b. FACS analysis

Cells were incubated with the fluorescent probe at room temperature in the dark for 5-15 minutes, and analysed on the FACScan machine. 10,000

events were recorded, and the results were further analysed by WINMDI software.

2.4 Immunoprecipitation

2.4.1 Reagents and buffers

Table 2.6 Reagents for immunoprecipitation

Products	Suppliers and Catalogue No.
Dynabeads Pan Mouse IgG	DYNAL Biotech, 110.41
Protease inhibitor cocktail	Sigma, P8340
Protein assay Kit	Bio-Rad, 500-0113/4
Spectrophotometer cuvettes	Sigma, C5416-100EA

Table 2.7 Buffers for immunoprecipitation

Buffers	Preparation
Washing buffer 1 (WB1, 50 ml)	• 50 mM Tris pH 7.5 (2.5 ml of 1 M stock) • 150 mM NaCl (1.5 ml of 5 M stock) • 1% Nonidet P40 (500 µl) • H_2O 45.5 ml
Washing buffer 2 (WB2, 50 ml)	• 50 mM Tris pH 7.5 (2.5 ml of 1 M

	stock) • 500 mM NaCl (5 ml of 5 M stock) • 0.1% Nonidet P40 50 µl • H_2O 42.45 ml
Washing buffer 3 (WB3, 50 ml)	• 50 mM Tris pH 7.5 (2.5 ml of 1 M stock) • 0.1% Nonidet P40 50 µl • H_2O 47.45 ml
CSH buffer (5×, 200 ml)	• 250 mM Tris pH 7.5 (50 ml of 1 M stock) • 1.25 M NaCl (50 ml of 5 M stock) • 5 mM EDTA (2 ml of 0.5 M stock) • H_2O 98 ml
Lysis buffer (2 ml, freshly made)	• CSH (5×) 400 µl • Triton 100 2 µl • Protease inhibitor cocktail 60 µl • H_2O 1.538 ml

2.4.2 Cell lysate preparation

Cells were washed with PBS twice and the pellet was frozen at -70ºC for at least 1 hr before being defrosted on ice. 30-50 µl of lysis buffer (see Table 7), depending on cell numbers, was added to the cell pellet and mixed by pipetting up and down gently (no air bubbles should be produced). They were then incubated on ice for 30 minutes before centrifuging at full speed, 4ºC for 20 minutes. The supernatant was carefully transferred to a clean pre-cooled microcentrifuge tube (on ice) and the amount of protein in the lysate was quantified using BioRad protein assay kit. The lysate was stored at -20ºC.

2.4.3 Protein quantification

The amount of protein in the lysate was quantified by BioRad Kit. 1 µl of lysate was diluted with 40 µl H_2O. 20 µl of the diluted lysate (using H_2O as blank), 100 µl Reagent A and 800 µl Reagent B were mixed together, vortexed and incubated at room temperature for 15 minutes before being transferred to a cuvette and analysed spectrophotometrically (Eppenforf, see 2.1), using the Bradford programme set up by BSA standards according to the manufacturer's guide.

2.4.4 Immunoprecipitation

i) Pan mouse IgG Dynabeads were mixed well before 50 µl per sample was transferred to an eppendorf and washed with 900 µl of WB1 (see Table 7) twice.

ii) The beads were then resuspended in 300 µl WB1 with the appropriate first antibody (2 µg/sample) and incubated at 4ºC overnight with agitation.

iii) The beads were washed with 1 ml of WB1 three times before being resuspended in 200 µl of WB1.

iv) The cell lysate was defrosted on ice. 20 µg protein was removed and set aside to save as an input control if immunoblotting was to be performed afterwards. An equal amount of protein for each sample (500-1000 µg)

was then added to the beads bound with the first antibody. 6 µl Protease inhibitor cocktail was added before incubation at 4ºC for 3 hours with agitation.

v) The beads bound with the first antibody and the cognate molecules were washed with 1 ml of WB1 for 15 minutes twice, 1 ml of WB2 (see Table 7) for 15 minutes twice and finally 1 ml of WB3 (see Table 7) for 15 minutes once at 4ºC before loading buffer was added and SDS-PAGE was performed (see 2.5).

2.5 Immunoblotting (Western blotting)

2.5.1 Reagents and materials

Table 2.8 Reagents and Materials for Western blotting

Products	**Suppliers and Catalogue No.**
Acrylamide/bisacrylamide	National Diagnostics, EC-890
Ammonium persulfate	Sigma, A3678
Blotting Paper	Sigma, P7769
BSA	Sigma, A7906

Hyper-film ECL	Amersham, RPN3103K
Protein Marker	New England Biolabs, P7702S
PVDF membrane	Sigma, P2813
Short plates	Bio-Rad, 165-3308
Spacer plates (0.75 mm)	Bio-Rad, 165-3310
SuperSignal Kit	PIERCE, 34080
Tween 20	Sigma, P1379

Table 2.9 Antibodies for Western blotting

Antibodies	**Suppliers and Catalogue No.**
beta-actin, mouse	Sigma, A5316
Bax (2D2), mouse	Santa Cruz, SC-20067
Bcl-2, mouse	Santa Cruz, SC-509
Bcl-2, rabbit	Abcam, ab7973
Bcl-x, mouse	BD PharMingen, 556499
Bid, rabbit	R&D Systems, AF846
Caspase-8, mouse	Alexis, ALX-804-242

Caspase-9, mouse	Stressgen, AAM-139
Fas, mouse	Immunotech, 1504
Galectin-3, mouse	R&D Systems, MAB1154
Galectin-3, mouse	BD PharMingen, 556904
I(kappa)B-alpha, mouse	Cell Signaling, 9247
Mouse IgG HRP, goat	BD Pharmingen, 554002
Penta-His, mouse	Qiagen, 34660
Phospho-Akt (Ser473), rabbit	Cell Signaling, 9271
Phospho-I(kappa)B-alpha (Ser32/36), mouse	Cell Signaling, 9246
Rabbit IgG HRP, goat	BD Pharmingen, 554021

Table 2.10 Buffers for Western blotting

Buffers	Preparation
1 M Tris pH 6.8 (500 ml)	• 60.57 g TRIZMA base was dissolved in 350 ml H_2O. • The pH was adjusted to 6.8 with HCl. • The volume was adjusted to 500 ml with H_2O.
1.5 M Tris pH 8.8 (500 ml)	• 90.855 g TRIZMA base was dissolved in 400 ml H_2O. • The pH was adjusted to 8.8 with HCl. • The volume was adjusted to 500 ml with H_2O.

<table>
<tr><td>Loading buffer (2×, 10 ml)</td><td>3%×2 SDS (6 ml of 10% stock)
10%×2 glycerol (2 ml)
50 mM×2 Tris pH 6.8 (1 ml of 1 M stock)
5%×2 2-mercaptoethanol (1 ml)
20 mg bromophenol blue
0.5 ml aliquots were stored at -20ºC</td></tr>
<tr><td>Running buffer (10×, 1 L)</td><td>0.25 M Tris (30.2 g)
1.92 M glycine (144 g)
1% SDS (100 ml of 10% stock, see Table 2)
H_2O (up to 1 L)</td></tr>
<tr><td>Transfer buffer (10×, 1 L)</td><td>0.25 M Tris (30.2 g)
1.92 M glycine (144 g)
H_2O (up to 1 L)</td></tr>
<tr><td>Working transfer buffer (3 L, freshly made)</td><td><table><tr><td></td><td>15% Methanol</td><td>20% Methanol</td></tr><tr><td>Transfer buffer (10×)</td><td>300 ml</td><td>300 ml</td></tr><tr><td>H_2O</td><td>2250 ml</td><td>2100 ml</td></tr><tr><td>Methanol</td><td>450 ml</td><td>600 ml</td></tr></table></td></tr>
<tr><td>Semi-dry transfer buffer (1 L)</td><td>48 mM Tris (5.82 g)
39 mM glycine (2.93 g)
1.3 mM SDS (3.75 ml 10% stock)
20% methanol (200 ml)
H_2O (up to 1 L)</td></tr>
<tr><td>TBST (0.5% Tween 20, 2 L)</td><td>40 ml 1 M Tris pH 7.5
60 ml 5 M NaCl
10 ml Tween 20
H_2O (up to 2 L)</td></tr>
<tr><td>TBS (500 ml)</td><td>10 ml 1 M Tris pH 7.5
15 ml 5 M NaCl
H_2O (up to 500 ml)</td></tr>
<tr><td>Washing buffer, 1 L</td><td>1% skimmed milk powder (10 g)
0.5% BSA (5 g, optional)
TBST (up to 1 L)</td></tr>
</table>

Stripping buffer 1 (for restaining with antibody of a different species, 200 ml)	• 3 g glycine (200 mM) was dissolved in ~150 ml H_2O and the pH was adjusted to 2.5 with HCl • H_2O (up to 200 ml) • Store at 4ºC
Stripping buffer 2 (for restaining with antibody of the same species, 250 ml)	• 100 mM 2-mercaptoethanol (1.75 ml) • 2% SDS (50 ml 10% stock) • 62.5 nM Tris (15.625 ml 1 M Tris pH 6.8) • H_2O (up to 250 ml) • Store at 4ºC

2.5.2 SDS-PAGE

a. Casting acrylamide gel

The BioRad gel cassette was assembled and the pre-gel solution (Table 2.11) was added using a Pasteur pipette. The space for stacking gel was filled with water. The resolving gel was left at room temperature for 30 minutes. The water was removed and the pre-gel solution (Table 2.12) was added. The comb was inserted and the stacking gel was left at room temperature for 30 minutes.

Table 2.11 Resolving gel (10 ml) for SDS-PAGE

	12%	10%
1.5 M Tris pH 8.8	2.5 ml	2.5 ml
H_2O	3.3 ml	4 ml
Acrylamide/bis-acrylamide (30%)	4 ml	3.3 ml
10% SDS	100 µl	100 µl
TEMED	4 µl	4 µl
10% APS (1 ml H_2O + 0.1 g APS, freshly made)	100 µl	100 µl

Table 2.12 Stacking gel (5%, 4 ml) for Western blotting

1 M Tris pH 6.8	0.5 ml
H_2O	2.753 ml
Acrylamide/bis-acrylamide (40%)	667 µl
10% SDS	40 µl
TEMED	4 µl
10% APS (1 ml H_2O + 0.1 g APS, freshly made)	40 µl

b. Sample loading and gel running

i) The gel cassette was assembled into the running tank and running buffer (see Table 10) was added to the inner and outer chamber to cover the gel. The comb was removed.

ii) A volume of lysate containing an equal amount of protein (20-50 µg) was transferred to a clean and cold eppendorf (on ice) and an equal volume of loading buffer (2×, see Table 2.10) was added.

iii) Final volume was adjusted to 20 µl (for 0.75 mm-thick gel) using loading buffer (1×, made by 1:2 dilution of the 2x loading buffer). 10 µl protein

marker plus 10 µl loading buffer (1×) was used as a reference for the molecular weight.

iv) Sample solution was heated at 100ºC for 3 minutes and incubated on ice for 5 minutes.

v) Sample solution was briefly centrifuged and loaded into the wells of the stacking gel.

vi) Empty wells were filled with 20 µl loading buffer (1×).

vii) The gel was run at constant current (voltage 60-70 V) for the stacking gel and constant voltage at 110 V for the resolving gel.

2.5.3 Blotting and staining

a. Tank transfer

i) The gel was washed in working transfer buffer (Table 2.10) for 30 minutes.

ii) PVDF membrane was cut, 8.5×5 cm for each gel, soaked in methanol for 20 seconds and washed in working transfer buffer for 15 minutes.

iii) The gel (close to the cathode) and the membrane (close to the anode) were put together in a folder, with blotting paper on each side and immersed in the transfer tank filled with cold working transfer buffer.

iv) Transfer was carried out at 30 V overnight.

v) Membrane was blocked with 5% milk TBST (Table 2.10, 100 ml TBST + 5 g skimmed milk powder) for 1 hour with agitation.

vi) The first antibody was added to 2 ml 1% milk TBST (100 ml TBST + 1 g skimmed milk powder) at 1:1000 to 1:200 dilution according to the supplier's instructions, and the membrane was stained in a 50 ml tube for 1 hour at room temperature with agitation.

vii) Membrane was washed in a small tray with washing buffer for 10 minutes × 6 with agitation.

viii) Secondary antibody (1:10 000) was added to 10 ml 1% milk TBST, and the membrane stained in a 50 ml tube for 45 minutes at room temperature with agitation.

ix) The membrane was washed in a small tray with TBST for 10 minutes ×6 with agitation.

x) The membrane was finally washed with TBS for 10 minutes ×2.

xi) The membrane was stained with SuperSignal Kit and exposed to a film which was then developed.

b. Semi-dry transfer

i) The PVDF membrane was placed in methanol.

ii) 6 blotting papers for each gel were placed in semi-dry transfer buffer (Table 2.10).

iii) The gel (close to the cathode) and membrane (close to the anode) were put into a semi-dry transfer chamber, with 3 blotting papers on each side. To get rid of air bubbles, rolling was performed between each layer.

iv) Transfer was carried out at constant current of 400 mA, maximum voltage of 25 V for 1 hour.

v) Membrane was stained with first antibody in 1% milk TBST at 4ºC overnight. The blocking and secondary staining was performed the following day as stated previously.

2.5.4 Stripping and restaining

a. Mild stripping (when using antibody from a different species)

i) Membrane was incubated with stripping buffer 1 (Table 2.10) for 20 minutes at room temperature with agitation.

ii) Membrane was equilibrated in 1 M Tris pH 7.5 for 5 minutes.

iii) Membrane was blocked and restained with the new antibody.

b. Harsh stripping (when using antibody from the same species)

i) Pre-warm stripping buffer 2 (Table 2.10) to room temperature.

ii) In a fume cupboard, the membrane was placed in 10 ml stripping buffer 2 in a small tray with a lid. The lid was sealed with parafilm.

iii) Membrane was incubated in 50ºC water bath for 5-30 minutes, depending on the affinity of the previous antibody.

iv) Membrane was washed in H_2O × 2 (in the fume cupboard).

v) Membrane was washed with TBST for 10 minutes × 2.

vi) Membrane was blocked and restained with the new antibody.

2.6 Molecular cloning

2.6.1 Reagents

Table 2.13 Reagents for molecular cloning

Products	**Suppliers and Catalogue No.**
ABI PRISM BigDye Kit	Applied Biosystems, 4336915
Agarose	Melford, MB1200
Ampicillin	Sigma, A9393
Calcium Chloride Dihydrate	BDH, 43705 5N
Calf Intestinal Alkaline Phosphatase (CIAP) Kit	Promega, M1821
$Ca_3(PO_4)_2.2H_2O$	BDH, 437053L
pCMV.Tag 4A vector	Stratagene, 211174
DH5a competent cells	Invitrogen, 18263-012
1 kb DNA ladder	New England Biolabs, N3232S
pEGFP-N3 vector	Clonetech, 6080-1
Formamide	BDH, 10326

Galectin-3 Image Clone 5755882	MRC geneservice, 12795-J11
HEPES	Sigma, H4034
Kanamycin	Sigma, K1876
LB agar (Lennox L Agar)	Gibco-BRL, 22700-041
$NaH_2PO_4 \cdot 2H_2O$	BDH, 301324Q
Na_2HPO_4	BDH, 102494C
QIAGEN Plasmid Maxi Kit	Qiagen, 12162
QIAprep Spin Miniprep Kit	Qiagen, 27104
QIAquick Gel Extraction Kit	Qiagen, 28704
PEI	Polysciences, 23966
pfu DNA polymerase	Promega, M7741
Taq DNA polymerase	Qiagen, 201205
Wizard DNA Clean-Up System	Promega, A7280

Table 2.14 Primers for molecular cloning

Primers	Sequence
Galectin-3 forward HindIII	5’-GAT CAA GCT TGC CAC CAT GGC AGA CAA TTT TTC GCT CC-3’
Galectin-3 reverse XhoI	5’-GAT CCT CGA GTA TCA TGG TAT ATG AAG CAC-3’
Bcl-2 forward HindIII	5’-GAT CAA GCT TGC CAC CAT GGC GCA CGC TGG GAG AAC AGG-3’
Bcl-2 reverse XhoI	5’-GAT CCT CGA GCT TGT GGC CCA GAT AGG CAC CCA G-3’

Galectin-3 reverse XhoI Stop	5'-GAT CCT CGA GTT ATA TCA TGG TAT ATG AAG CAC-3'
Bcl-2 reverse XhoI Stop	5'-GAT CCT CGA GTC ACT TGT GGC CCA GAT AGG CAC CCA G-3'

Table 2.15 Plates, medium and buffers for molecular cloning

Plates or solution	Preparation
Agar plates	32 g LB agar was dissolved in 1 L H_2O and autoclaved. When cool antibiotic of choice was added (25 µg/ml kanamycin, 100 µg/ml ampicillin). The pre-gel mixture was poured into 10 cm petri dishes and allowed to set at room temperature by a flame. Air bubbles were removed using the flame.
CIAP stop buffer (10 ml)	• 10 mM Tris pH 7.5 (100 µl of 1 M Tris pH 7.5) • 1 mM EDTA (20 µl of 0.5 M stock) • 200 mM NaCl (400 µl of 5 M stock) • 0.5% SDS (500 µl of 10% stock) • H_2O, 8.98 ml, up to 10 ml
DNA loading buffer (6X, 100 ml, for agarose gel)	• 50% glycerol, 60 ml • 0.5 M EDTA pH8, 12 ml • 1 M Tris pH7.5, 6 ml • 0.5-1 mg xylenol orange • H_2O, up to 100 ml • Aliquoted and stored at -20ºC.
DNA sequencing buffer (2.5×)	• 200 mM Tris • 5 mM $MgCl_2$ • pH 9 at room temperature
HBS (2×) stock:	• 280 mM NaCl (1.64 g) • 50 mM HEPES (1.19 g) • 1.5 mM $Na_2HPO_4 \cdot 7H_2O$ (400 µl of 1 g in 10 ml) or 1.5 mM $Na_2HPO_4 \cdot 2H_2O$ (400 µl of 0.67 g in 10 ml)

	• H_2O, up to 60 ml • Adjusted to pH 7.05 – 7.1 (exact) using 1 M NaOH. • H_2O, up to 100 ml • Aliquoted in 5 ml×20 and stored at -20ºC.
LB medium (1 L)	• 10 g bactotryptone • 5 g yeast extract • 5 g NaCl • H_2O, up to 1 L • Autoclaved and stored at 4ºC
TE buffer (1 L)	• 10 mM Tris (10 ml of 1 M Tris pH7.5) • 1 mM EDTA (2 ml of 0.5 M EDTA) • H_2O was added and pH was adjusted to 8, volume to 1 L
TBE buffer (10×, 1 L)	• 108 g Tris-Base • 55 g boric acid • 7.44 g EDTA • H_2O, up to 1 L
PCR mix (general, 10 µl)	• 1 µl Buffer (10×, 15 mM $MgCl_2$) • 1 µl Primer 1 (forward) • 1 µl Primer 2 (reverse) • 1 µl dNTPs (2.5 m?) • 0.05 µl *Tag* polymerase (5 U/µl) / 0.2 µl *pfu* • H_2O, up to 9 µl • 1 µl DNA • Programme: 95ºC 1'? (94ºC 30"? 55ºC 30"? 72ºC 2')×30? 72ºC 10'? 4ºC
PEI (25 kD) stock solution (125 mM)	This was made assuming the molecular weight was 44. 275 mg PEI was dissolved in 40 ml H_2O and heated in 70ºC water-bath with stirring until all dissolved. The pH was adjusted to 7 using HCl. The volume was adjusted to 50 ml. The solution was aliquoted and stored at -20ºC.
PEI working solution (10 mM)	1 ml stock (125 mM) was mixed with 11.5 ml H_2O, aliquoted and stored at -20ºC.
Sequencing reaction mix (10 µl)	• 0.5 µl (10-20 ng) DNA sample • 0.32 µl primer (from 5 µM stock) • 2 µl sequencing buffer (2.5×)

	• 2 µl BigDye 3 (ABI PRISM BigDye Kit) • 5.18 µl H_2O • Programme: (96ºC 10”? 50ºC 5”? 60ºC 4’) × 24 ? 4ºC

2.6.2 Galectin-3/Bcl-2 cDNA plasmid preparation

a. Agar stabs of galectin-3 and Bcl-2 cDNA plasmid clones were subcultured on ampicillin-containing agar plates at 37ºC overnight.
b. A single colony from each plate was innoculated in ampicillin-containing LB medium and grown in a shaking incubator at 37ºC overnight.
c. Glycerol stocks were made by adding 500 µl LB culture to 250 µl 50% filter-sterilised glycerol, vortexed and stored at -80ºC.
d. Plasmid DNA was extracted from the bacterial cells using QIAprep Spin Miniprep Kit.
e. DNA from both plasmid clones (galectin-3 and Bcl-2) was sequenced to confirm that the expected cDNA inserts were present and correct using ABI PRISM BigDye Kit.
f. Sequencing reactions were cleaned as follows:
   i) 10 µl reaction product, 1 µl 3 M NaOAc and 25 µl cold ethanol (-20ºC) were mixed together and incubated on ice for 10 minutes.
   ii) Product was centrifuged at 14,000 RPM, 4ºC for 20 minutes.

iii) Product was washed with 125 µl cold 70% ethanol (-20ºC) at 14,000 RPM, 4ºC for 5 minutes.

iv) Pellet was air dried at 37ºC for 3 minutes before being re-suspended in 10 µl Hi-di formamide and denatured at 95ºC for 4 minutes.

v) Product was analysed on the ABI 3100 sequencing machine (Applied Biosystems).

2.6.3 Cloning

a. PCR amplification of galectin-3 and Bcl-2 full length cDNA.

PCR primers were designed to amplify the full length cDNA of galectin-3 and Bcl-2 from the plasmid clones. Restriction sites were incorporated into the 5' ends of the forward primer (HindIII) and the reverse primer (XhoI) compatible with the cloning sites of the pCMV.Tag 4A vector. The PCR amplification (20 µlx5) was carried out using *pfu* polymerase. The PCR products were cut and extracted from the gel using QIAquick Gel Extraction Kit.

b. Restriction digestion of galectin-3 and Bcl-2 PCR products of pCMV.Tag 4A vector.

i) PCR products extracted from the gel and the pCMV.Tag 4A vector were digested with the restriction enzymes HindIII and XhoI (New England

Biolab, the buffer and BSA were provided with the enzyme kit) at 37ºC overnight as follows.

Table 2.16 Digestion buffer

	DNA extracted from the gel	Vector
DNA	200 ng (24 µl)	5 µg (5 µl)
Buffer 2	5 µl	1 µl
BSA	5 µl	1 µl
Hind???(20 U/ml)	5 µl	1 µl
Xho?(20 U/ml)	5 µl	1 µl
H_2O	6 µl (up to 50 µl)	1 µl (up to 10 µl)

ii) Digestion products were purified using Wizard DNA Clean-up System (Promega).

iii) DNA in the purified product was quantified.

c. Dephosphorylation of the vector

i) The total number of 5'- and 3'-ends in the digested vector was calculated as follows:

1 µg 1 kb = 1.52 pmol DNA

1 µg 4.3 kb (e.g. pCMV.Tag 4A is 4.3 kb) = 1.52/4.3 = 0.353 pmol DNA

The purified product was 50 µl 330 ng/ul DNA. Therefore the total amount of DNA was 0.33 × 50 = 16.5 µg = 5.82 pmol (16.5 × 0.353).

The amount of 5'- and 3'-ends in 25 µl (i.e. 2.91 pmol, 5.82/(50/25)), was 2.91 × 2 = 5.82 pmol.

0.01 U/pmol calf intestinal alkaline phosphatase (CIAP kit, Promega) was used, i.e. 0.06 U CIAP for 5.82 pmol.

ii) 1:100 dilution of the 1 U/µl CIAP stock was made by adding 1 µl stock in 99 µl 1×buffer (1:10 dilution of the 10x buffer provided with the Kit). 50 µl reaction was made by mixing 25 µl vector, 5 µl 10×buffer, 6 µl CIAP dilution (0.01 U/µl) and 14 µl H_2O, as calculated above.

iii) The reaction mix was incubated at 37ºC for 30 minutes

iv) 6 µl of 0.01 U/µl CIAP was added to the reaction.

v) The reaction mix was incubated at 37ºC for another 30 minutes.

vi) 300 µl CIAP stop buffer (see Table 15) was added to the reaction.

vii) The product was purified with Wizard DNA Clean-up System (Promega).

viii) The amount of DNA in the product was quantified.

d. Ligation

Digested galectin-3 and Bcl-2 products were ligated into the digested and dephosphorylated pCMV.Tag 4A vector. Vector:insert ratio of 1:3 was used.

Table 2.17 Vector:insert ratio for ligation

	kb	ng/µl	ratio	ng	µl
Vector	4.3	256	1	100	100/256=0.4
Insert	0.9	36	3	100×0.9×3/4.3=63	63/36=1.75

10 µl reactions were set up by mixing 0.4 µl vector (100 ng), 1.75 µl insert (1:3), 1 µl 10×buffer, 1 µl T4 DNA ligase (New England Biolab) and 5.85 µl H_2O (up to 10 µl). Ligation was carried out at 14ºC overnight.

e. Transformation

i) DH5a competent cells were thawed on ice and gently mixed by tapping.

ii) 50 µl cells and 10 µl ligation product were incubated on ice for 30 minutes.

iii) Cells were heat-shocked in 42ºC water bath for 45 seconds without shaking.

iv) Cells were incubated on ice for 2 minutes.

v) 0.9 ml S.O.C. medium (provided with the competent cells by Invitrogen) was added using sterile tips.

vi) Cells were shaken at 37ºC for 1 hour.

vii) Cells were centrifuged briefly. Most of the supernatant (leave 100 µl) was discarded.

viii) Cells were resuspended by gentle tapping, transferred to a LB agar plate containing ampicillin and spread evenly by a sterile spreader by a flame.

ix) Cells were incubated at 37ºC overnight.

f. Screening transformation reaction for colonies containing galectin-3 and Bcl-2 insert.

i) Colonies were screened by PCR using galectin-3 or Bcl-2 primers (Table 2.14).

ii) Positive colonies were inoculated in 5 ml LB medium with ampicillin in a shaking incubator at 37ºC overnight.

iii) A glycerol stock of each positive colony was made by mixing 500 µl LB culture and 250 µl 50% glycerol. This was vortexed and stored at -70ºC. The rest was mini-preped using QIAprep Spin Miniprep Kit (Qiagen).

iv) 20 ng plasmid DNA was used in a sequencing reaction (Table 2.15) with Sp6 and T7 primer to determine whether or not the expected insert had been cloned.

v) If the sequence was correct, a maxi-prep of the cDNA clone was prepared by subculturing from the glycerol stock and using QIAGEN Plasmid Maxi Kit for transfection experiments (see 2.6.4 & 2.6.5).

2.6.4 Transfection I using $Ca_3(PO_4)_2$ precipitation

i) Hek293 cells were subcultured on 10 cm TC plates from TC flasks at appropriate densities, e.g. cells in a 150 cm^2 TC flask with 70% confluence were seeded on 10 plates.

ii) Top medium was changed with 10 ml fresh DME medium after 24-hour culture and the plates were cultured for additional 2 hours.

iii) HBS (2×) buffer (Table 2.15) was thawed on ice.

iv) 10 ml 2 M $CaCl_2$ solution was made in a 10 ml measuring cylinder by adding 2.94 g calcium chloride dihydrate (BDH, Table 2.13) and H_2O slowly to 10 ml.

v) 10 µg DNA, 68.2 µl 2 M $CaCl_2$ solution and H_2O, up to 550 µl, were mixed in a 1.5 ml eppendorf and dropped into 550 µl HBS (2×) slowly with vortexing. This was incubated at room temperature for 20 minutes before being dropped evenly and slowly to cells on the plate.

vi) Cells were cultured for 24 hours before the medium was refreshed and collected at 48-hour after transfection.

2.6.5 Transfection II using Polyethyleneimine (PEI)

i) HEK293 cells were subcultured on 10 cm TC plates at appropriate densities 24-hour before transfection. One additional plate was made to

be transfected with pEGFP-N3 vector as a control for transfection efficiency.

ii) Medium (6 ml/10 cm plate) was refreshed at least 1 hour before transfection.

iii) PEI solution was made by mixing 550 µl 150 mM NaCl and 150 µl 10 mM PEI.

iv) DNA solution was made by mixing 10 µg DNA and 150 mM NaCl (up to 700 µl).

v) PEI solution was dropped into DNA solution slowly with vortexing. This was incubated at room temperature for 30 minutes.

vi) The PEI and DNA mix was dropped on cells slowly while shaking the plate and cultured for 5 hours before the medium was refreshed.

vii) GFP expression was checked after 24 hours using a UV-laser microscope by the presence of green fluorescence in the transfected cells. Transfection efficiency was checked by flow cytometry by analysing the percentage of green fluorescent cells.

viii) Cells were collected after 48-72 hours.

2.7 Immunofluorescent imaging

2.7.1 Reagents and materials

Table 2.18 Reagents used for immunofluorescent imaging

Reagents	Suppliers, Catalogue No. / preparation
Anti-Bcl-2, rabbit	Abcam, ab7973
Anti-galectin-3, rabbit	Abcam, ab2473
ImmEdge Pen	Vector Laboratories, H-4000
Anti-Mouse-IgG AlexaFluor 488, donkey	Molecular Probe, A-21202
Anti-Mouse-IgG PE, goat	Sigma, P9287
Anti-Rabbit-IgG PE, goat	Sigma, P9795
0.1% PBST	1 ml Tween 20 was added to 1 L PBS solution.
2% PFA (fixative, toxic, 500 ml)	• 2% PFA (10 g) • 100 mM NaCl (50 ml 5 M stock) • 300 mM sucrose (51 g) • 3 mM $MgCl_2$ (1.5 ml of 1 M stock) • 1 mM EGTA (5 ml of 100 mM stock) • 10 mM PIPES pH 6.8 (5 ml of 1 M stock) • H_2O (up to 500 ml) • Heated in 60ºC water-bath for 0.5 hour to dissolve. • Aliquoted and stored at -20ºC.

2.7.2 Imaging of suspension cells

i) Slides were labelled for different samples. For each secondary antibody / cell line / combination of secondary antibodies and cell line, a non-specific-binding control with cells stained with only secondary antibody / combination of secondary antibodies was labelled and made. The fluorescence of this control was to be deducted from the background.

ii) Cytospin preparations were made. 150-200 µl confluent cells were spun onto glass slides at 500 RPM, 5 minutes.

iii) Cells were air dried for 1-2 minutes at room temperature.

iv) Cell pellet was circled completely with the ImmEdge pen.

v) Cells were fixed with 2% PFA (Table 2.18) for 10 minutes.

vi) Cells were permeabilised with cold 0.1% PBST (Table 2.18) for 5 minutes.

vii) Cells were blocked with 3% FCS in PBS for 30 minutes, and stained with primary antibody at 4ºC overnight.

viii) Cells were washed with 0.1% PBST 5 minutes × 6, and stained with secondary antibody at room temperature in the dark for 2 hours.

ix) Cells were washed with 0.1% PBST 5 minutes × 4, and stained with DAPI (1:10 000) for 5 minutes.

x) Cells were washed with 0.1% PBST 5 minites × 2 before mounted and and air dried.

2.8. Real-time Quantitative PCR

All reagents and primers were purchased from Applied Biosystems unless otherwise stated.

Nox-5 primers are PDAR (predeveloped assay reagent, 20x) from Applied Biosystems (proprietary-product ID Hs00225846_m1).

The primer/probe sequences for c-ABL were as follows:

c-ABL Forward: 5'-TGGAGATAACACTCTAAGCATAACTAAAGG-3'

c-ABL Reverse: 5'-GATGTAGTTGCTTGGGACCCA-3'

c-ABL Probe (Eurengentech): FAM5'-CCATTTTTGGTTTGGGCTTCACACCATT-3'TAMRA.

Total RNA was extracted from 1-10x10^6 cells using RNeasy Mini Kit (Qiagen), and reverse transcribed using Superscript reverse transcriptase (Invitrogen).

2.8.1 Reverse transcription

i) 1 µl Random primer (200 ng/µl), 1 µl dNTP mix (10 mM of A, T, C, G type) and RNA free H_2O (to make up the final volume of 12 µl with the volume of RNA samples deducted) were mixed together.

ii) Total RNA samples (no more than 5 µg) were added in triplicate.

iii) One sample was specified as RT-blank control containing all of the above reagents with any one of the RNA samples but no Superscript (RT-enzyme).

iv) Samples were Incubated at 65ºC for 5 minutes, cooled down on ice and flash spun.

v) 4 µl First-strand Buffer (5x), 2 µl 0.1 M DTT and 1 µl RNase Out (40 U/µl) were added to each sample.

vi) Samples were Incubated at 25ºC for 10 minutes.

vii) 1 µl Superscript were added to each sample except the RT-blank control.

viii) Samples were incubated at 42ºC for 50 minutes, 70ºC for 15 minutes to inactivate the enzyme and stored at -20ºC.

2.8.2 Quantitative PCR (qPCR)

i) 20 µl qPCR reaction was prepared by mixing 10 µl TaqMan Master Mix (2x, containing *Taq*, dNTP, etc), 1 µl PDAR (predeveloped assay reagent, 20x), 100 ng cDNA sample and H_2O (up to 20 µl).

ii) 20 µl c-ABL control reaction (endogenous control) was prepared by mixing 10 µl TaqMan Master Mix (2x), 0.6 µl c-ABL forward primer, 0.6 µl c-ABL reverse primer, 0.4 µl c-ABL probe (final primer/probe concentrations 900 nM and 250 nM, respectively), H_2O (up to 20 µl) and 100 ng cDNA sample.

iii) A standard curve was made by using standardized cDNA samples with increasing concentration of 0.1, 1, 10 and 100 ng of cDNA. Negative controls with no DNA were also included. Samples were prepared in triplicate to reduce the statistical error.

iv) Thermal cycling was performed on the ABI7700 under the standard conditions: 94ºC 10 minutes ? 94ºC 15 seconds ? 60ºC 1 minute, 40 cycles.

v) Ct values were normalized relative to c-ABL.

2.9 Statistical Analysis

Statistical analysis was performed using MINITAP 15.0 software. One-way ANOVA was used to evaluate the statistical differences between experiments repeated in at least triplicate. When p values <0.05, the differences are considered significant.

Chapter 3 Apoptosis induction by GCS-100

Apoptosis can be induced via many mechanisms. It is known that modified citrus pectin can antagonize the anti-apoptotic action of galectin-3 and induce apoptosis in tumour cells (see 1.7). GCS-100 is a novel type of modified citrus pectin and induces apoptosis in human multiple myeloma cells (Chauhan, Li *et al.* 2005). It has also shown anti-tumour effects in patients with colorectal and pancreatic cancers (Cotter 2004; Gunzburg and Salmons 2001). When these studies were initiated in 1998, GCS-100 was attracting interest as a therapeutic agent in clinical trial for cancer treatment. It seemed pertinent therefore, to study its effects on apoptosis in human malignant B-lineage cell lines, primary CLL cells, normal human lymphocytes and certain additional human cell lines. The interference of GCS-100 in the mitochondrial apoptotic pathway was focused and changes in mitochondrial staining and plasma membrane were measured as a correlate of apoptosis. The effects of concurrent treatment with GCS-100 and VP-16, a chemotherapeutic drug for leukaemia, were also studied in order to determine if they act synergistically in leukaemia cells. Caspase activation, one of the major events in the apoptotic cascade, was examined in GCS-100- and/or VP-16- treated cells to further confirm their pro-apoptotic effects, both alone and in combination.

3.1 Effects of GCS-100 in DOHH2 and primary lymphocytes.

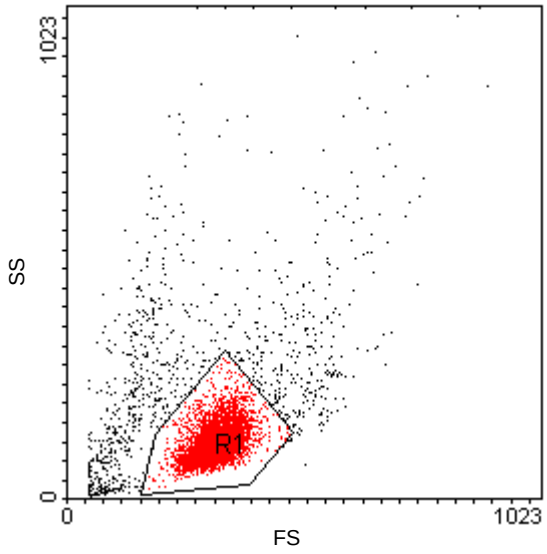
As stated above, GCS-100 induces apoptosis in multiple myeloma cells (Chauhan, Li *et al*. 2005). Whether or not it has the same effects in human mature B cells has not been investigated. Here the pro-apoptotic effects of GCS-100 were examined in DOHH2, a B-lineage cell line, and primary lymphocytes, including cells from CLL patients and normal donors. This was achieved by measuring changes in mitochondrial transmembrane potential (DiOC6(3) staining) and disorders in plasma membrane (MC540 staining), both typical events in apoptosis, and widely used for investigations of this type in cells of diverse origin (Laakko, King *et al.* 2002; Ozgen, Savasan *et al*. 2000).

3.1.1 Effects of GCS-100 in DOHH2 cells

DOHH2 is a mature B cell line which expresses high levels of Bcl-2 and is resistant to many chemotherapeutic drugs (Kluin-Nelemans, Limpens *et al*. 1991). These cells were treated with GCS-100 and stained with DiOC6(3) and MC540. An increase in the MC540 staining detected on FL-2 channel, consistent with perturbation in plasma membrane, and a decrease in the DiOC6(3) staining detected on FL-1 channel, consistent with changes in mitochondrial inner membrane potential (DeltaPsi(m)), were seen in GCS-

100-treated cells. Data from an individual experiment are illustrated in Figure 3.1, by way of exemplification. Complete data for this cell line, based on a minimum of 3 experiments, are presented in Figure 3.2 and Figure 3.3. Here, MC540 positive and DiOC6(3) negative cells were quantified using WinMDI software, and were found to be significantly increased (p<0.01) after GCS-100 treatment.

(A)



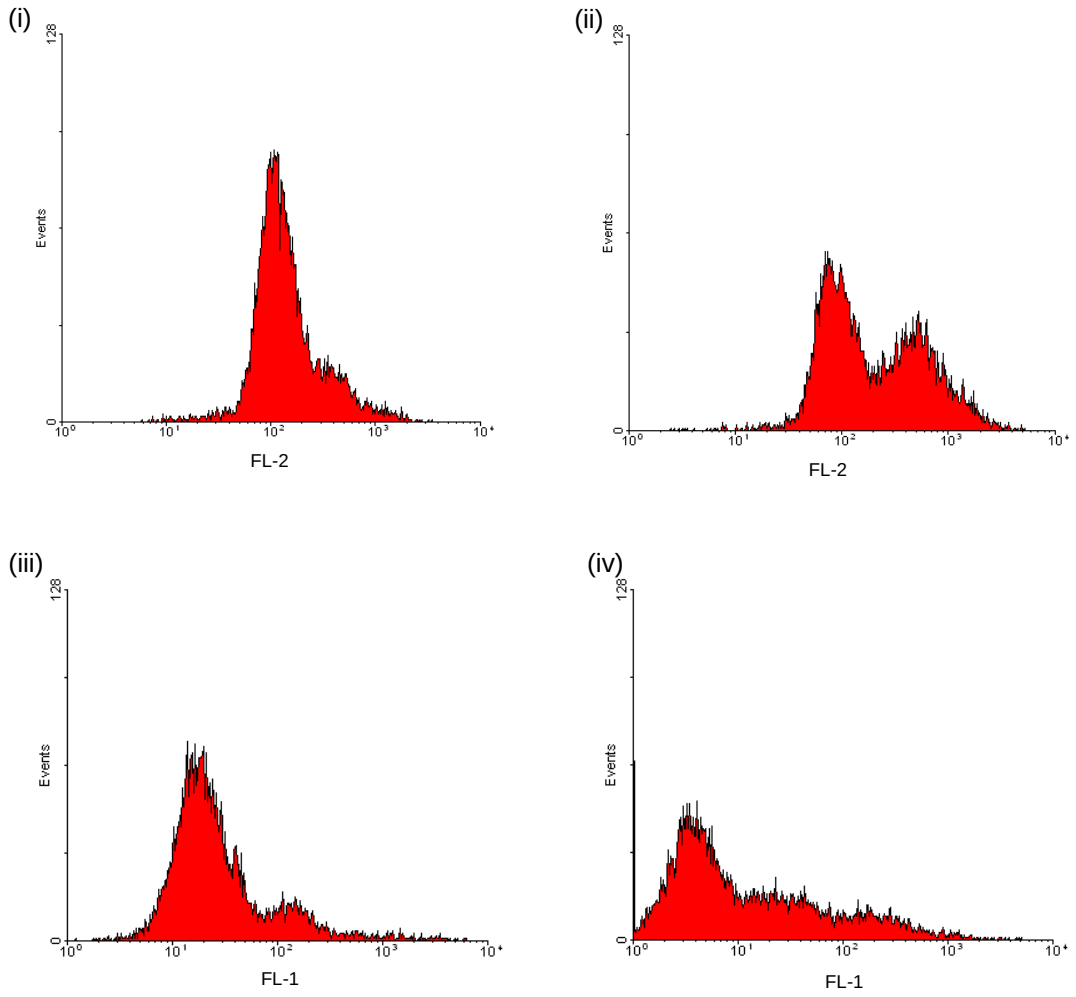


Figure 3.1 Flow analysis of the pro-apoptotic effects of GCS-100 in DOHH2 cells. Cells were treated with or without (control) 40 µg/ml GCS-100 for 24 hours and then stained with either MC540 or DiOC6(3) before flow analysis. (A) Cell debris was gated out on the FS/SS plot. (i) non-treated cells stained with MC540; (ii) GCS-100-treated cells stained with MC540, showing a marked positive shift on FL-2 channel; (iii) non-treated cells stained with DiOC6(3); (iv) GCS-100-treated cells stained with DiOC6(3), showing a marked negative shift on FL-1 channel. R1, region 1; FS, forward scatter; SS, side scatter.

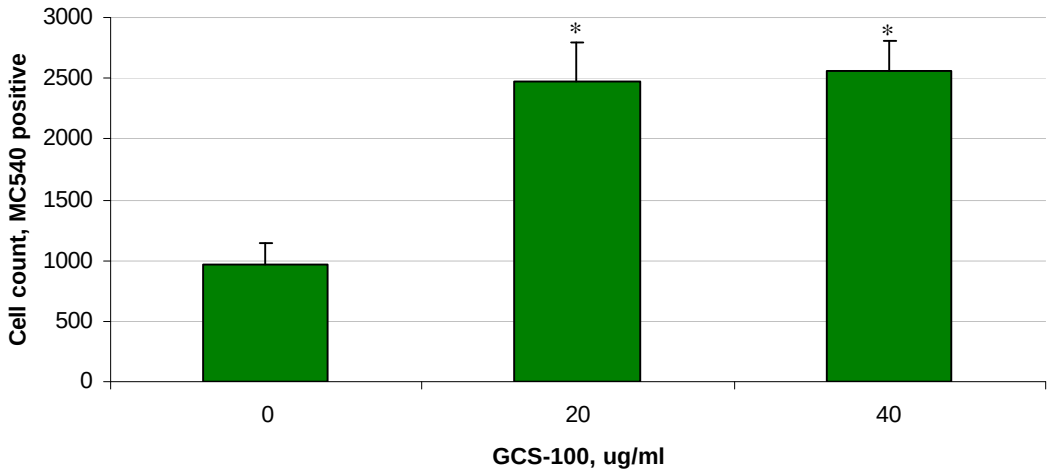


Figure 3.2 Flow analysis of the effect of GCS-100 on membrane integrity in DOHH2 cells. Cells were treated with or without 20 or 40 µg/ml GCS-100 for 24 hours and then stained with MC540 before flow analysis. Data were quantified and analysed as described in Chapter 2. GCS-100 treatment with both doses increased the MC540 positive population significantly. *, $p<0.01$ (n=3).

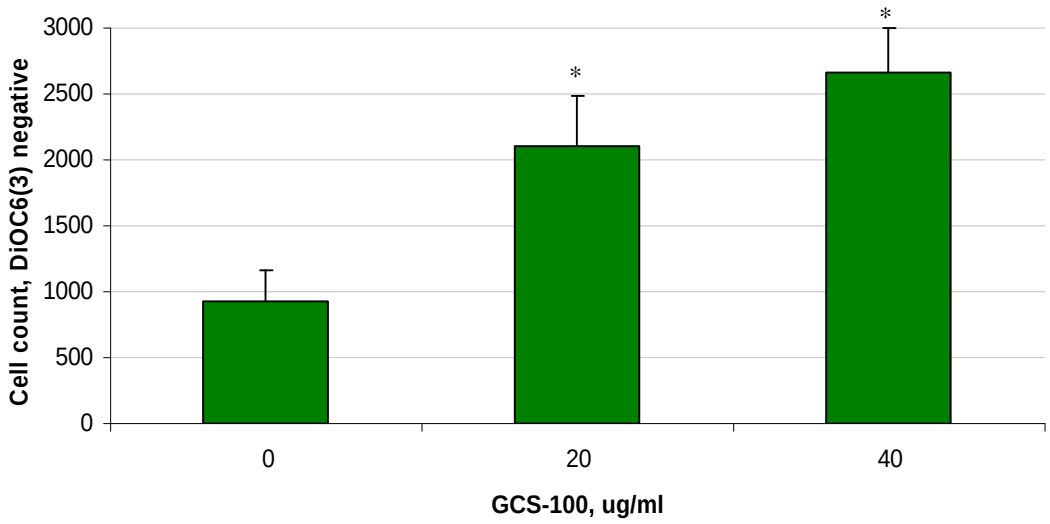


Figure 3.3 Flow analysis of mitochondrial DeltaPsi(m) dissipation induced by GCS-100 in DOHH2 cells. Cells were treated with or without 20 or 40 µg/ml GCS-100 for 24 hours and then stained with DiOC6(3) before flow analysis. Data were quantified and analysed as described in Chapter 2. GCS-100 treatment with both doses increased the DiOC6(3) negative population significantly. *, *p<0.01* (n=3).

3.1.2 Effects of GCS-100 in primary CLL cells.

While GCS-100 clearly induces apoptosis in human primary multiple myeloma cells and B cell lines, the effect of the agent in primary chronic lymphocytic leukaemia (CLL) cells has not been reported. Data on the lymphocytes of CLL patients studied are summarised in Appendix I. Thus, using freshly isolated lymphocytes from whole blood samples of CLL patients

with white cell counts in excess of $100x10^9$/L, the pro-apoptotic effects of GCS-100 were investigated by DiOC6(3) and MC540 staining and flow cytometry. As with the cell line DOHH2, CLL cells were treated with increasing doses of GCS-100 for 24 hours prior to MC540 and DiOC6(3) staining and flow analysis. By contrast with the cell lines, there was much greater variability in the numbers of cells undergoing apoptosis in the untreated (control) population shown, for clarity, as individual values in Figure 3.5(A) and correspondingly reflected in the values for the treated CLL samples. Nevertheless, a right-shift on FL-2 channel was demonstrated in MC540-stained cells after GCS-100 treatment which was clearly dose-related as exemplified in Figure 3.4. Regardless of the level of pretreatment apoptosis (*i.e.* that observed in the controls), all samples were characterised by an increase in the MC540-positive population on GCS-100 exposure. Data from CLL cells (from 8 patients), collectively quantified, are summarised and statistically analysed in Figure 3.5(B). Significant differences in the MC540-positive population were found between treated and non-treated cells, confirming the pattern of findings exemplified in Figure 3.4.

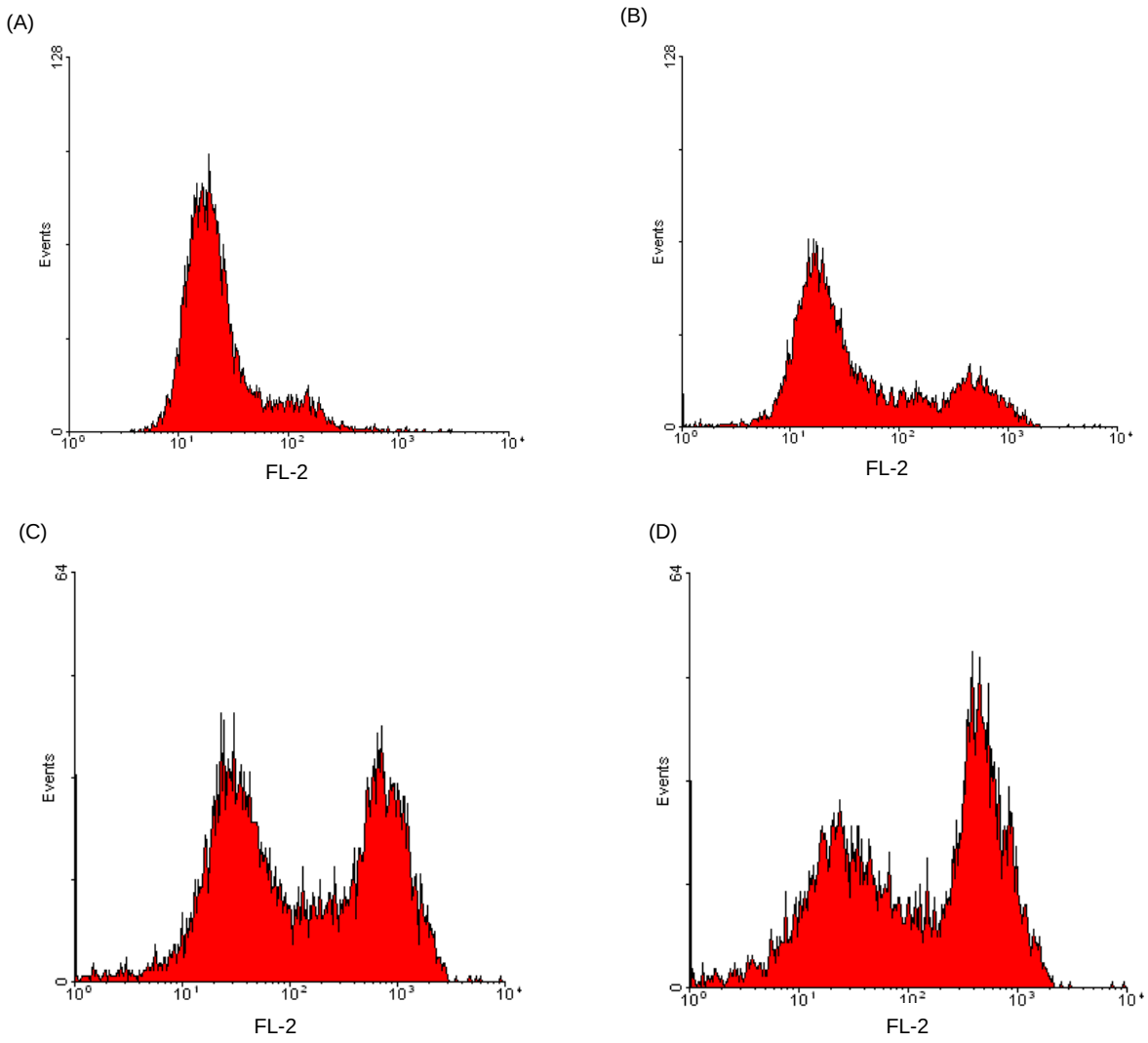
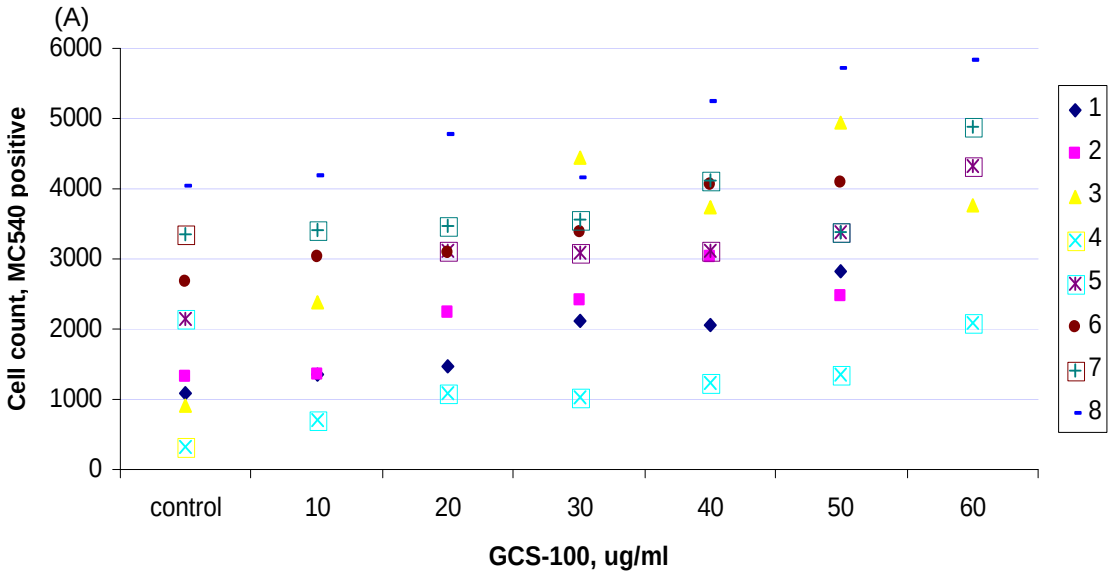


Figure 3.4 Flow analysis of the effects of GCS-100 in primary CLL cells using MC540 staining. Freshly isolated CLL cells were treated with or without different doses of GCS-100 for 24 hours prior to MC540 staining and flow analysis. A marked right-shift on FL-2 channel was detected in GCS-100-treated cells. (A) non-treated; (B) 10 µg/ml GCS-100-treated; (C) 30 µg/ml GCS-100-treated; (D) 50 µg/ml GCS-100 treated.



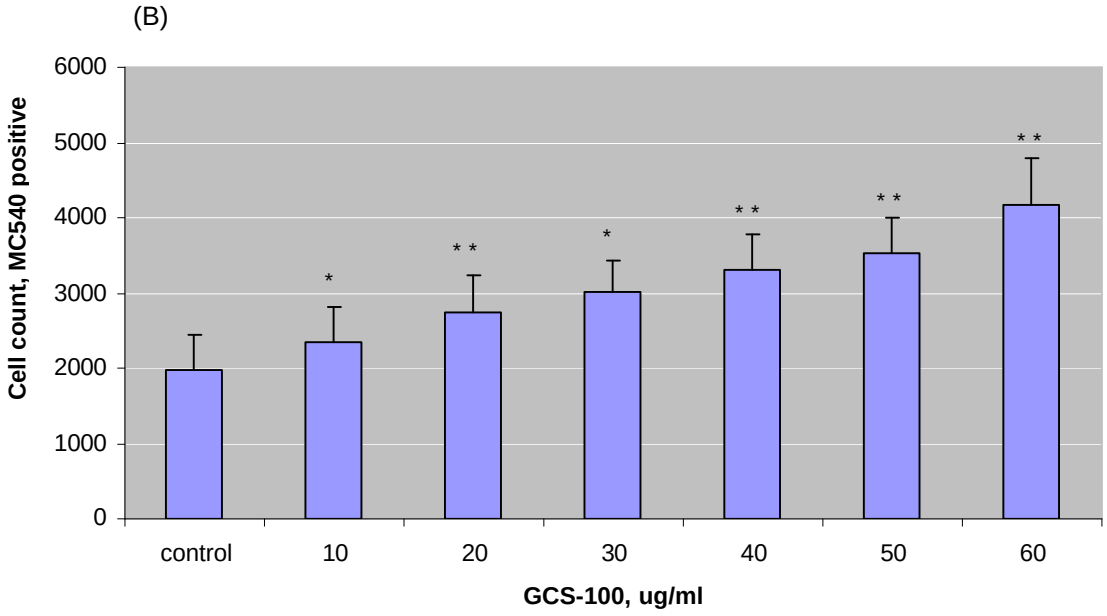


Figure 3.5 Summary of GCS-100 effects in primary CLL cells using MC540 staining and flow cytometry. Lymphocytes from eight samples of CLL patients (see Appendix I) were treated with or without different doses of GCS-100 for 24 hours prior to MC540 staining and flow analysis. Significant changes in the MC540-positive population between treated and non-treated cells were observed. (A) Summarised data from eight CLL samples; (B) Means

and standard errors for these data. Data were analysed as previously described in Chapter 2. *, *p<0.05; **, p<0.01; n=8*.

Similar effects of GCS-100 were seen in DiOC6(3)-stained cells where a marked left-shift on FL-1 channel was detected after GCS-100 treatment as exemplified in Figure 3.6. Data quantification and statistical analysis revealed significant differences in the DiOC6(3)-negative population between treated and non-treated cells at all doses with the exception of 30 µg/ml (Figure 3.7). Notwithstanding this unexplained result, there was a statistically significant trend for GCS-100-treated CLL cells to exhibit a greater degree of apoptosis than untreated controls.

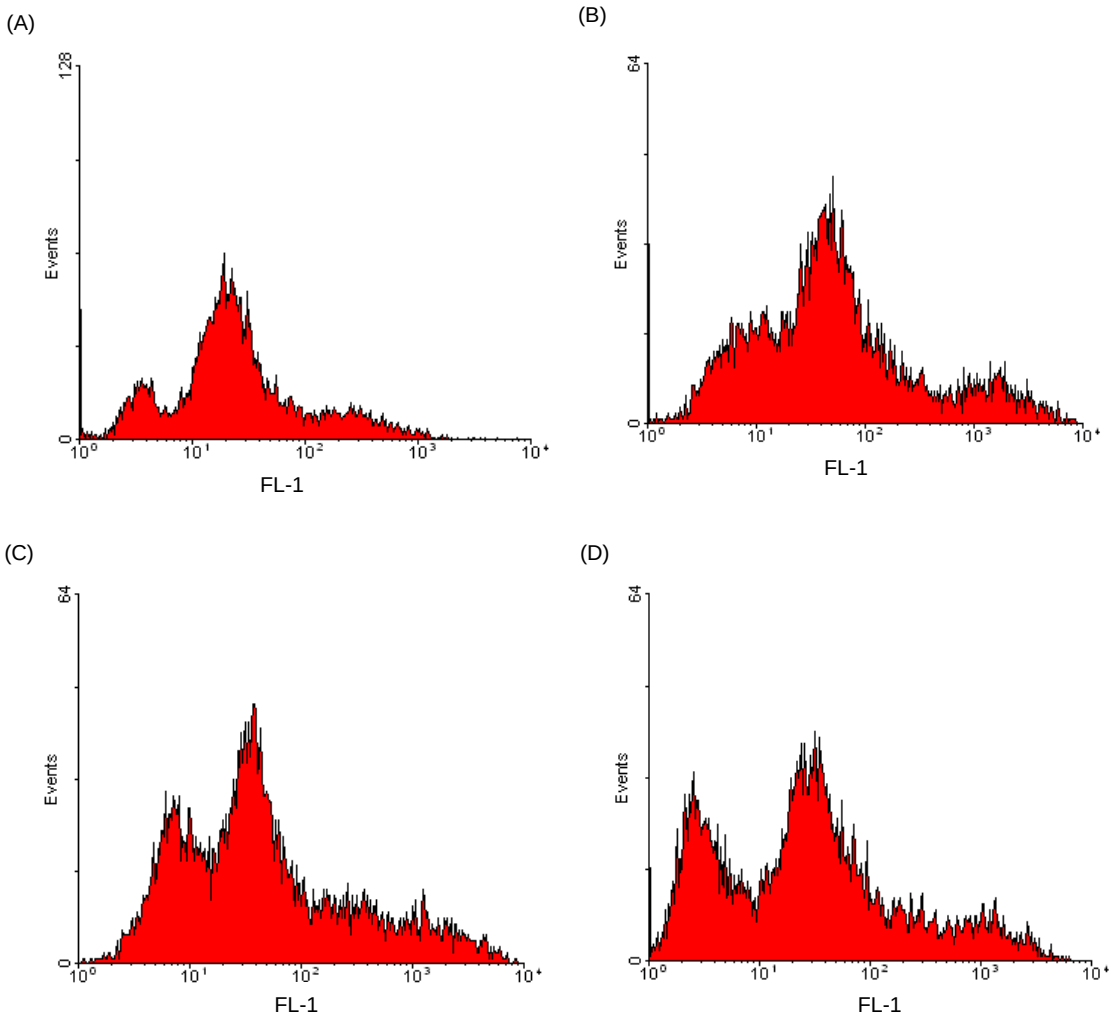


Figure 3.6 Flow analysis of the effects of GCS-100 in primary CLL cells using DiOC6(3) staining. Freshly isolated CLL cells were treated with or without different doses of GCS-100 for 24 hours prior to DiOC6(3) staining and flow analysis. A marked left-shift on FL-1 channel was detected in GCS-100-treated cells. (A) non-treated; (B) 10 µg/ml GCS-100-treated; (C) 30 µg/ml GCS-100-treated; (D) 50 µg/ml GCS-100-treated.

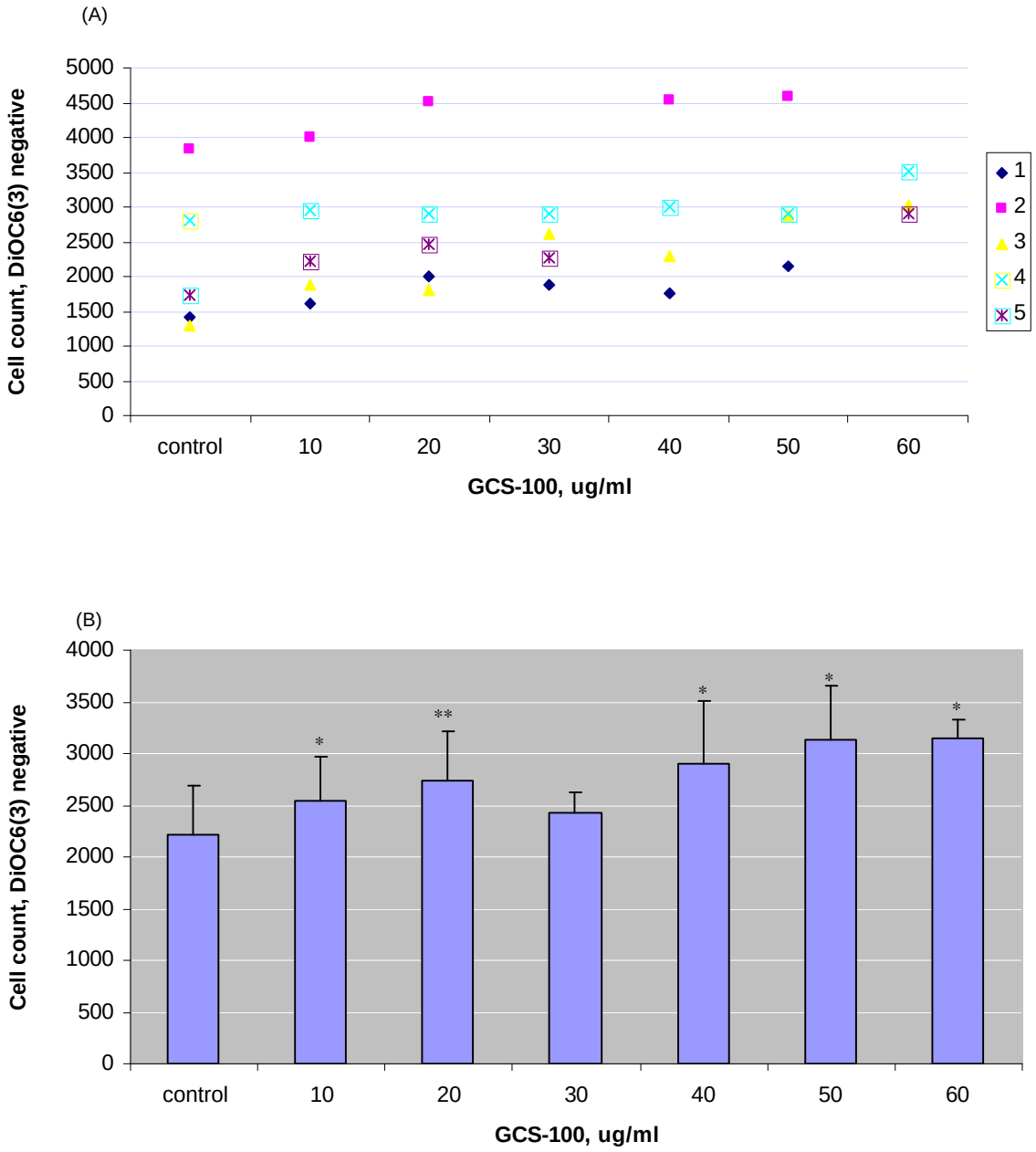


Figure 3.7 Summary of GCS-100 effects in primary CLL cells using DiOC6(3) staining and flow cytometry. Lymphocytes from five CLL patients (see Appendix I) were treated with or without different doses of GCS-100 for 24 hours prior to DiOC6(3) staining and flow analysis. Significant changes in the DiOC6(3)-negative population between treated and non-

treated cells were shown with the exception of the 30 µg/ml dose. (A) Summarised data from five CLL patients; (B) Means and standard errors for these data. *, *p<0.05; **, p<0.01; n=5*.

Notwithstanding the demonstration of apoptosis induction by GCS-100 *in vitro* in CLL cells in the foregoing experiments, the number of CLL samples examined for this property was too small to make any correlations with immunological and genetic parameters or treatment status of the patients listed in Appendix I.

3.1.3 Effects of GCS-100 in human normal lymphocytes.

The cytotoxic effects of GCS-100 in human normal lymphocytes were studied previously using cell viability assays (Chauhan, Li *et al*. 2005). However, any potential pro-apoptotic effect of the agent in these cells has not been reported. Therefore, using freshly isolated lymphocytes from three normal donors, the effects of GCS-100 on mitochondrial transmembrane potential were investigated. Ideally, for comparison with transformed B cells (as in CLL), such experiments should have been conducted with purified B cells or at least populations enriched for the B cell compartment. However, due to the limited availability of samples, it was impossible to recover sufficient pure B cells for this study. Any potential effect of GCS-100 on normal B cells was thus inferential and based on the following reasoning: It is known that typically 5-10% of normal peripheral blood mononuclear cells are B cells (Splawski,

Lipsky *et al.* 1997). If GCS-100 had the same pro-apoptotic effects on normal B cells as on CLL cells, which showed an approximately 10% difference in DeltaPsi(m) collapse between control and treated cells at the highest dose (60 µg/ml, see Figure 3.7), only a 0.5-1% (5-10%x10%) difference between control and treated cells might have been expected, a difference outside the capacity of the assay for reliable detection. However, in the event, at all doses the trend was toward a decrease in the DiOC6(3)-negative population, *i.e*. the number of apoptotic cells decreased. This was particularly marked at the highest dose of 200 µg/ml for 48 hours where the population in DeltaPsi(m) collapse was lower *even than the controls*. On these grounds it is concluded that induction of an apoptotic effect in normal B cells by GCS-100 is unlikely, and that the effect of the agent on transformed B cells is selective to that extent.

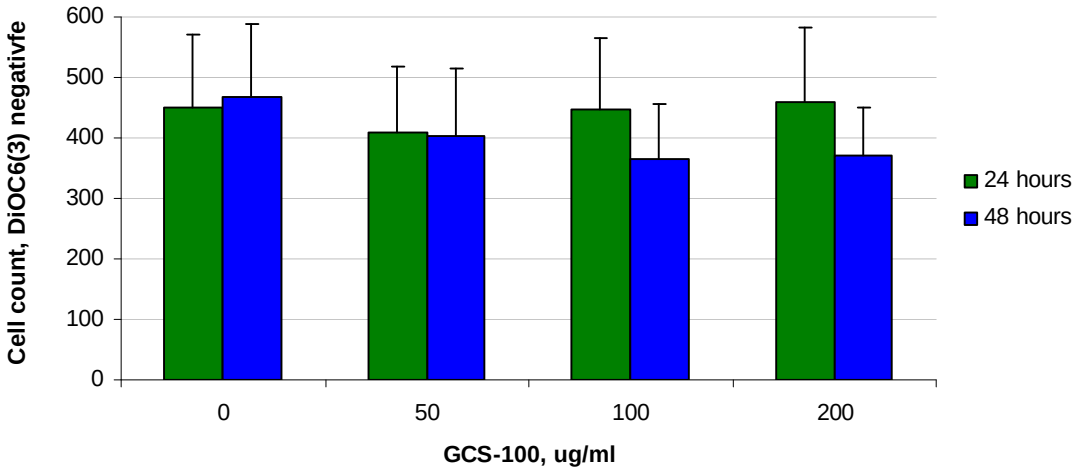


Figure 3.8 Flow analysis of the pro-apoptotic effects of GCS-100 in unfractionated human normal lymphocytes. Lymphocytes were freshly isolated from three normal

peripheral blood samples and incubated with 0-200 µg/ml GCS-100 for 24 or 48 hours prior to flow cytometry analysis with DiOC6(3) staining. Statistical analysis was undertaken as previously described (see legend in Figure 3.2). No significant differences ($p>0.05$, $n=3$) were found between control and treated cells. By contrast with CLL cells, there was a downward trend in the DiOC6(3)-negative population.

3.2 Effects of VP-16 in GCS-100-treated cell lines.

VP-16, or etoposide, is a semi-synthetic derivative of podophyllotoxin that exhibits anti-tumour activity. VP-16 inhibits DNA synthesis by forming a complex with topoisomerase II and DNA and has been used for the treatment of haematopoietic malignancies (Martins, Mesner *et al*. 1997). However, the cytotoxic effects of the drug have restricted its use at high doses for treatment of these neoplasms. It was pertinent therefore to ask the question whether GCS-100 might facilitate the pro-apoptotic effects of VP-16 as a potential basis for its use in a lower dose regimen. The effect of combined treatment with the drugs in human haemapoietic cell lines was therefore investigated using the methodologies previously applied.

3.2.1 Effects of GCS-100 and VP-16 in DOHH2 cells.

Dose-response curves for VP-16 were derived with or without (control) pre-incubation with GCS-100. VP-16 alone induced apoptosis in DOHH2 cells in a dose-dependent manner (Figure 3.9, red). As anticipated from earlier experiments, pre-incubation for 24 hours with GCS-100 at both doses induced a level of apoptosis which exceeded that in the untreated controls. However, this was enhanced by subsequent exposure to VP-16 in a dose-dependent manner, especially at concentrations higher than 1 μM. Thus, pre-incubation with GCS-100 shifted the dose-response curve of VP-16 to the left, significantly ($p<0.05$, $n=3$) enhancing the pro-apoptotic effects of VP-16 (Figure 3.9, blue and green). EC50 values were calculated by GraphPad Prizm software and statistically analysed, as shown in Table 3.1.

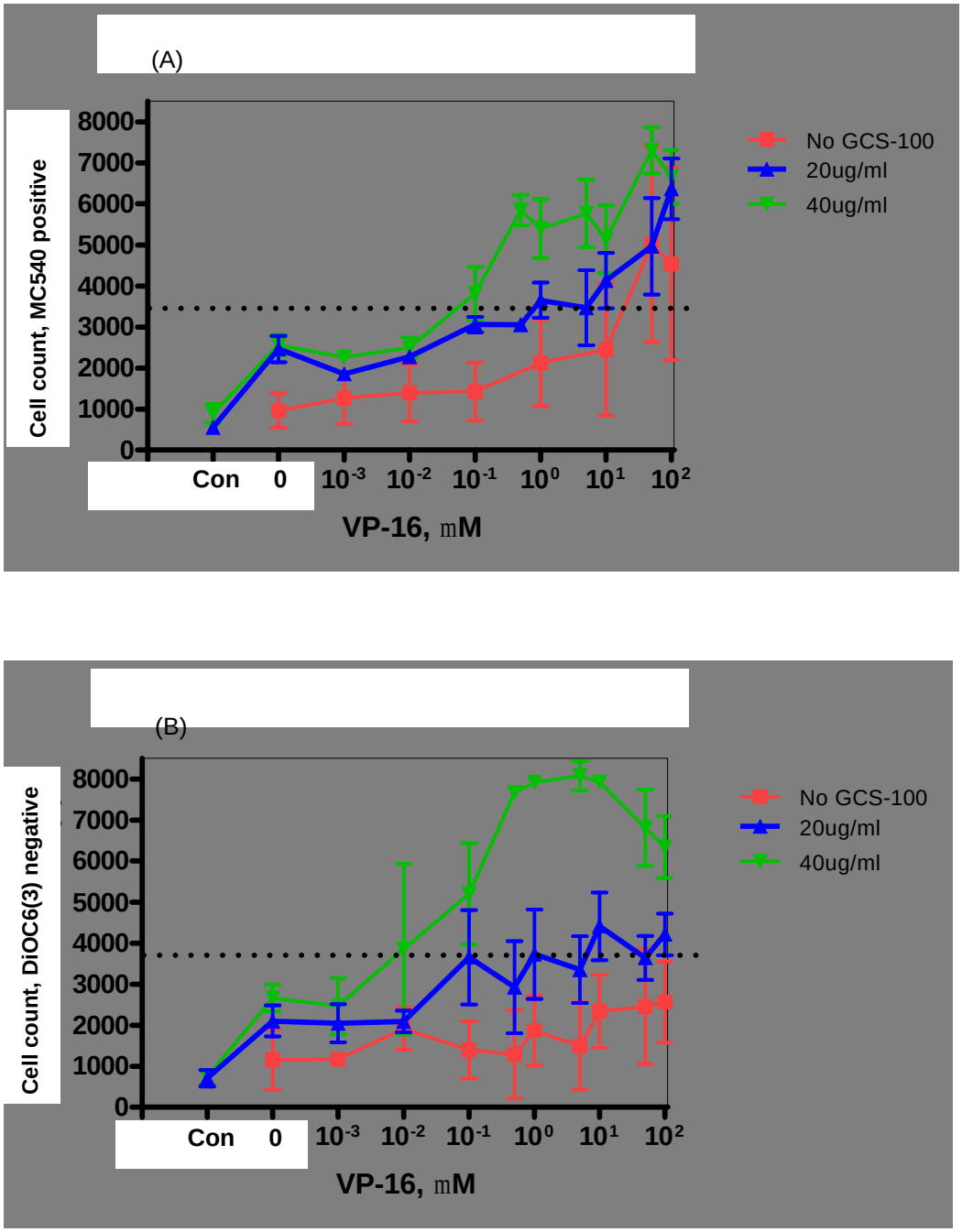


Figure 3.9 Flow analysis of effects of combined treatment of DOHH2 cells with GCS-100 and VP-16. Cells were treated with or without (control) GCS-100 for 24 hours prior to incubation with different doses of VP-16 for 20 hours. Cells were then stained with (A) MC540 and (B) DiOC6(3) and analysed by flow cytometry. GCS-100 reduced the EC50 value

for VP-16 and therefore facilitated the pro-apoptotic effects of VP-16 (see Table 3.1, statistical analysis was undertaken as described in Chapter 2, n=3).

Table 3.1 EC50s of GCS-100/VP-16 treatment in DOHH2 cells.

Cell Line	GCS-100 (μg/ml)	EC_{50} (μM, MC540)	*P* value	EC_{50} (μM, DiOC6(3))	*P* value
DOHH2	0	>10	<0.05	>10	<0.01
	20	0.8		0.1	
	40	0.05		0.007	

3.2.2 Effects of GCS-100 and VP-16 in RAMOS cells.

RAMOS is a B lymphoma cell line which expresses a p53 mutation (see 2.2.2). As DOHH2 cells have wild type p53, the question whether p53 mutations could alter the cell response to GCS-100/VP-16 was addressed, using the same methodology to determine pro-apoptotic effects. RAMOS cells differed from DOHH2 cells in their greater sensitivity to apoptosis induction by GCS-100 alone, a factor which appeared to contribute to their much enhanced sensitivity, relative to DOHH2, to the combination of drugs (Figure 3.10), as evident from the EC50 calculations (Table 3.2). Greater sensitivity to apoptosis induction in a cell line containing a p53 mutation could be argued as counter-intuitive, but caution needs to be exercised about such limited data.

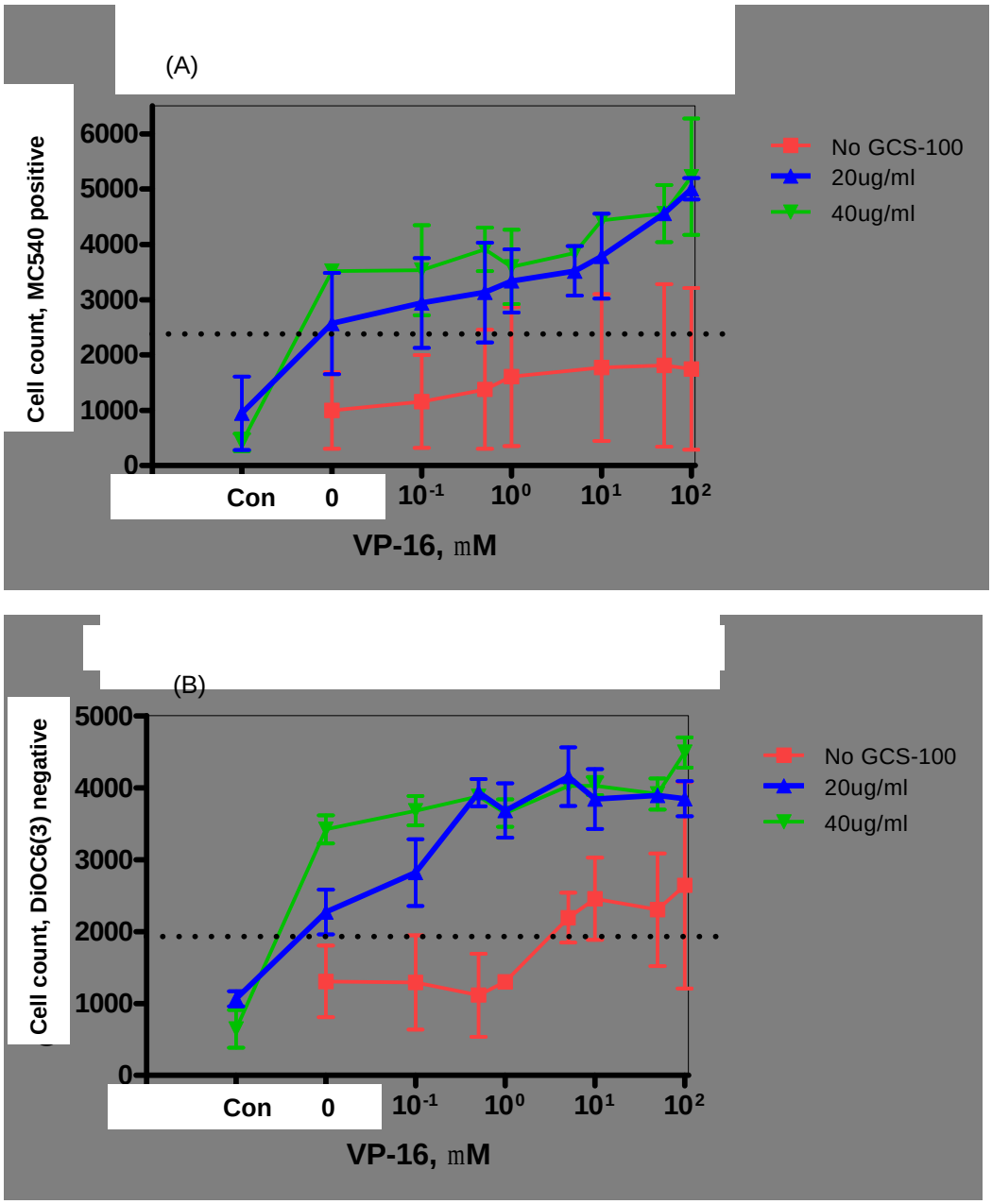


Figure 3.10 Flow analysis of effects of combined treatment of RAMOS cells with GCS-100 and VP-16. Cells were treated with or without GCS-100 for 24 hours before incubation with different doses of VP-16 for 20 hours. Cells were then stained with (A) MC540 and (B) DiOC6(3) and analysed by flow cytometry. GCS-100 reduced the EC50 value for VP-16 and therefore facilitated the pro-apoptotic effects of VP-16 (see Table 3.2, statistical analysis was undertaken as described in Chapter 2, n=3).

Table 3.2 EC50s of GCS-100/VP-16 treatment in RAMOS cells

Cell Line	GCS-100 (μg/ml)	EC_{50} (μM, MC540)	*P* value	EC_{50} (μM, DiOC6(3))	*P* value
RAMOS	0	>10	<0.01	3.2	<0.01
	20	0.008		0.005	
	40	0.004		0.003	

3.2.3 Effects of GCS-100 and VP-16 in K562 cells.

The K562 cell line is derived from myeloid leukaemia (see 2.2.2) and, by contrast with DOHH2 and RAMOS cells, showed greater resistance to both VP-16 and GCS-100 treatment alone (Figure 3.11). However, GCS-100 at 40 μg/ml still reduced the EC50 of VP-16, exhibiting greater pro-apoptotic effects for the drugs in combination, even in this relatively resistant cell line.

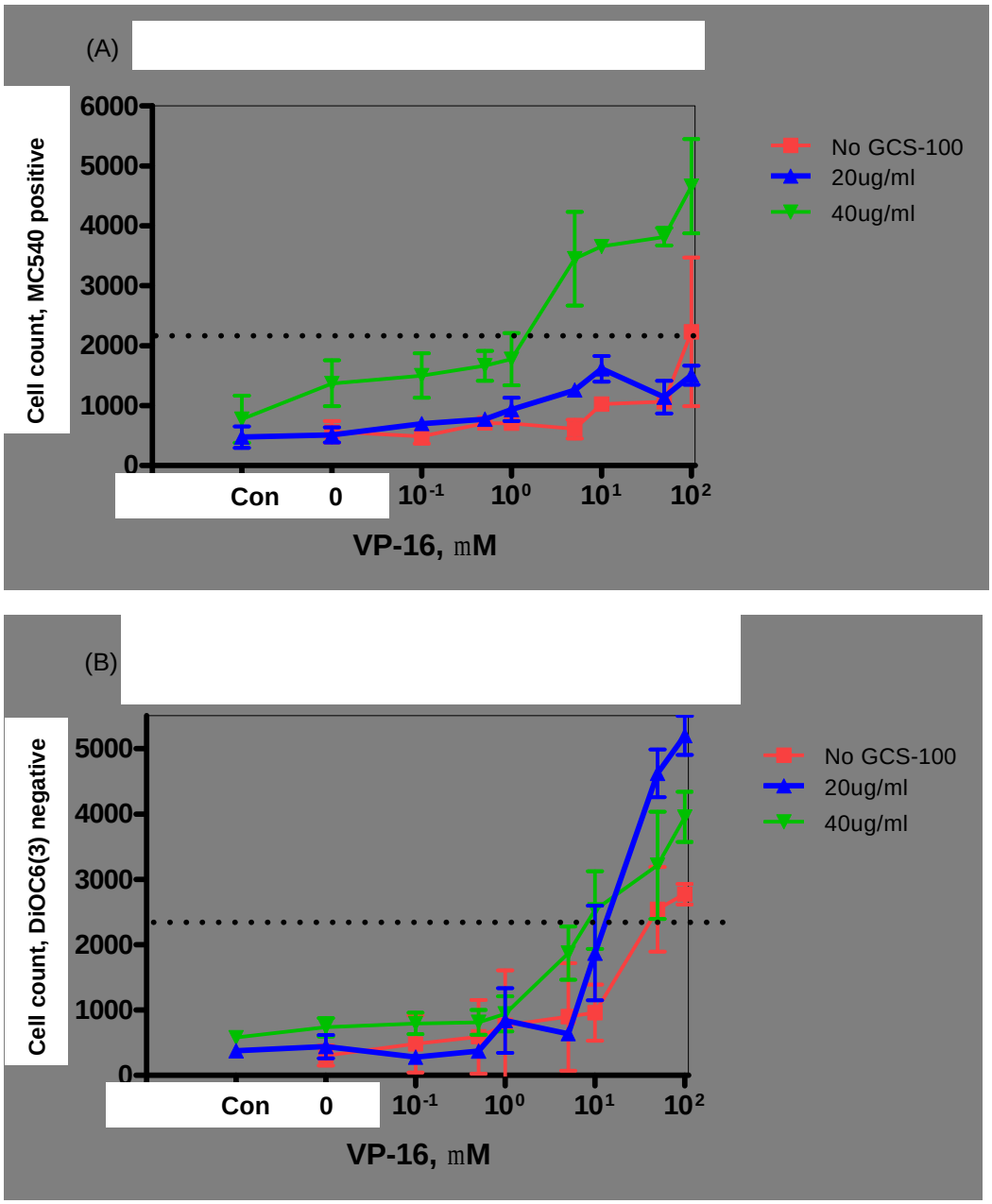


Figure 3.11 Flow analysis of effects of combined treatment of K562 cells with GCS-100 and VP-16. Cells were treated with or without (control) GCS-100 for 24 hours prior to incubation with different doses of VP-16 for 20 hours. Cells were then stained with (A) MC540 and (B) DiOC6(3) and analysed by flow cytometry. GCS-100 reduced the EC50 value for VP-16 and therefore facilitated the pro-apoptotic effects of VP-16 (see Table 3.3, statisitcal analysis was undertaken as described in Chapter 2, n=3).

Table 3.3 EC50s of GCS-100/VP-16 treatment in K562 cells.

Cell Line	GCS-100 (μg/ml)	EC_{50} (μM, MC540)	*P* value	EC_{50} (μM, DiOC6(3))	*P* value
K562	0	>10	<0.01	>10	<0.05
	20	>10		>10	
	40	1.5		6.8	

The above data from 3.2.1 to 3.2.3 showed that apoptosis could be induced by GCS-100/VP-16 in human haematopoietic malignant cells, with differential potency depending on the cell line. Relatively speaking, DOHH2 and RAMOS cells appeared to be more sensitive to apoptosis induction by GCS-100/VP-16 than K562 cells. In VP-16 treated cell lines, pre-treatment with GCS-100 enhanced the induction of apoptosis to different degrees, depending on the cell type.

3.3 GCS-100 and the mitochondrial apoptotic pathway.

It has been established herein that GCS-100 induces apoptosis either alone or in combination with VP-16 in human malignant B cells (see 3.1 & 3.2). The next part of this project set out to determine the effect of the agent on the apoptotic pathway. As most apoptosis inducers act via either caspase-8 or caspase-9, immunoblotting analysis was performed (see 2.5) to determine

which of these two enzymes was involved in the effect of GCS-100. Such an experiment would also confirm the pro-apoptotic effects of GCS-100 shown in 3.1 and 3.2, as distinct from an artefact of flow cytometric methodology, since activation of either caspase is one of the major defining events in the apoptotic cascade (see 1.2).

Caspase-8 activation is involved in the extrinsic apoptotic pathway (see 1.2) and was first analysed in GCS-100/VP-16-treated DOHH2 cells. This enzyme must become cleaved in order to produce its effects (see 1.2.1). However, cleavage of caspase-8 was not detected in DOHH2 cells after 24-hour incubation with GCS-100 at a dose of up to 100 µg/ml (Figure 3.12(A)). By contrast, cleavage of caspase-9, the enzyme involved in the intrinsic apoptotic pathway (see 1.2), was clearly seen in cells treated with either GCS-100 alone or in combination with VP-16 (Figure 3.12(B)).

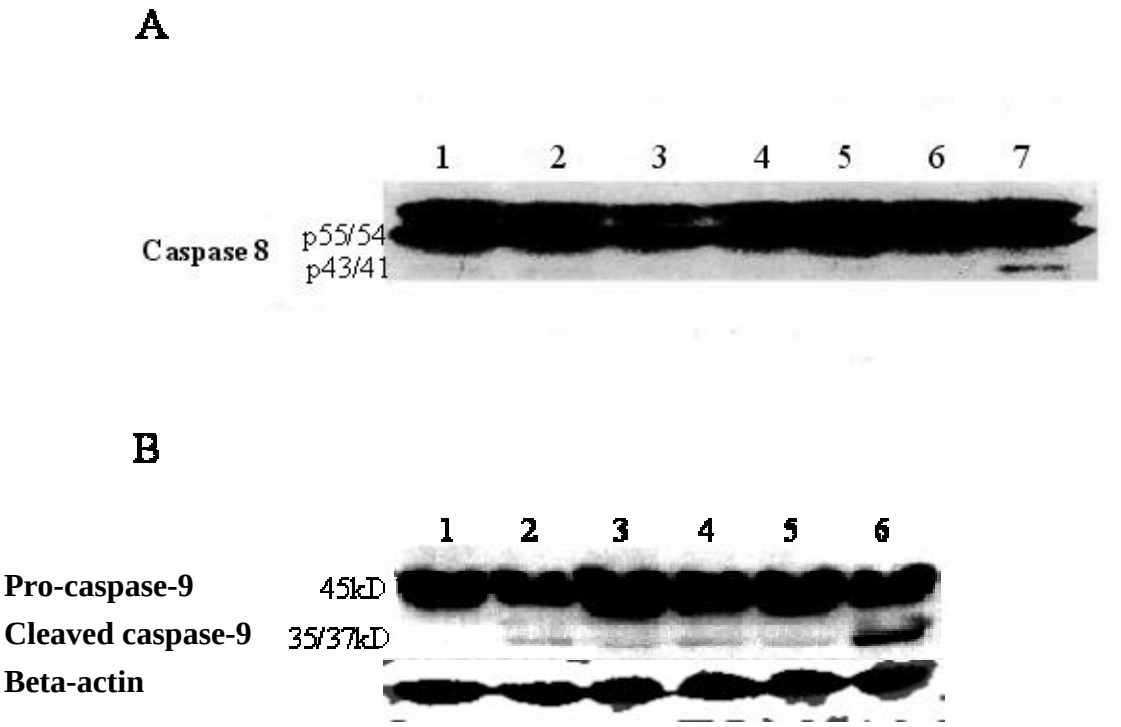


Figure 3.12 Western analysis of caspase-8 and -9 activities in DOHH2 cells after incubation with GCS-100, either alone or in combination with VP16. Western blotting was performed as stated above (see 2.5). Whole cell lysates were prepared from $10x10^6$ cells incubated with medium alone, GCS-100 at various concentrations from 40 to 100 µg/ml, VP-16 1 µM, or GCS-100 and VP-16 together, and analysed by Western blotting for activation of (A) caspase-8 and (B) caspase-9. (A) The anti-caspase-8 antibody is directed against the p18 subunit which detects pro-caspase 8 (p55/54) and the intermediate cleavage products of 43 kD and 41 kD (p43/41). No cleavage products were detected after incubation with GCS-100, VP-16 or the two in combination. Lane 1, control with no treatment; Lane 2, VP16 1 µM for 16 hours; Lane 3, GCS-100 40 µg/ml; Lane 4, GCS-100 80 µg/ml; Lane 5, GCS-100 100 µg/ml, all for 24 hours; Lane 6, GCS-100 40 µg/ml 6 hours + VP16 1 µM for 16 hours; Lane 7, anti-FAS 100 ng/ml for 4 hours, as a positive control for caspase-8 activation. (B) The anti-caspase-9 antibody detects the inactive pro-caspase-9 (approximately 46 kD) and following activation cleaved caspase-9 of approximately 35 and 37 kD. Although GCS-

100 and VP-16 alone both induced caspase-9 cleavage, this effect was enhanced when the two were used in combination. Lane 1, control with no treatment; Lane 2, VP16 1 μM for 16 hours; Lane 3, GCS-100 40 μg/ml; Lane 4, GCS-100 80 μg/ml; Lane 5, GCS-100 100 μg/ml, all for 24 hours; Lane 6, GCS-100 40 μg/ml 6 hours + VP16 1 μM for 16 hours.

3.4 GCS-100 and ROS generation.

ROS generation is an important mechanism for apoptosis induction, in particular when the mitochondrial apoptotic pathway was involved (see 1.2.2). To further investigate the possible mechanism of apoptosis induction by GCS-100, the question whether this agent has ROS-generating effect was explored. To address this question, DOHH2 cells, among the most susceptible of all the cell lines studied for the apoptosis-inducing effect of GCS-100, were treated with various doses of GCS-100 and ROS production was measured by dihydroethidium (DHE) staining and flow cytometry (see 2.3). No significant enhancement ($p>0.05$, $n=3$) of intracellular ROS was detected at doses of up to 160 μg/ml for 24 hours (Figure 3.13), conditions under which apoptosis was readily induced in this cell line (see 3.1.1).

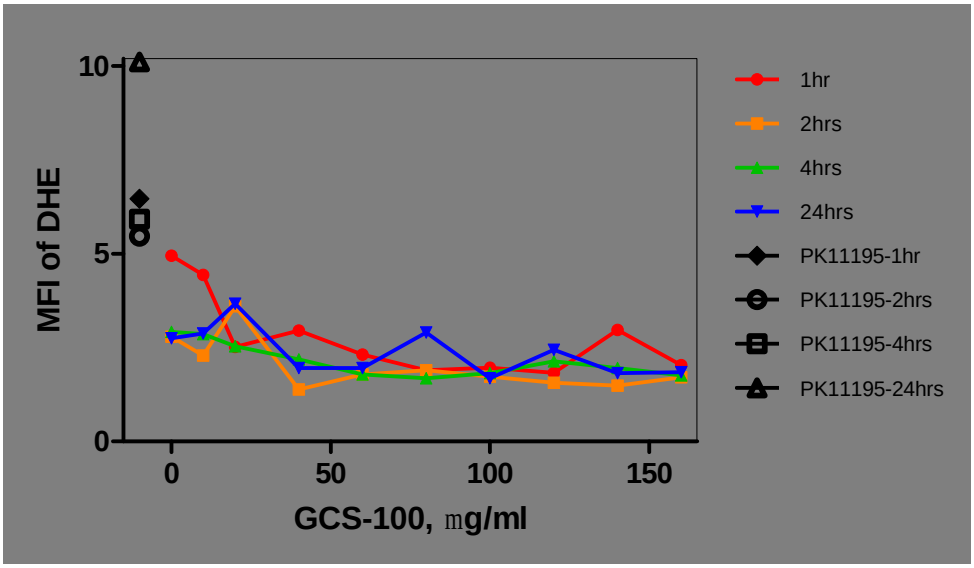


Figure 3.13 Flow analysis of the ROS-inducing effect of GCS-100 in DOHH2 cells. Cells were incubated with 0 (control) – 160 µg/ml GCS-100 for 1, 2, 4 or 24 hours. ROS generation was measured by flow cytometry with DHE staining (see 2.3). 100 µM PK11195-treated cells were used as positive control for ROS. No significant ($p>0.05$, $n=3$) ROS production was detected in GCS-100-treated cells. MFI, mean of fluorescence intensity; DHE, dihydroethidium; ROS, reactive oxygen species.

Summary

DiOC6(3) and MC540 staining are commonly used as surrogate measures of apoptosis (Laakko, King *et al.* 2002; Ozgen, Savasan *et al*. 2000). DiOC6(3) probes mitochondrial inner membrane potential (DeltaPsi(m)) changes by staining non-apoptotic mitochondrial membrane. When DeltaPsi(m) collapses – which happens during early apoptosis – the uptake of DiOC6(3) by a cell is

reduced which can be detected by flow cytometry. MC540 measures cell membrane integrity and lipid asymmetry, alterations which are associated with the membrane blebbing during apoptosis (see 2.3). These approaches have been successfully adopted to measure, in reproducible fashion, the apoptosis-inducing effects of GCS-100.

In addition, the effect of GCS-100 on apoptosis has been demonstrated by immunoblotting studies on the mitochondrial apoptotic pathway. Thus two independent methodologies have established the pro-apoptotic properties of GCS-100 in cell types beyond that of multiple myeloma originally described in the literature.

In this chapter, data have been presented which have shown unequivocally that the modified citrus pectin, GCS-100, is capable of inducing apoptosis in a dose-dependent manner in a variety of malignant cell lines of B cell provenance, as well as primary CLL cells. To the extent that apoptosis is not observed under similar conditions in normal lymphocytes, the effect would also appear to be selective for cells of the transformed state. GCS-100 also significantly ($p<0.05$, $n=3$) enhanced the pro-apoptotic effect of VP-16 in these cells, acting via the mitochondrial apoptotic pathway as evidenced by activation of caspase-9. The above findings suggest a basis for a novel, effective and putatively selective way to approach the treatment of haemotopoietic malignancies with GCS-100 *per se*, or more likely through

lowering the thresholds for apoptosis induction with established anti-tumour agents such as VP-16.

In conclusion,

i) GCS-100 induces apoptosis in human malignant B cells, including primary CLL cells, though it was not possible to relate this property to any of the patients' laboratory parameters or clinical status (see Appendix I). DOHH2 and RAMOS, the human malignant B-cell lines, showed the greatest sensitivity to GCS-100 in comparison with another cell line, K562, used in this study. The sensitivity of the RAMOS cells, which carries a p53 mutation, could be considered counter-intuitive since p53 mutant tumour cells might generally be considered to be more resistant to apoptosis induction.

ii) GCS-100 does not readily induce apoptosis in normal human lymphocytes.

iii) GCS-100 significantly ($p<0.05$, $n=3$) enhances the pro-apoptotic effect of VP-16 in malignant cell lines.

iv) GCS-100 acts via the mitochondrial caspase-9 pathway but not the caspase-8 pathway.

GCS-100 is theoretically an inhibitor of galectin-3 (see 1.7), thus the role that galectin-3 plays in the apoptosis-inducing effect of GCS-100 became of intrinsic interest and was investigated in Chapters 4 and 5.

Chapter 4 Interaction of galectin-3 and Bcl-2

Having shown that GCS-100 induces apoptosis in malignant cells via the mitochondria-caspase-9 pathway, this chapter aims to investigate whether or not galectin-3 is a direct target for GCS-100 and how galectin-3 interacts with Bcl-2 proteins in order to exert its effects on the caspase-9 pathway. Bcl-2 proteins exert their effects through the mitochondrial permeability transition pore, controlling the release of apoptogenic factors (see 1.2.3 & 1.3.1). It has been shown *in vitro* that galectin-3 and Bcl-2 physically bind and co-immunoprecipitate, possibly through their homologous *NWGR* motif in the carbohydrate-binding domain and the BH1 domain, respectively (Yang, Hsu *et al*. 1996). Here the interaction of galectin-3 and Bcl-2 and the possible influence of GCS-100 on this interaction were investigated in human cell lines.

GCS-100 is suggested to function by binding to galectin-3 across its carbohydrate-binding domain and acts as an antagonist due to its abundance of galactoside residues (see 1.7). The carbohydrate-binding domain of galectin-3 contains the *NWGR* motif of the BH1 domain, which it shares with some members of the Bcl-2 family proteins including Bcl-2 itself (see 1.3).

4.1 Expression of galectin-3 and Bcl-2 family proteins in tumour cells

For an interaction to occur between two molecules and be amenable to study, they must exist, either naturally or reconstitutively, at a detectable level and be accessible to each other within the cell. The expression levels of galectin-3 and Bcl-2 proteins were therefore determined in all studied cell lines and primary CLL cells by immunoblotting analysis (see 2.5). The intracellular localization of Bcl-2 and galectin-3 was studied by immunofluorescent imaging (see 2.7).

4.1.1 Galectin-3 and Bcl-2 protein expression

Bcl-2 was highly expressed in DOHH2, JURKAT, SUD4, U266, RPMI8226 cell lines and primary CLL cells (Figure 4.1). Galectin-3 was highly expressed in U266 and CLL cells and at lower levels in RPMI8226 and HEK293, but was not seen in DOHH2, JURKAT, K562, RAMOS and SUD4 cells (Figure 4.1). Bax was generally expressed at high levels in all cells tested, except JURKAT cells which showed a relatively low level of expression (Figure 4.1). Bcl-xl expression was detected at higher levels in U266, K562, RAMOS and SUD4 cells and at lower levels in JURKAT, DOHH2 and RPMI8226 cells (Figure 4.1). Primary CLL cells from a single patient and HEK293 cells did not show

any expression of Bcl-xl (Figure 4.1). Beta-actin was used as a control to determine protein loading in each of the lanes.

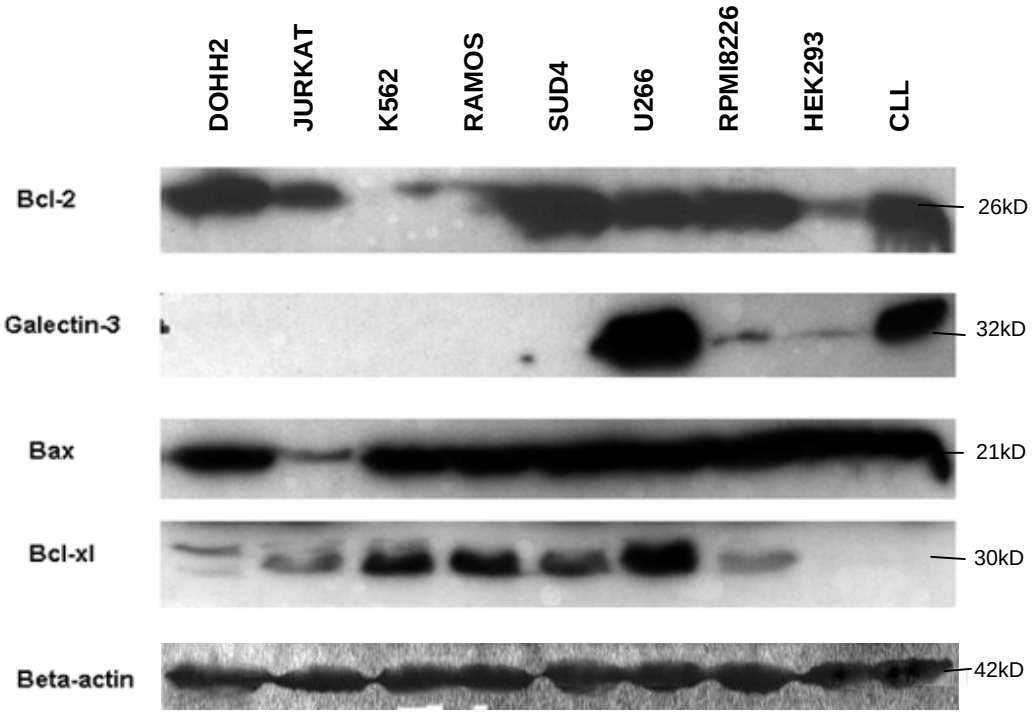


Figure 4.1 Western analysis of Bcl-2, galectin-3, Bax and Bcl-xl in human cell lines and primary CLL cells. Whole cell lysates were analysed by immunoblotting (for details see 2.5) for cellular expression of Bcl-2, galectin-3, Bax and Bcl-xl. Beta-actin was used as the loading control.

These findings do not support the theory that GCS-100 must bind galectin-3 to induce pro-apoptotic effects, as in DOHH2, RAMOS and, to a lesser extent, K562 cells where GCS-100 was found to be an effective apoptosis-inducer, galectin-3 was not detectable by immunoblotting (Figure 4.1).

This experiment revealed that U266 and RPMI8226 are the only two haematopoietic cell lines which naturally co-express galectin-3 and Bcl-2. Therefore, despite their resistance to GCS-100, U266 cells were used in the following study to determine the cellular localisation of galectin-3 and Bcl-2.

4.1.2 Galectin-3 expression in primary CLL cells.

Galectin-3 expression in CLL was further investigated using primary lymphocytes isolated from 8 CLL patients. The result (Figure 4.2) indicated that galectin-3 expression was heterogeneous amongst CLL patients, ranging from intense staining (patient 8) to being virtually indetectable (patient 6).

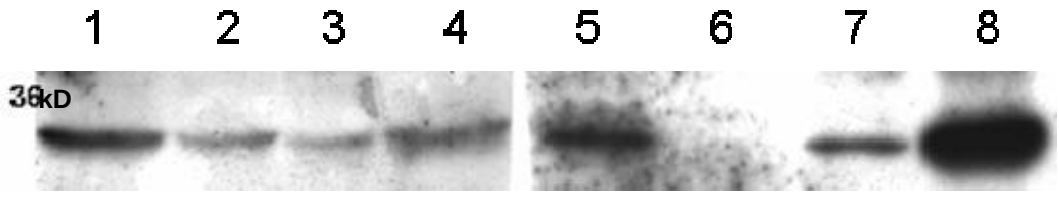


Figure 4.2 Western blotting analysis of galectin-3 expression in primary CLL cells. Lymphocytes were isolated (see 2.2) from fresh peripheral blood samples of eight CLL patients (Lane 1 – 8, see Appendix I). Whole cell lysates were analysed for galectin-3 expression by Western blotting (see 2.5).

4.1.3 Intracellular distribution of galectin-3 in RPMI8226 cells.

RPMI8226 is a human myeloma cell line with constitutive expression of both galectin-3 and Bcl-2 (see Figure 4.1). The intracellular localisation of galectin-3 was analysed in these cells using an immunofluorescent imaging technique (see 2.7). Galectin-3 was detected mainly as diffuse staining in the cytoplasm. No nuclear staining was observed (Figure 4.3).

(A) (B)

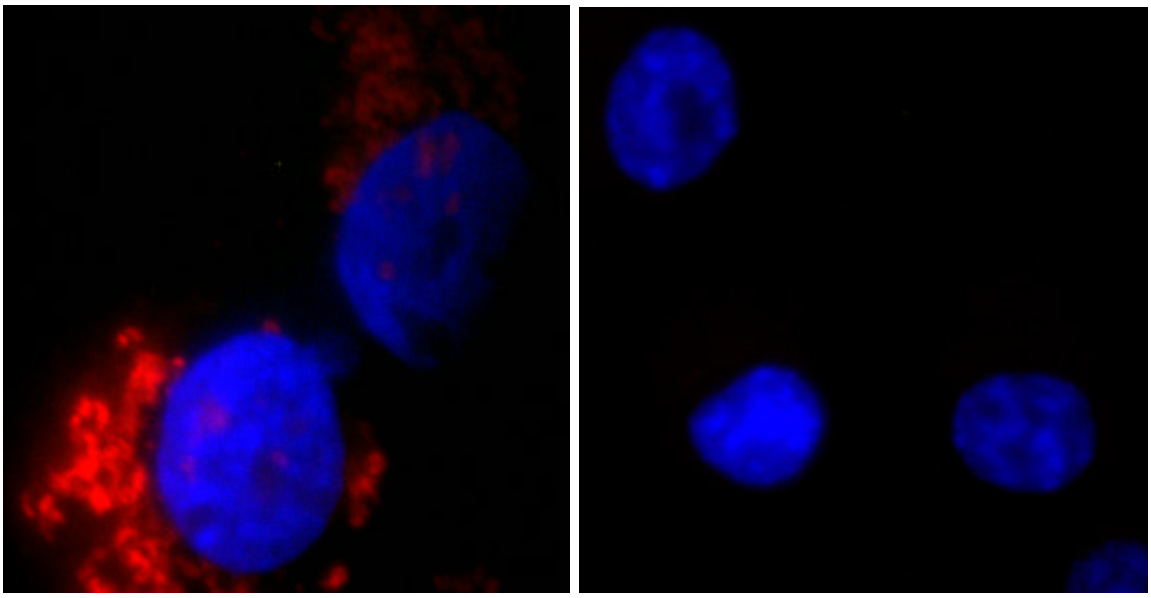


Figure 4.3 Fluorescent imaging of intracellular distribution of galectin-3 in RPMI8226 cells. Cells were stained with a mouse mononuclear anti-galectin-3 antibody and then a PE-conjugated goat-anti-mouse antibody (red, see 2.7). Cell nuclei were stained with DAPI (blue). (A) Diffuse staining of intracellular galectin-3; (B) Control image showing cells stained

with secondary antibody only. 100X magnification. PE, phycoerythrin; DAPI, 4,6-diamino-2-phenylindole.

4.1.4 Intracellular distribution of Bcl-2 in RPMI8226 cells.

A further question was whether Bcl-2 was also a cytoplasmic protein accessible for interaction with galectin-3. Bcl-2 was expressed in RPMI8226 cells (see Figure 4.1), and its intracellular distribution was investigated by immunofluorescent imaging (see 2.7). Bcl-2 was also found mainly in the cytoplasm. As with galectin-3, no nuclear localisation of Bcl-2 was observed (Figure 4.4).

(A) (B)

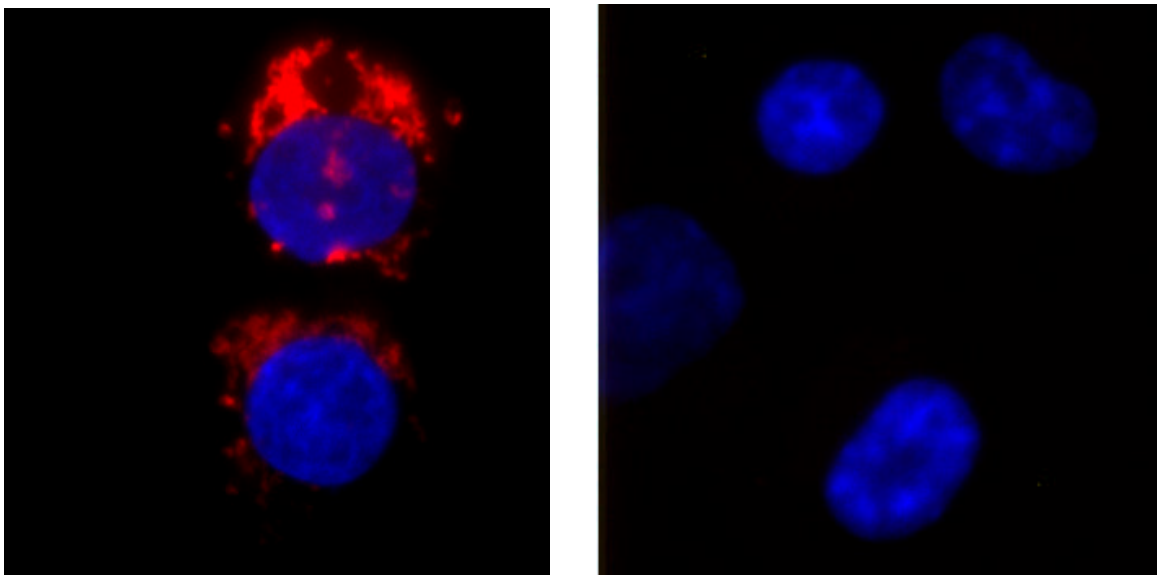


Figure 4.4 Fluorescent imaging of intracellular distribution of Bcl-2 in RPMI8226 cells. Cells were stained with a polyclonal rabbit anti-Bcl-2 antibody and then PE-conjugated goat-

anti-rabbit antibody (see 2.7). Cell nuclei were stained with DAPI (blue). (A) Intracellular distribution of Bcl-2; (B) Control image with secondary antibody staining only. 100X magnification. PE, phycoerythrin; DAPI, 4,6-diamino-2-phenylindole.

4.2 Interaction of galectin-3 and Bcl-2.

Following the demonstration that in some cell types (*e.g*. RPMI8226) at least, galectin-3 and Bcl-2 appear to be constitutively co-expressed (see Figure 4.1) and that these two molecules are both cytoplasmic (see Figures 4.3 & 4.4), the direct physical interaction between Bcl-2 and galectin-3 was investigated. To maximise the opportunity for interaction, cell transfection was selected as the methodology of choice. For this purpose, a cell model with high expression of both galectin-3 and Bcl-2 was artificially established. The interaction between these two molecules was then investigated by immunoprecipitation. This was further confirmed by immunofluorescence imaging.

4.2.1 Galectin-3 and Bcl-2 co-transfection and immuno-precipitation.

Galectin-3 and Bcl-2 expression vectors were cloned and transfected in HEK293 cells, chosen for their normally high transfection efficiency and, coincidentally, their low level of constitutive expression of each protein of interest. Thus the expression of these two molecules was elevated artificially in the same cell so as to raise the heteroligomer level of galectin-3 and Bcl-2, assuming that they bound interactively. Human *galectin-3* and *bcl-2* genes were cloned into the pCMV.Tag 4A expression vector with or without the 6×His Tag (see 2.6). HEK293 cells were then transfected with the cloned *galectin-3* or *bcl-2* vectors (see 2.6). A GFP-vector control was included in each transfection to determine the transfection efficiency (>80% as positive) by flow cytometry (see 2.6 & Figure 4.5). Expression levels of the transfected genes was further confirmed by immunoblotting analysis (see 2.5 & Figure 4.6) before immunoprecipitation was performed.

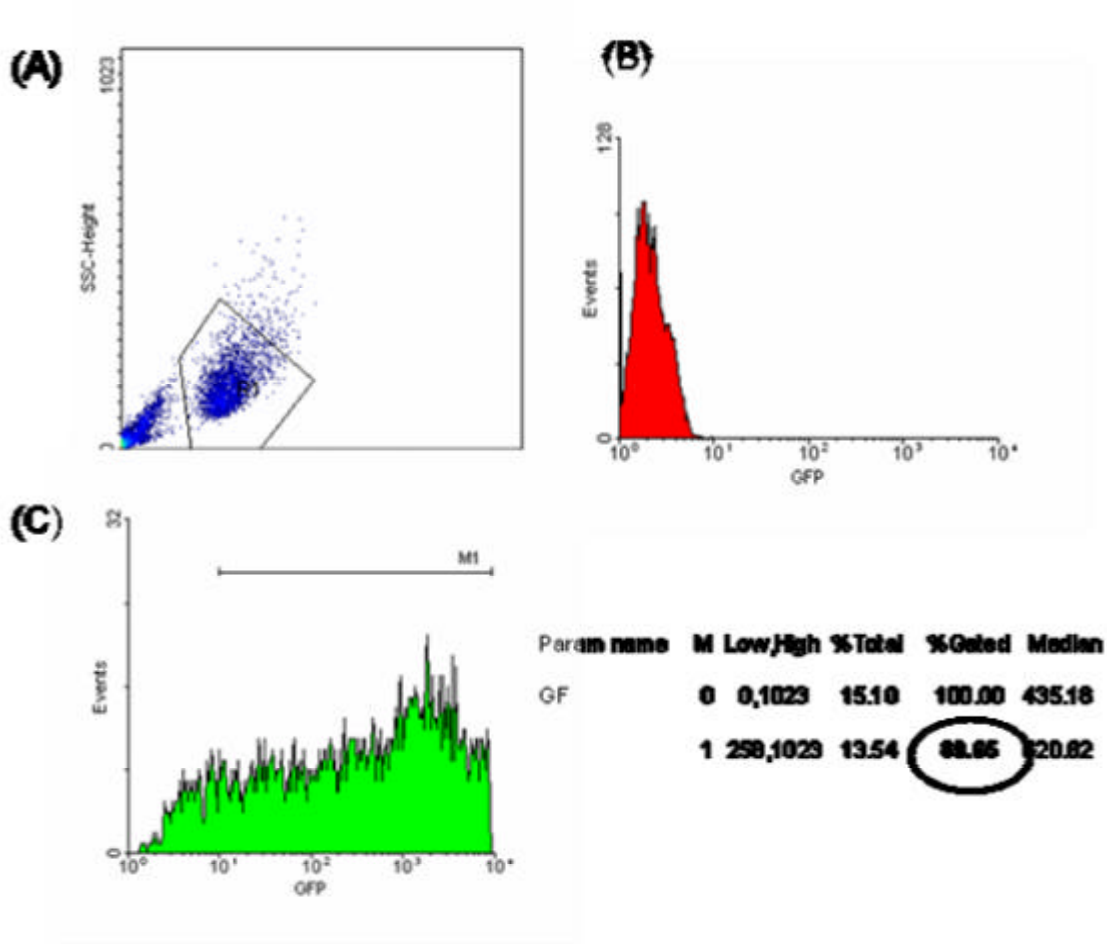


Figure 4.5 Flow cytometry analysis of the transfection efficiency in HEK293 cells. For each experiment, cells were transfected with pEGFP-N3 vector (Clontech) (see 2.6) and analysed by flow cytometry to check the transfection efficiency. (A) Cell debris was gated out. (B) Vector-control: cells transfected with empty vector had no GFP expression. (C) GFP-control: cells transfected with pEGFP-N3 vector showed strong GFP expression. Data analysis indicated that the transfection efficiency was >80%.

HEK293 cells express relatively low levels of galectin-3 and Bcl-2 constitutively (see Figure 4.1), as detected in vector control cells (Figure 4.6,

Lane 1). *Galectin-3*-transfected cells showed greatly increased galectin-3 expression (Figure 4.6, Lanes 2 & 3). Similarly, Bcl-2 expression level was also highly elevated in *bcl-2*-transfected cells (Figure 4.6, Lane 4 & 5). Some of the vectors were tagged with the 6xHis sequence and detected by the Penta-His antibody (Figure 4.6, Lanes 2 & 4).

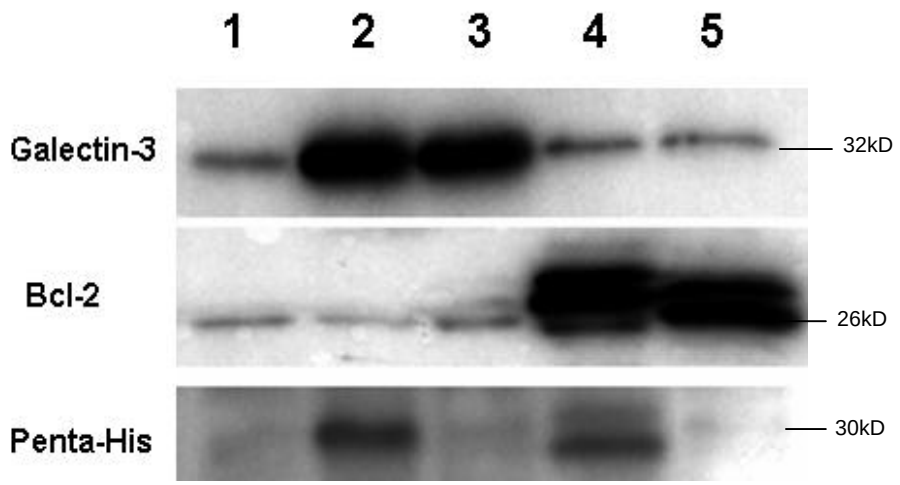


Figure 4.6 Western blotting analysis of galectin-3 and Bcl-2 transfection in HEK293 cells. Cells were transfected using the PEI method (see 2.6) with different expression vectors as indicated and collected at 72 hours post-transfection. Whole cell lysates were analysed by immunoblotting (see 2.5) for galectin-3 and Bcl-2 expression. Lane 1, empty pCMV vector-control; Lane 2, *galectin-3*-6xHis-tagged; Lane 3, *galectin-3* without tag; Lane 4, *bcl-2*-6xHis-tagged; Lane 5, *bcl-2* without tag. PEI, polyethyleneimine.

After HEK293 cells were successfully co-transfected with both galectin-3 and bcl-2 vectors, immunoprecipitation was performed on whole cell lysates using monoclonal antibodies against galectin-3 and Bcl-2 separately.

Immunoblotting was then performed with anti-Bcl-2 and anti-galectin-3 antibodies respectively.

Galectin-3 was detected in the anti-Bcl-2-precipitated product (Figure 4.7A, Lane 4). Treatment with GCS-100 at an extremely high dose of 500 µg/ml for 24 hours did not significantly alter this co-immunoprecipitation (Figure 4.7A, Lane 5). This suggests that an interaction between galectin-3 and Bcl-2 exists but that GCS-100 does not appear to affect it.

Reciprocally, Bcl-2 was detected in the anti-galectin-3-precipitated product (Figure 4.7B, Lane 4). Treatment with 500 µg/ml GCS-100 for 24 hours again did not affect this interaction (Figure 4.7B, Lane 5). The quantity of Bcl-2 detected on the blot was much less than the galectin-3 detected in the anti-Bcl-2-precipitated product. This was attributable to the different affinity of antibodies used in the immunoprecipitation.

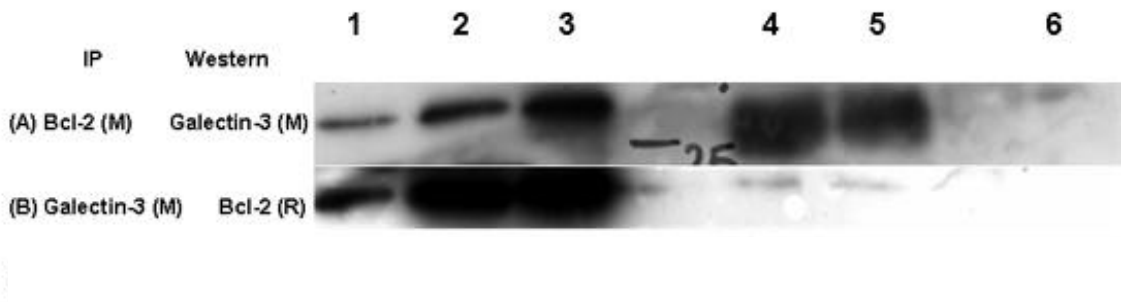


Figure 4.7 Co-immunoprecipitation of galectin-3 and Bcl-2 in transfected HEK293 cells. Immunoprecipitation and subsequent immunoblotting (see 2.4 & 2.5) were performed in whole cell lysate with different sets of antibodies as indicated. (A) Two monoclonal antibodies, anti-Bcl-2 and anti-galectin-3; (B) Two antibodies from different species, *i.e*. monoclonal anti-galectin-3 and rabbit polyclonal anti-Bcl-2. Lane 1, Vector-control whole cell lysate; Lane 2, *galectin-3*-6xHis-tagged and *bcl-2* co-transfected whole cell lysate control; Lane 3, *galectin-3*-6xHis-tagged and *bcl-2* co-transfected whole cell lysate with 500 µg/ml GCS-100 treatment for 24 hours; Lane 4 & 5, samples as Lane 2 & 3 after immunoprecipitation, respectively; Lane 6, antibody-control: the antibody used in the immunoprecipitation. Galectin-3 antibody was purchased from BD Bioscience, Bcl-2 monoclonal from Santa Cruz, Bcl-2 polyclonal from Abcam (see Chapter 2). M, monoclonal; R, rabbit polyclonal; IP, immunoprecipitation.

4.2.2 Co-localization of galectin-3 and Bcl-2 in RPMI8226 cells.

RPMI8226 constitutively expresses both galectin-3 and Bcl-2 (see Figure 4.1) and, based on previous experiments (see 4.1.3 & 4.1.4), immunofluorescent imaging (see 2.7) was performed in order to further investigate the interaction

between galectin-3 and Bcl-2 in the constitutive setting with RPMI8226 cells, on account of the technical limitations imposed with adherent HEK293 cells in fluorescent studies (see Chapter 7). Cells were co-stained with galectin-3 and Bcl-2 antibodies from different species as previously described (see 4.1.3 & 4.1.4), and then with species-specific secondary antibodies conjugated with different fluorochromes (see 2.7). In most cells there appeared to be overlap (Figure 4.8), which was most obvious in regions of the cytoplasm where individual expression was greatest. Such co-localisation is consistent with an interaction between Bcl-2 and galectin-3, as demonstrated in 4.2.1 and previously in the literature (Yang, Hsu *et al*. 1996). However, not all cellular co-expressed galectin-3 and Bcl-2 proteins may be mutually bound.

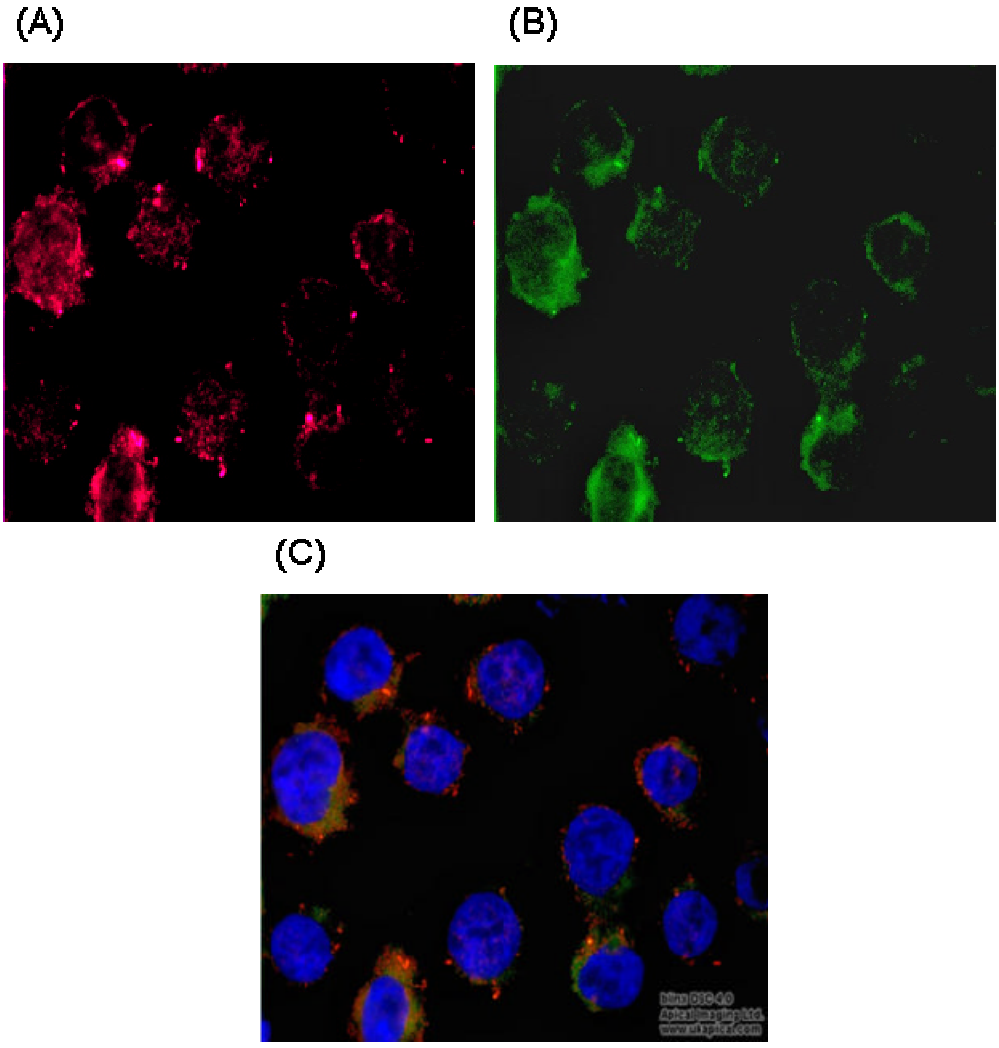


Figure 4.8 Fluorescent imaging of galectin-3 and Bcl-2 in RPMI8226 cells. Cells were stained with both anti-galectin-3 (mouse, monoclonal) and anti-Bcl-2 (rabbit, polyclonal) followed by secondary antibodies staining, *i.e*. Alexa Fluor 488 donkey anti-mouse (green) for galectin-3 and PE-conjugated goat anti-rabbit (red) for Bcl-2 (see 2.7). (A) intracellular distribution of Bcl-2; (B) intracellular distribution of galectin-3; (C) galectin-3 and Bcl-2 co-localization. 100X magnification. PE, polyethyleneimine.

Summary

The aim of this chapter was to explore the interplay between galectin-3 and Bcl-2, and whether any interaction was influenced by GCS-100. The following is a summary of the conclusions.

i) Galectin-3 and Bcl-2 interact intracellularly, as demonstrated by immunoprecipitation in transfected adherent cell lines with elevated expression of these two proteins, and by immunofluorescent imaging in human malignant B-lineage cell lines. Due to the technical difficulties experienced when using these two different techniques, two distinct cell lines were selected to perform these experiments. Therefore the data presented herein cannot be extrapolated to other B cell types.

ii) Notwithstanding the shared *NWGR* motif in the carbohydrate-binding domain and BH1 domain of galectin-3 and Bcl-2, respectively, GCS-100 does not significantly affect the interaction between the two proteins at a dose of up to 500 µg/ml for 24 hours.

If GCS-100 does not act on the oligomerisation of galectin-3 and Bcl-2, other mechanisms for its apoptosis-inducing effect must exist. The next chapter explores the action of GCS-100 on other survival mechanisms in malignant B cells.

Chapter 5 PI3K/Akt/NF-?B signalling pathway

The PI3K/Akt/NF-?B signalling transduction pathway is another survival mechanism operative in tumour cells (see 1.3). This chapter aims to investigate the involvement of the pathway in apoptosis and how it contributes to the mechanism of the pro-apoptotic action of GCS-100 in human haematopoietic cell lines.

5.1 Effects of GCS-100/VP-16 on Akt activity.

Akt is an important protein kinase involved in cell proliferation, survival and the apoptotic pathway (see 1.3.3). It is phosphorylated and activated downstream of phosphoinositide 3-kinase (PI3K). Hyperactivity of Akt correlates with tumour progression and inhibition of its activation induces apoptosis in tumour cells (see 1.3.3). The involvement of Akt activity in the apoptotic pathway was investigated in DOHH2 cells by immunoblotting analysis using an antibody specific for the phosphorylated (activated) form of Akt (see 2.5). Significant downregulation of phosphorylated Akt was observed following treatment with GCS-100 (Figure 5.1). This downregulation was detected following 6-hour GCS-100 treatment at 500 µg/ml (Figure 5.1, Lane 3) and increased significantly after 24-hour treatment (Figure 5.1, Lane 5). Interestingly, 1 µM VP-16 alone did not reduce the Akt activity even after 24 hours of exposure (Figure 5.1, Lane 6). However, combined treatment with both VP-16 and GCS-100 caused a much greater decrease in Akt activity

(Figure 5.1, Lane 7). Beta-actin confirmed consistent protein loading apart from Lane 6 which had slightly higher protein levels, which was also reflected in the pAkt band. However, this was probably insufficient to obscure any inhibitory effect of VP16 alone.

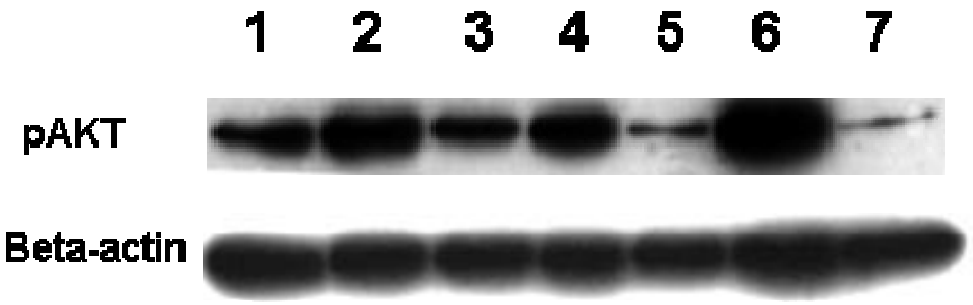


Figure 5.1 Western analysis of Akt downregulation by GCS-100 in DOHH2 cells. Cells were treated with or without (as control) 500 µg/ml GCS-100 (Batch.6527) or 1 µM VP-16 and collected at the time point indicated. Whole cell lysates were analysed by Western blotting (see 2.5) for phosphorylated Akt. Lane 1, Time-0 control; Lane 2, 6-hour control; Lane 3, GCS-100-treated, 6 hours; Lane 4, 24-hour control; Lane 5, GCS-100-treated, 24 hours; Lane 6, VP-16-treated, 24 hours; Lane 7, VP-16- and GCS-100-treated, 24 hours.

Akt knockdown by GCS-100 in DOHH2 cells was seen only at high doses relative to those needed to induce apoptosis as measured by flow cytometry. This new action of GCS-100, which has not been described elsewhere in the literature, appears to contribute to the pro-apoptotic effect of GCS-100. However, the extent of this contribution is difficult to quantify because differences in assay sensitivity could account, at least in part, for the

differential GCS-100 activity. It is beyond the scope of this thesis to investigate independently. However, another factor may also be implicated. Hitherto, all experiments in this thesis were conducted on a single batch of GCS-100 (*viz*. 876284-HO1, supplied by GlycoGenesys, see 2.1). The experiments on the Akt pathway were conducted with a new batch (*viz*. 6527), the activity of which was less by a factor of approximately 5-fold than 876284-HO1. Any effects on the Akt pathway will thus have been underestimated.

To investigate the effect of GCS-100 on this pathway further, experiments were conducted in additional cell lines and effects on markers downstream of Akt were also analysed.

5.2 GCS-100 and NF-(kappa)B pathway.

Akt is an activator of the NF-(kappa)B pathway, downstream of PI3K activation (see 1.3.3). The effects of GCS-100 on the NF-(kappa)B pathway were therefore investigated.

Various cell lines as well as a single sample of primary CLL cells were screened for Akt phosphorylation by immunoblotting (see 2.5). The JURKAT cell line (derived from human T-leukaemia cells, see 2.2) showed the highest

constitutive activation of Akt (Figure 5.2, Lane 1) and was therefore selected for this experiment. The other cell line with significant phosphorylated Akt expression was HEK293 (Figure 5.2, Lane 7). SUD4 cells also showed some constitutive Akt activity (Figure 5.2, Lane 4). No Akt activity was detected in the single sample of CLL cells tested (Figure 5.2, Lane 8).

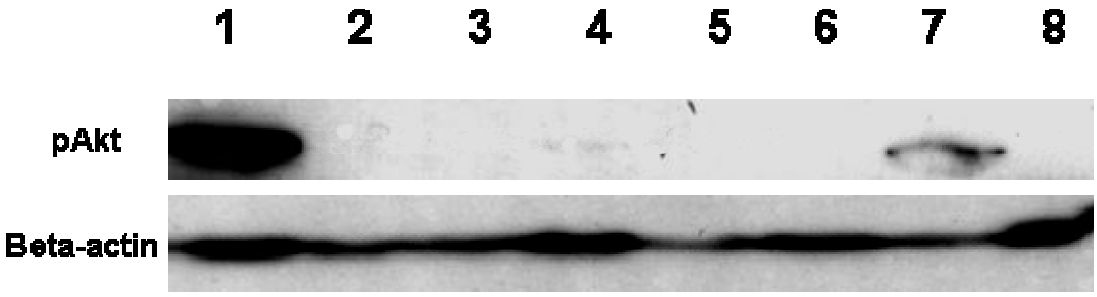


Figure 5.2 Western analysis of constitutive Akt phosphorylation in cell lines and primary CLL cells. Whole cell lysates were analysed by Western blotting (see 2.5) for phosphorylated Akt level in each cell type. Beta-actin control was used to ensure equal protein loading. Lane 1, JURKAT cells; Lane 2, K562 cells; Lane 3, RAMOS cells; Lane 4, SUD4 cells; Lane 5, U266 cells; Lane 6, RPMI8226 cells; Lane 7, HEK293 cells; Lane 8, primary CLL cells from the patient whose data are exhibited in Appendix I.

JURKAT cells were treated with 500 µg/ml GCS-100 for 24 or 48 hours and the intracellular level of phosphorylated Akt and the 65 kD subunit (p65) of NF-(kappa)B were analysed by immunoblotting (see 2.5). Downregulation of Akt activity was observed after 24-hour treatment with 500 µg/ml GCS-100 (Figure 5.3, Lane 2), in a similar manner to DOHH2 cells (Figure 5.1, Lane 5)

although the effect did not appear to be so marked. Longer incubation (48 hours) with GCS-100 did not further reduce the level of Akt phosphorylation (Figure 5.3, Lane 3). No significant effect of GCS-100 on NF-(kappa)B-p65 protein level was seen in this experiment (Figure 5.3, Land 2 & 3).

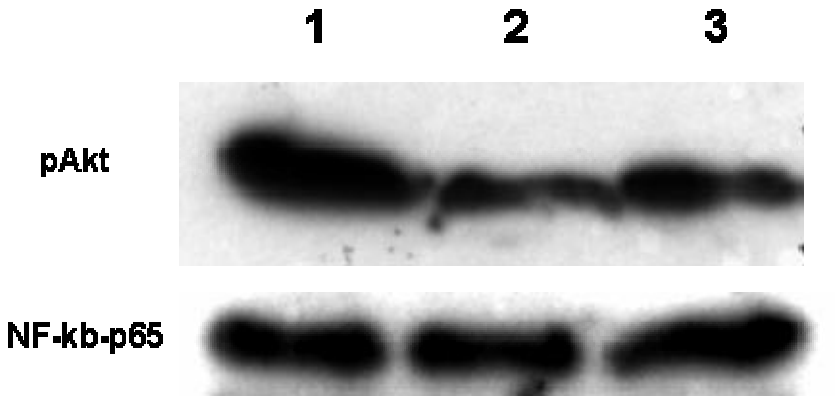


Figure 5.3 Western analysis of GCS-100 effect on Akt/NF-(kappa)B pathway in JURKAT cells. Cells were treated with or without (as control) 500 µg/ml (Batch.6527) GCS-100 for 24 or 48 hours. Whole cell lysates were analysed by Western blotting (see 2.5) for Akt activity and the 65 kD subunit (p65) of NF-(kappa)B. Lane 1, control; Lane 2, GCS-100-treated, 24 hours; Lane 3, GCS-100-treated, 48 hours.

The effect of GCS-100 on I(kappa)B, the negative regulator of NF-(kappa)B pathway, was analysed by immunoblotting (see 2.5) for I(kappa)B-alpha and its phosphorylation. The level of phosphorylated I(kappa)B-alpha appeared to

be moderately upregulated following 24-hour treatment with 300 µg/ml GCS-100 in DOHH2 cells (Figure 5.4, Lane 2).

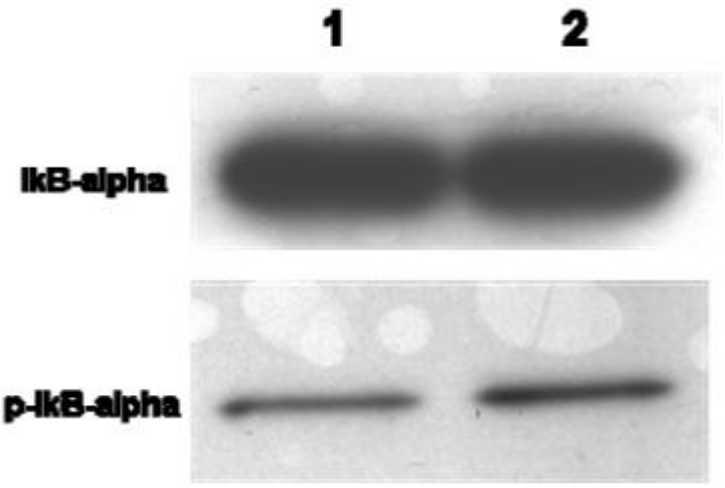


Figure 5.4 Western analysis of GCS-100 effect on I(kappa)B-alpha and its phosphorylation in DOHH2 cells. Cells were treated with or without (as control) 300 µg/ml GCS-100 (Batch.6527) for 24 hours. Whole cell lysates were analysed by Western blotting (see 2.5) for I(kappa)B-alpha and its phosphorylation. Lane 1, control; Lane 2, GCS-100-treated, 24 hours.

5.3 Galectin-3/Bcl-2 expression and Akt activity.

It has been shown in a breast cancer cell line that dephosphorylation of Akt occurs when galectin-3 is transfected (Lee, Song *et al*. 2003). In contrast, overexpression of galectin-3 increases the level of phosphorylated Akt in a bladder carcinoma cell line (Oka, Nakahara *et al.* 2005). These controversial

findings led to the following investigation of the effect of intracellular galectin-3 as well as Bcl-2 levels on Akt phosphorylation in HEK293 cells, a human embryonic kidney cell line, when transfected with galectin-3 or Bcl-2 expression vectors, *i.e.* the same transfected cell systems used in earlier experiments (see 4.2.1. especially Figure 4.6). Immunoblotting was performed to compare the level of Akt phosphorylation in cells transfected with empty vector (Figure 5.5, Lane 2), *galectin-3* vectors (Figure 5.5, Lanes 3 & 4) and *bcl-2* vectors (Figure 5.5, Lanes 5 & 6). No significant difference of Akt activity was seen between cells with wild-type (Figure 5.5, Lanes 1 & 2) and overexpressed galectin-3 (Figure 5.5, Lanes 3 & 4). The Bcl-2 expression level did not appear to be related to Akt activity either (Figure 5.5, Lanes 5 & 6).

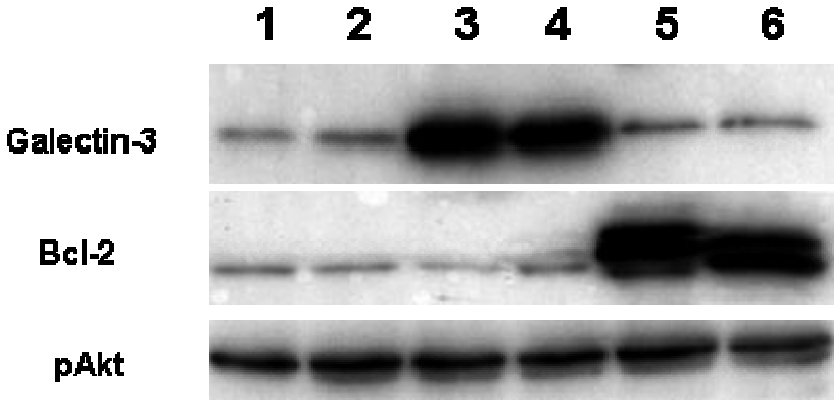


Figure 5.5 Western analysis of intracellular galectin-3/Bcl-2 levels and Akt activity in transfected HEK293 cells. Cells were transfected with GFP vector, empty pCMV vector,

galectin-3 or *bcl-2* vectors using PEI method (see 2.6). The GFP transfection efficiency was above 80% as a result of flow cytometry analysis (see 4.2.2). Whole cell lysates were then analysed by immunoblotting (see 2.5) for galectin-3, Bcl-2 and phosphorylated Akt levels, i.e. the same samples in Figure 4.6 were probed for Akt activity. Lane 1, GFP vector control; Lane 2, empty pCMV vector control; Lane 3, *galectin-3*-6xHis-transfected; Lane 4, *galectin*-3-transfected; Lane 5, *bcl*-2-6xHis-transfected; Lane 6, *bcl-2*-transfected. PEI, polyethyl-eneimine.

Summary

i) Akt phosphorylation is significantly downregulated by a relatively high dose of GCS-100, but not by VP-16 in DOHH2 cells. JURKAT cells with high constitutive expression of pAkt were also responsive to GCS-100 but to an apparently lesser extent than DOHH2 cells.
ii) The p65 unit of NF-(kappa)B was not affected by GCS-100 treatment at a dose up to 500 µg/ml for 48 hours.

The Akt knockdown effect of GCS-100 explains in part its pro-apoptotic effect, but the contribution of this component relative to other mechanisms is difficult to quantify on account of differential assay sensitivity and the use of a different batch of GCS-100 for reasons beyond the investigators' control. The data as described may thus have underestimated the role of pAkt downregulation. Akt has been suggested to be a significant factor in survival

pathways for tumour cells (see 1.3.3). The findings of this study (*i.e*. downregulation of Akt leading to induction of apoptosis) add weight to this hypothesis. This effect was apparently more significant in DOHH2 than JURKAT cells, suggesting that DOHH2 cells are relatively more sensitive to GCS-100.

Chapter 6 ROS and apoptosis

This thesis aims to analyse the underlying mechanisms for tumour cell survival in human B lymphoid malignancies by studying apoptosis in the context of two disparate apoptosis-inducing agents which have been subject of clinical investigation. As has been reported above, GCS-100 targets the mitochondrial apoptotic pathway and downregulates Akt phosphorylation (see Chapters 3, 4 and 5). By contrast, PK11195 induces apoptosis via induction of reactive oxygen species (ROS).

ROS are natural products from mitochondria during cell respiration. They can induce cell apoptosis if not converted to other non-toxic products by the natural defence mechanisms of the cell. Many drugs that generate ROS can also induce apoptosis. PK11195 induces apoptosis in many haematopoietic malignant cells (see 1.8), but the link between its pro-apoptotic effects and its role in intracellular ROS production has not been investigated. In this chapter the mechanism of the pro-apoptotic effect of PK11195 was examined in depth, with a focus on its ROS-generating role and expression level of Nox-5, the enzyme which catalyses the generation of ROS in lymphoid tissues.

PK11195 is a small synthetic molecule that induces apoptosis in many types of tumour cells with low toxicity for their normal counterparts. Both *in vitro* and *in vivo* it has the ability to increase the sensitivity of tumour cells to chemotherapy-induced apoptosis (Banker, Cooper *et al*. 2002; Chauhan, Li *et al.* 2004; Fennell, Corbo *et al*. 2001; Gonzalez- Polo, Carvalho *et al*. 2005;

Okaro, Fennell *et al.* 2002). It has been described as a high-affinity ligand for the mitochondrial peripheral benzodiazepine receptor (mPBR, see 1.2.2.a). However, its mechanism of action in the apoptotic pathway has not been fully clarified and is the aim of this chapter. As in previous studies, the effect of PK11195 on apoptosis was investigated, together with the role of ROS generation in its mechanism in human haematopoietic cell lines.

6.1 PK11195 and apoptosis induction.

The effect of PK11195 was studied in the DOHH2 cell line with high levels of Bcl-2 protein and consequent resistance to apoptosis. 75 µM PK11195 treatment for 24 hours induced significant mitochondrial transmembrane potential (DeltaPsi(m)) collapse which was partially reversed by MnTBAP, a ROS scavenger. MnTBAP alone did not induce DeltaPsi(m) collapse (Figure 6.1). This indicates that ROS generation was a key mechanism for the pro-apoptotic effects of PK11195.

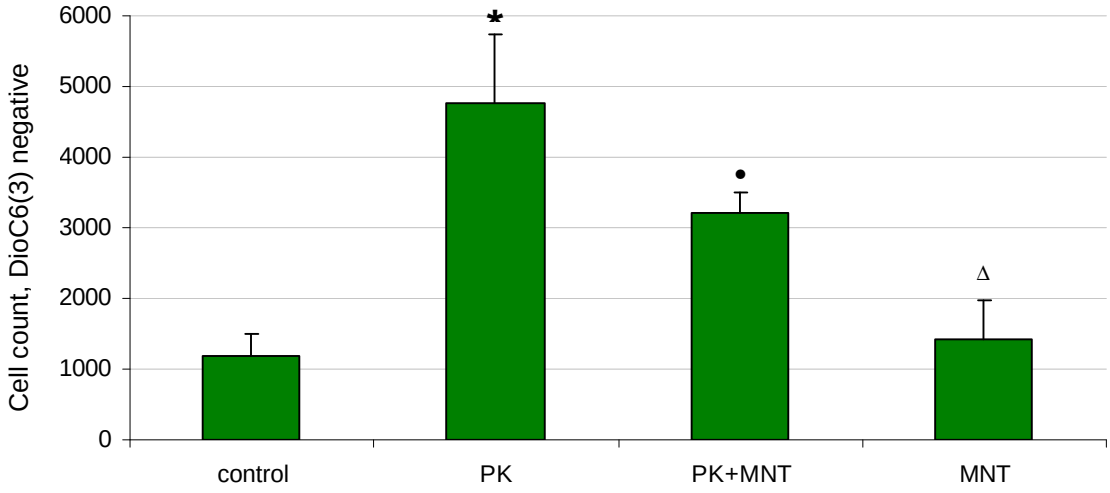


Figure 6.1 Flow analysis of the pro-apoptotic effects of PK11195 in DOHH2 cells. Cells were treated with or without (control) 75 µM PK11195 for 24 hours in the presence/absence of 100 µM MnTBAP, prior to DiOC6(3) staining and flow cytometry analysis. PK11195 alone caused a significant increase (*, $p<0.01$, $n=3$) in the DiOC6(3)-negative population, which was partially reversed by MnTBAP (•, $p<0.05$, $n=3$),. MnTBAP alone did not induce DeltaPsi(m) collapse (Δ, $p>0.05$, $n=3$). PK, PK11195; MNT, MnTBAP.

6.2 PK11195 and ROS induction.

ROS generation has been implicated in the mechanism of apoptosis induction by PK11195 in multiple myeloma cells (Chauhan, Li, *et al.* 2004). Thus the role of intracellular ROS in the pro-apoptotic effects of PK11195 was studied by DHE staining and flow cytometry analysis (see 2.3) in DOHH2 cells.

PK11195 treatment alone (75 μM) caused an increase of DHE fluorescence which was visualised on the FL-3 channel by flow cytometry, suggesting generation of ROS (Figure 6.2).

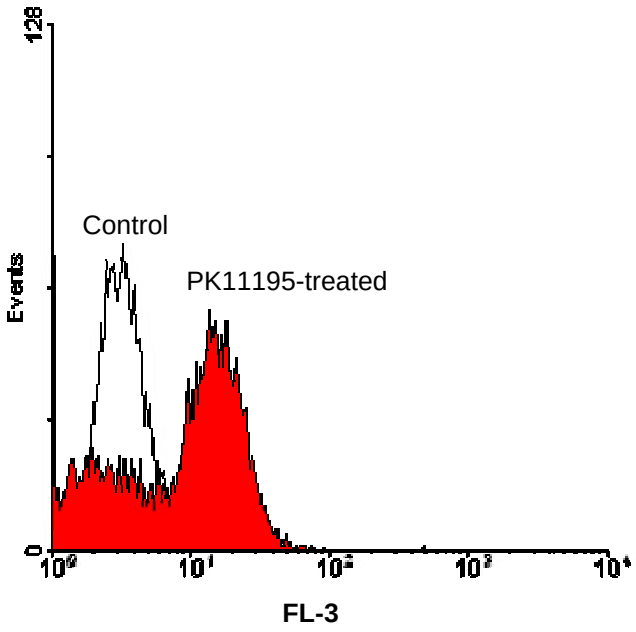


Figure 6.2 DHE staining and flow analysis of the ROS-generating effects of PK11195 in DOHH2 cells. Cells were treated with 75 μM PK11195 for 24 hours before DHE staining and flow analysis. PK11195 treatment caused an increase of fluorescence on the FL-3 channel, which indicates ROS generation. DHE, dihydroethidium; ROS, reactive oxygen species.

It has been shown that PK11195 induced apoptosis in DOHH2 cells (see 6.1). Whether or not ROS induction was involved in this process was investigated in the same cell type. The enhancement of intracellular ROS by PK11195 was observed with the same dose (75 μM) and time course (24 hours). These

cells showed a significantly increased level of ROS following 24-hour treatment with PK11195 (Figure 6.3).

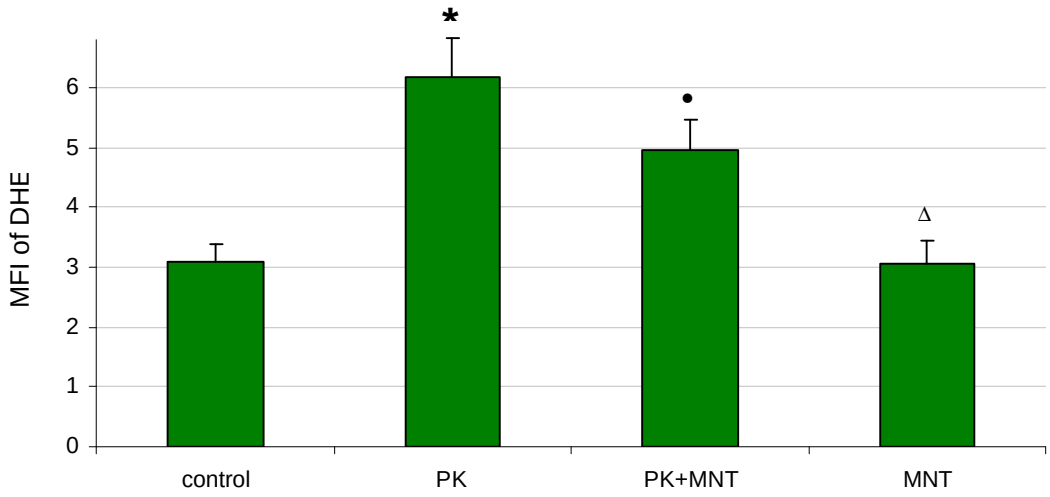


Figure 6.3 Flow analysis of the ROS-generating effects of PK11195 in DOHH2 cells. Cells were treated with or without (control) 75 μM PK11195 for 24 hours in the presence/absence of 100 μM MnTBAP, before DHE staining and flow cytometry analysis were performed. PK11195 alone caused a significant increase (*, $p<0.01$, n=3) of DHE fluorescence, which was partially reversed by MnTBAP (•, $p<0.05$, n=3). MnTBAP alone did not induce ROS generation (Δ, $p>0.05$, n=3). PK, PK11195; MNT, MnTBAP; MFI, mean of fluorescent intensity; DHE, dihydroethidium.

6.3 Nox-5 expression in a PK11195-resistant cell line.

It is understood that the reaction of intracellular ROS production in lymphoid tissues is mainly catalysed by Nox-5, a member of the NADPH oxidase family (see 1.2.2.b). As PK11195 raised the intracellular ROS level in various cell lines with different sensitivity, whether or not this was related to cell resistance to PK11195 was examined by comparing Nox-5 levels in PK11195-sensitive and -resistant cell lines. A PK11195-resistant cell line was established by culturing RAMOS cells with increasing doses of PK11195 over time (see 2.2.6). These cells were used to study the effect of PK11195 on Nox-5 expression in comparison with its sensitive counterpart. The Nox-5 mRNA level in the PK11195-resistant cells was significantly lower than in the sensitive cells (Figure 6.4), suggesting that Nox-5 level could be related to cell sensitivity to PK11195.

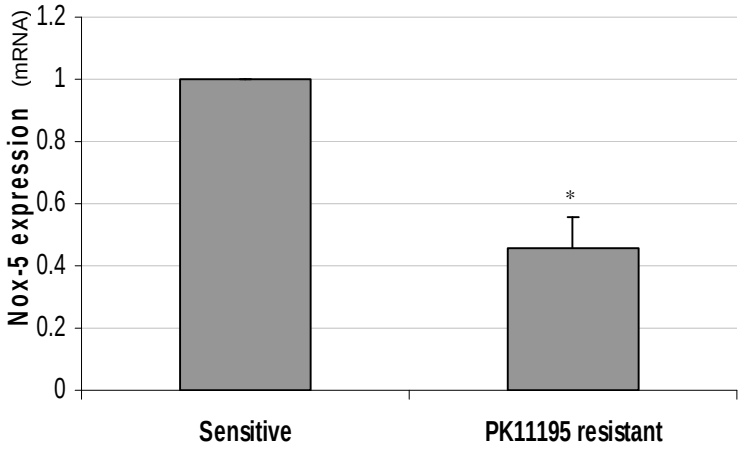


Figure 6.4 Different Nox-5 levels in PK11195-sensitive and -resistant cells. Three independent experiments were performed in PK11195-sensitive and -resistant cells to compare their Nox-5 mRNA levels by real-time PCR (see 2.8). The Nox-5 expression was significantly lower (*, $p<0.01$, $n=3$) in resistant cells than in sensitive cells.

6.4 PK11195 and Nox-5 expression.

The previous experiments suggested that ROS generation plays an important part in the mechanism of PK11195 (see 6.2). Since it had previously been hypothesised that PK11195 functioned as a mPBR ligand, this study resolved the mPBR role by investigating the effect of PK11195 in JURKAT cells which lack the mPBR (Canat, Carayon *et al.* 1993; Carayon, Portier *et al.* 1996; Hans, Wislet-Gendebien *et al.* 2005) . The response was as strong as that for other cell lines which possessed the mPBR (see 6.2). Therefore, ROS

generation would appear to be the major factor for the apoptosis induction by PK11195. This was further studied by comparing Nox-5 expression before and after PK11195 treatment in JURKAT cells using real-time PCR (see 2.8). A dose of 75 μM PK11195 for 24 hours enhanced the Nox-5 level significantly in comparison with the non-treated cells (Figure 6.5). This suggests that PK11195 interferes with the Nox-5 level and thus ROS generation. Taken together, the above data (6.3 & 6.4) imply that the NADPH oxidase pathway is important for PK11195-induced apoptosis in tumour cells.

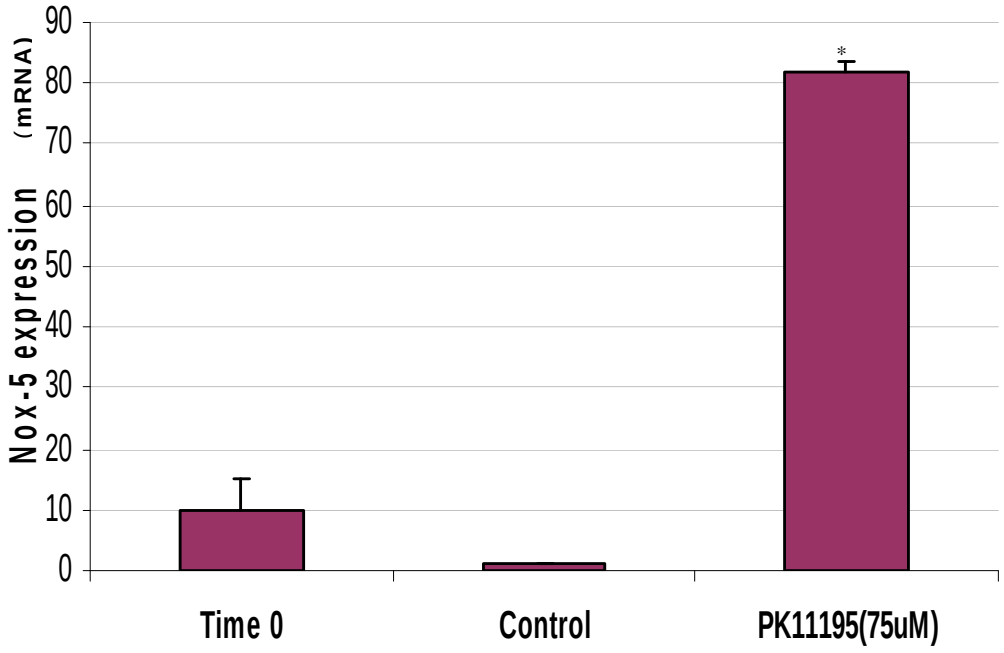


Figure 6.5 Effects of PK11195 on Nox-5 expression in JURKAT cells. Cells were treated with or without (control) 75 μM PK11195 for 24 hours before the intracellular Nox-5 level was analysed by real-time PCR (see 2.8). Time 0 was cells before the 24-hour treatment was performed. The control level of Nox-5 was set as 1. In non-treated cells, the 24-hour

incubation reduced the Nox-5 expression, *i.e*. the difference between Time 0 and control. PK11195 treatment significantly enhanced the Nox-5 level (*, $p<0.01$, $n=3$).

Summary

This chapter has demonstrated that the synthetic compound PK11195 has three effects on haematopoietic malignant cells: apoptosis induction via the mitochondrial pathway, ROS generation, and enhancement of Nox-5 expression. Apoptosis induction by PK11195 can be inhibited by MnTBAP, a ROS antagonist. Taken together, these data support the essential role of intracellular ROS, generated by NADPH oxidase, in the apoptosis-inducing effects of PK11195, suggesting a new mechanism of apoptosis induction in tumour cells. The mechanism would appear to be differentiated from that of GCS-100, notwithstanding the fact that the mechanism of action of the latter agent has not been fully elucidated. The mitochondrial NADPH oxidase pathway is a potential target for cancer therapy.

Chapter 7 Discussion

Galectin-3, Bcl-2 and the PI3K/Akt/NF-?B pathway are implicated in three major survival mechanisms in tumour cells amongst others, often resulting in defective apopotic pathways as well as abnormal cellular proliferation. Interference with these mechanisms can result in the induction of apoptosis and thereby point to potential strategies for cancer treatment. Other mechanisms of apoptosis induction include reactive oxygen species (ROS) generation. This thesis has studied two structurally different anti-cancer drug candidates, GCS-100 and PK11195, for their role in surmounting the anti-apoptotic mechanisms in human malignant B cells, and the possible molecular mechanisms underlying their apoptosis-inducing effects.

GCS-100 is a type of modified citrus pectin derived by acid/base hydrolysis of citrus pectin isolated from the skin and pulp of citrus fruits (GlycoGenesys, Inc, Boston, MA). A series of clinical trials on this drug candidate have been carried out in different types of cancer patients in the U.S.A. (Cotter 2004; Daliani, Pratt *et al*. 1999; Grous, Redfern *et al*. 2006; Gunzburg and Salmons 2001; Springgate, Cartwright *et al*. 2001; Staddon, Bonnem *et al.* 1999; Stuart, Kindler *et al.* 2001). It induces apoptosis in human multiple myeloma cells and is currently in Phase II clinical trial in multiple myeloma, colorectal and pancreatic cancers (see 1.7). Its pro-apoptotic effects are thought to be connected with its ability to bind and inhibit galectin-3, the anti-apoptotic molecule frequently overexpressed in tumour cells. In this project, it is shown for the first time that GCS-100 induced apoptosis in a number of human

malignant B cells which express high levels of Bcl-2 but do not have detectable galectin-3 expression, indicating that GCS-100 can overcome the apoptosis-resistant survival mechanisms induced by Bcl-2 overexpression, but also that galectin-3 may not be instrumental in apoptosis induction by this agent. Further investigation has shown that GCS-100 targets the mitochondrial apoptotic pathway by inducing cleavage of caspase-9, the key enzyme activated by apoptogenic factors released from mitochondria. Another important mechanism of GCS-100 action provisionally shown herein is downregulation of Akt activity, the crucial molecule in the PI3K signalling pathway, suggesting that, notwithstanding the need to establish the relative role of different mechanisms more quantitatively, GCS-100 has multi-faceted mechanisms for its apoptosis-inducing effects, with the ability to target a broad range of pathways leading to programmed cell death.

By contrast with GCS-100, PK11195 is a synthetic ligand (isoquinoline carboxamide) for the peripheral benzodiazepine receptor (PBR) with high affinity (K_D<20 nM) (Casellas, Galiegue *et al*. 2002). It induces apoptosis in human acute promyelocytic leukaemia cells which is thought to be irrelevant to its PBR-binding capacity, as the doses required for apoptosis induction are much higher than those required for receptor engagement (Fennell, Corbo *et al.* 2001). In parallel with the GCS-100 study, this part of the project examined the pro-apoptotic effect of PK11195 and its ROS-generating role, as well as any potential link between these two mechanisms. The data showed that

PK11195 induced apoptosis in human malignant B cell lines and intracellular ROS levels were involved in this process. In addition, Nox-5, the enzyme that catalyses production of ROS intracellularly, was found to be related to PK11195's apoptosis-inducing effect, confirming the involvement of ROS generation in the mechanism underlying the action of PK11195.

7.1 GCS-100 is pro-apoptotic

GCS-100 consistently induced apoptosis in a variety of human cell lines, alone or in combination with VP-16, one of the most potent chemotherapeutic drugs used in clinical cancer therapy (Montecucco and Biamonti 2007), although the sensitivity (EC50) varied between cell types. GCS-100 treatment (20 or 40 µg/ml) shifted the dose-response curve of VP-16 to the left, reducing the EC50 values by 2-4 logfold in DOHH2 and RAMOS cells (see 3.1 & 3.2), indicating that GCS-100 can act synergistically with VP-16 for apoptosis induction in human malignant B cell lines.

To address the same issue in primary B cells, blood samples from a panel of up to 8 patients with chronic lymphocytic leukaemia (CLL) were used. In common with B cell lines, GCS-100 caused significant mitochondrial inner membrane potential (DeltaPsi(m)) collapse and cell membrane perturbation in CLL cells, both typical events in the apoptotic cascade. Although a significant

amount of haematological and other data were available on the CLL patients (courtesy of Heartlands Hospital), the scope of this study was insufficient to seek any potential correlations between parameters associated with apoptosis, *e.g.* ZAP-70 expression (see 1.6), or prognosis, *e.g*. V_H mutation status (see 1.6), and sensitivity to apoptosis induction *in vitro* by GCS-100. Any such correlations would require a dedicated study in a much larger patient population and more extensive *in vitro* studies, including GCS-100 in combination with drugs routinely used in the management of CLL.

Notwithstanding the relatively small size of the B cell compartment in normal unfractionated lymphocytes, it was possible to conclude that a pro-apoptotic effect in the latter, with concomitant implications for unwanted toxicities, was unlikely on the grounds that on treatment with a much higher dose (up to 200 µg/ml) and for a longer incubation time (up to 48 hours), a contrary trend in the DiOC6(3)-negative population was observed. These results are consistent with published data on the selectivity of GCS-100 for multiple myeloma (Chauhan, Li *et al*. 2005).

Two types of technique were used as widely-accepted and well-established measurements of apoptosis: (i) DiOC6(3) and MC540 staining with flow cytometry measuring DeltaPsi(m) collapse and cell membrane integrity, respectively (Laakko, King *et al*. 2002; Ozgen, Savasan *et al*. 2000); and (ii)

immunoblotting analysis for caspase activation, which defines apoptosis at the molecular level (see 1.2).

DOHH2 cells are known to overexpress Bcl-2 which renders certain cancer cells drug resistant (see 1.3.1). These cells showed high sensitivity to GCS-100 and only 20 μg/ml treatment for 24 hours was required to observe a significant increase in DeltaPsi(m) collapse and membrane blebbing (Figures 3.2 & 3.3). Thus, GCS-100 has the potential to overcome Bcl-2-overexpression-mediated anti-apoptosis, at least in some cell lines.

In comparison with DOHH2 cells which express wild type p53, the tumour suppressor protein, RAMOS cells have mutated p53 which contributes to tumour progression and chemo-resistance (see 2.2.2). If GCS-100/VP-16 acted through p53 to induce apoptosis, the expectation would be that RAMOS cells might be more resistant to GCS-100/VP-16 than DOHH2 cells. In fact, the EC50s for concurrent treatment with GCS-100 and VP-16 were lower in RAMOS cells than in DOHH2 cells under the same treatment conditions (Tables 3.1 & 3.2). This implies that RAMOS cells are inherently more sensitive to GCS-100/VP-16 treatment and that p53 status is not critical for their action.

It has been reported that GCS-100 (formerly designated GBC-590), as a type of modified citrus pectin, binds and inhibits galectin-3, the anti-apoptotic

carbohydrate-binding protein widely believed to be overexpressed in tumour cells (see 1.3.2), and that this is the key mechanism for its pro-apoptotic effects. This hypothesis was not supported by the data presented herein, as DOHH2 and RAMOS cells did not reveal any detectable galectin-3 expression (Figure 4.1), which is consistent with published data on the non-detectable level of galectin-3 expression in Burkitt lymphoma and follicular lymphoma cell lines and patient samples (Hoyer, Pang *et al*. 2004). However, they still responded to GCS-100 (see Chapter 3). This leads to the important conclusion, hitherto not described, that galectin-3 at least was not the only mechanism for the apoptosis-inducing action of GCS-100.

7.2 GCS-100 acts via mitochondria.

It is known that VP-16 induces apoptosis in cancer cells via the mitochondrial apoptotic pathway (Montecucco and Biamonti 2007). As shown in 3.2, GCS-100 acted synergistically with VP-16 to exert its pro-apoptotic effects. Therefore it was pertinent to ask whether GCS-100 might also work via the mitochondrial apoptotic pathway. DiOC6(3) staining already established that mitochondrial inner membrane potential (DeltaPsi(m)) collapsed due to the effect of GCS-100 (see 3.1 & 3.2), indicating that mitochondria were involved in its apoptosis-inducing effect. This was further confirmed by showing that caspase-9, but not caspase-8, was activated in GCS-100-treated cells (Figure

3.12), as caspase-9 acts downstream of mitochondria and is cleaved by apoptogenic factors released from mitochondria (see 1.2.2).

Combined treatment of GCS-100 and VP-16 caused caspase-9 activation to a much greater extent than either agent alone (Figure 3.12), which was consistent with previous data showing that these two drugs were more effective for apoptosis induction if applied concurrently to malignant cells (see 3.2). These data support the conclusion that GCS-100 induces apoptosis via the intrinsic apoptotic pathway, activating caspase-9 as the downstream target.

7.3 Galectin-3 interacts with Bcl-2.

Galectin-3 and Bcl-2 are both anti-apoptotic proteins overexpressed in cancer cells, structurally sharing the *NWGR* motif within the carbohydrate-binding domain of galectin-3, where GCS-100 binds, and the BH1 domain of Bcl-2. Point mutation studies have shown that this sequence is crucial for the anti-apoptotic effect of both Bcl-2 and galectin-3 (see 1.3). Physical binding between these two molecules has been demonstrated previously *in vitro* (Yang, Hsu *et al*. 1996). Using an immunofluorescence technique, data presented herein showed that both molecules were detectable in the cytoplasm (see 4.1.3 and 4.1.4). Based on the above observations, the

possible interaction between galectin-3 and Bcl-2 was investigated using two techniques, immunoprecipitation and immunofluorescence.

For immunoprecipitation, HEK293 cells were co-transfected with expression vectors of galectin-3 and Bcl-2. Transfection of this adherent cell line ensured that conditions were optimal for detection of the respective proteins and any interaction between them, especially by the relatively insensitive technique of immunoprecipitation. Flow cytometry for GFP expression (see 4.2.1) and immunoblotting showed that expression of both molecules was indeed highly elevated after transfection. Whole cell lysates were prepared and galectin-3 or Bcl-2 were precipitated using specific antibodies prior to immunoblotting analysis for the other molecule. In the anti-Bcl-2-precipitated products, galectin-3 was detected. Reciprocally, Bcl-2 was detected in the anti-galectin-3-precipitated products. However, the interaction between galectin-3 and Bcl-2 was not affected by GCS-100 treatment (500 µg/ml, 24 hours, see 4.2.1).

Immunofluorescence is another well-established technique widely used for co-localisation studies with the advantage of greater inherent sensitivity than immunoprecipitation and immunoblotting. However, in our experience, this technique was less reliable when applied to adherent cells (HEK293) than to cytospin preparations of RPMI8226 cells, in which co-expression of galectin-3 and Bcl-2 is constitutive, albeit at a lower level than in transfectants. Thus, in the immunofluorescence experiments, RPMI8226 cells were co-stained with

anti-Bcl-2 (rabbit) and anti-galectin-3 (mouse). Secondary antibodies with different fluorescent labels, anti-rabbit-PE (red) and anti-mouse-Alexa Fluor 488 (green) were then applied. The microscopic images showed that, where Bcl-2 (red) and galectin-3 (green) co-localised, the emission wavelength of fluorescence changed to orange. These findings further confirmed the data obtained by immunoprecipitation.

The above data provide strong evidence that interaction between galectin-3 and Bcl-2 exists in tumour cells. However, as no marked effect of GCS-100 on this interaction could be demonstrated, it is concluded that this may not be the main mechanism of its action on the apoptotic pathway.

7.4 Akt knockdown by GCS-100.

A number of studies have validated the association between increased Akt activity and cell survival (see 1.3.3). However, the underlying molecular basis for this has been poorly defined. It has been reported that galectin-3 overexpression inactivates Akt by dephosphorylation in a breast cancer cell line (Lee, Song *et al.* 2003), while in contrast, high levels of galectin-3 promote Akt activation in a bladder carcinoma cell line (Oka, Nakahara *et al*. 2005). GCS-100 has the ability to downregulate the galectin-3 level in

multiple myeloma cells (Chauhan, Li *et al*. 2005). These findings show that the link between GCS-100, galectin-3 and Akt is still unclear.

In this study, the intracellular level of phosphorylated Akt was significantly reduced after GCS-100 treatment in DOHH2 and JURKAT cells (Figures 5.1 & 5.3). DOHH2 cells, the more sensitive cell line to GCS-100, showed clear inhibition of Akt activity following 6-hour treatment. The Akt level continued to fall up to 24 hours of incubation (Figure 5.1). In contrast, JURKAT cells did not show significant Akt downregulation until 48 hours after treatment (Figure 5.3). VP-16 treatment alone of DOHH2 cells did not alter Akt phosphorylation. Thus the effectiveness of GCS-100 against malignant cells appears to be related to its action on Akt. As expected, combined treatment with both GCS-100 and VP-16 induced a more profound reduction of Akt phosphorylation, which correlates with the effectiveness of the apoptosis-inducing role of the combination.

Both DOHH2 and JURKAT cells showed reduced Akt activity following GCS-100 treatment although they did not express detectable levels of galectin-3 (Figure 4.1), which suggests that galectin-3 alone did not appear to mediate the effect of GCS-100 on Akt. In addition, HEK293 cells with elevated levels of galectin-3 failed to show any altered Akt activity (Figure 5.5), further supporting this hypothesis. The above data all indicate that GCS-100 can interfere with Akt phosphorylation, but that much of this effect is not mediated

by galectin-3. PI3K, a lipid kinase upstream of Akt that can be activated by various cell surface receptors, is a possible target of GCS-100. More studies are needed to investigate this possibility. The PI3K/Akt pathway is more commonly activated in tumour cells (Samuels and Ericson 2006) than expression of galectin-3. If the former is a major target for GCS-100, a broader application might be expected for this agent in cancer therapy.

A caveat to the above data on Akt downregulation discussed in 5.1 is that a different batch of GCS-100 had to be deployed for these experiments. The potency of the second batch was approximately 5-fold less than that used for the vast majority of experiments described in this thesis. Data on Akt downregulation must therefore be interpreted in the awareness that the extent of the phenomenon is underestimated. The fact that Akt downregulation was observed at all, strongly supports the conclusion that this is a real event. The issue that the weaker batch of GCS-100 therefore raises, is that of the relative contribution of Akt downregulation to the overall mechanisms of action of GCS-100, not whether it occurs.

7.5 Akt and the apoptotic pathway.

GCS-100 downregulates Akt phosphorylation and also interferes with the mitochondrial apoptotic pathway. It is thus not unreasonable to consider that

connections between Akt and the mitochondrial pathway may contribute to GCS-100's apoptosis-inducing effects.

Akt can act as an inhibitor of apoptosis and block mitochondrial apoptotic events, *e.g*. cytochrome c release (Kennedy, Kandel *et al*. 1999). This protein kinase exerts influence intracellularly at multiple levels and its substrates are involved in a wide range of signal transduction pathways, including that of apoptosis. The Bad protein, a pro-apoptotic member of the Bcl-2 family, is one of its direct targets and phosphorylation of Bad by Akt releases Bcl-xl, an anti-apoptotic Bcl-2 family protein. Bcl-xl then dimerizes with Bax to promote cell survival (see 1.3.3). GCS-100 mediates dephosphorylation and inactivation of Akt, possibly reducing Bad phosphorylation indirectly. Dephosphorylated Bad oligomerizes with Bcl-xl and frees Bax to form homodimers which cause the subsequent DeltaPsi(m) collapse, apoptogenic factor release and activation of caspases.

Caspase-9 is another direct substrate of Akt. Activated Akt phosphorylates and inactivates caspase-9 and interrupts the mitochondrial amplification loop (see 1.3.3). This suggests that Akt can block the apoptotic cascade even downstream of cychrome c release. GCS-100 treatment in DOHH2 cells caused activation of caspase-9 (Figure 3.12), which may in part be due to Akt inactivation by GCS-100 with subsequent dephosphorylation of caspase-9.

The third direct target of Akt that may be related to the effect of GCS-100 is I(kappa)B kinase (IKK). IKK catalyses the phosphorylation and degradation of Inhibitor of NF-(kappa)B (I(kappa)B), the negative regulator of NF-(kappa)B. Akt phosphorylates and activates IKK, which in turn upregulates the NF-(kappa)B activity (see 1.3.3). Bcl-2, IAP and FLIP are among the pro-survival genes under NF-(kappa)B regulation (see 1.3.3). GCS-100 promotes dephosphorylation and inactivation of Akt and therefore inhibits NF-(kappa)B activity as well as the subsequent transcription of the pro-survival genes. Although this is theoretically possible, the NF-(kappa)B activity did not show significant changes following GCS-100 treatment in JURKAT cells (Figure 5.3). The I(kappa)B-alpha phosphorylation level was found to be only slightly increased in DOHH2 cells (Figure 5.4). In addition, it has been reported that GCS-100 decreases cell viability in an I(kappa)B-mutated lymphoma cell line (Chauhan, Li *et al*. 2005), suggesting that I(kappa)B is not predominantly involved in the pro-apoptotic effect of GCS-100.

Akt can prevent Forkhead transcription factors from entering the nucleus and the subsequent transcription of FasL gene (Datta, Brunet *et al*. 1999). It is therefore possible that GCS-100 inhibits Akt activity and thus enhances the expression of FasL and Fas-related apoptosis. However, caspase-8 activation was not observed (Figure 3.12) and would have been expected if this were the case.

Akt may also influence mitochondrial energy production and the electron transduction chain (Gottlob, Majewski *et al*. 2001) where alterations in ROS generation would be expected. No significant elevation of intracellular ROS level could be detected following GCS-100 treatment in DOHH2 cells (Figure 3.13), suggesting that this is not a viable explanation.

It is concluded that GCS-100 has a multi-faceted role in the apoptotic pathway. The mechanism of its pro-apoptotic effect involves several signal transduction pathways activated directly or indirectly by GCS-100. Akt can prevent apoptosis upstream of mitochondria, bypassing mitochondria via direct phosphorylation of caspase-9, or through regulation of other transcriptional pathways. The data presented here support the involvement of Akt activity and caspase-9 activation in the mechanism of GCS-100 and provide some understanding for the complex and intriguing role of GCS-100 and further insights for future experiments.

7.6 PK11195, ROS and apoptosis

ROS generation is a physiological process that inevitably occurs during cell respiration (see 1.2.2.b). The association between ROS and apoptosis has been studied extensively. It is believed that tumour cells experience more oxidative stress than normal cells and have generally enhanced intracellular

ROS levels, a key feature involved in transformation, proliferation and tumourigenesis (Pelicano, Carney *et al*. 2004). However, excessive ROS also kill malignant cells, as it is often a concomitant phenomenon with apoptosis when collapse of mitochondrial inner membrane potential (DeltaPsi(m)) and the demise of energy production and electron transduction chain occur. Mitochondria are the major source of intracellular ROS (Paradies, Petrosillo *et al*. 2001; Pelicano, Carney *et al*. 2004). Such significant enhancement of intracellular ROS by PK11195 is likely to be due to its effects on mitochondria. Data herein have clearly demonstrated induction of ROS generation by PK11195 in DOHH2 cells and concomitant DeltaPsi(m) collapse (Figures 6.1 & 6.3). The two apoptotic events are tightly linked and, based on previous studies in the literature as stated above, they can be both cause and consequence of each other. This is also suggested by data presented here showing that enhancement of intracellular ROS level was detected at the same dose and time point after PK11195 treatment as DeltaPsi(m) collapse.

The effects of PK11195 shown here appear to be independent of the mitochondrial peripheral benzodiazepine receptor (mPBR) based on the following findings.

i) PBR gene knockout by specific antisense does not abolish the effects of PK11195 (Gonzalez-Polo, Carvalho *et al*. 2005).

ii) JURKAT cells, naturally lacking the mPBR expression, responded to PK11195 (Figure 6.5).

iii) The dose of PK11195 (μM) required to induce apoptosis or ROS generation is several orders of magnitude higher than its K_D for the PBR (nM) (Casellas, Galiegue *et al*. 2002).

Nox-5 is a possible downstream target for PK11195. 24-hour treatment with 75 μM PK11195 raised the Nox-5 mRNA expression level over eight fold in JURKAT cells (Figure 6.5), consistent with the extent of ROS-level enhancement by the same dose of PK11195 in the same cell type (see 1.8). Repeated treatment of RAMOS cells with increasing doses of PK11195 reduced Nox-5 expression and caused resistance to PK11195 in these cells (Figure 6.4), indicating the existence of a negative feedback loop that downregulates Nox-5 after long exposure to PK11195.

Nox-5 acts as a sensor of intracellular [Ca^{2+}] through its N-terminal EF-hand domains (see 1.2.2.b). It could be suggested that PK11195 interferes with the intracellular [Ca^{2+}] balance, a key factor in the apoptotic pathway. This subsequently alters the Nox-5 function as well as induces apoptosis. The relationship between Nox-5/intracellular [Ca^{2+}] and PK11195/ROS/apoptosis remains to be fully clarified.

MnTBAP is a synthetic manganese superoxide dismutase (MnSOD) mimetic and has comparable SOD activity (Gauuan, Trova *et al*. 2002). It scavenges intracellular ROS potently and can inhibit Fas-induced apoptosis in liver cells (Malassagne, Ferret *et al.* 2001). Thus, its effect on PK11195-induced ROS generation and apoptosis was studied. Without inducing ROS or apoptosis on its own, MnTBAP significantly reduced ROS as well as apoptosis induced by PK11195 in DOHH2 cells (Figures 6.1 & 6.3). This further provides evidence that the intracellular ROS level is crucial for the apoptosis inducing effect of PK11195.

Summary: Implications for cancer therapy

It is axiomatic that tumour cells have survival advantages over normal cells. Two widely disparate synthetic molecules, GCS-100 and PK11195, can each overcome these advantages at least *in vitro*, and induce apoptosis in certain malignant cells, via different mechanisms. GCS-100, the putative galectin-3 inhibitor currently in Phase II clinical trial for the treatment of various malignant diseases (see 1.7), selectively induces apoptosis in B-lineage tumour cells without appearing to affect normal lymphocytes. It also significantly enhances the pro-apoptotic effect of the chemotherapeutic agent VP-16. GCS-100 mediates two major mitochondrial apoptotic events: mitochondrial inner membrane potential (DeltaPsi(m)) collapse and caspase-

9 activation. Further investigations reveal that GCS-100 causes dephosphorylation and inactivation of Akt, a serine/threonine kinase that promotes cell survival by regulating transcriptiton factors and proteins that control apoptosis. PK11195, a small synthetic molecule with high affinity for mPBR (see 1.8), induces intracellular ROS generation and subsequent apoptosis in malignant B cells. This appears to be due to its effect on Nox-5, the member of NADPH oxidase family in lymphocytes which catalyses intracellular ROS production. As such, the mechanisms of action of the respective agents are fundamentally different.

Interference with cell signal transduction has been extensively explored for potential therapeutic strategies for the treatment of malignant disease. A number of anti-cancer drugs with the ability to induce apoptosis have exhibited sucessful clinical utility, *e.g.* imatinib in the treatment of chronic myeloid leukaemia (CML) (Ohno 2006). However, as more drugs enter the clinical arena, *intrinsic drug resistance*, due to a plethora of apoptosis-resistant mechanisms pre-existing in cancer cells, and/or *acquired drug resistance* to the original molecular target, remain a major issue (Frame 2007). Therefore, superior drugs that work via novel mechanisms on selective targets to overcome cell survival, alone or in combination with existing chemotherapeutic drugs, will be required for as long as cytotoxic agents are the mainstay of the oncologists' armoury.

The data presented here in the context of agents GCS-100 and PK11195 have revealed the complexity of the underlying mechanisms of cell survival and apoptosis induction in B cell malignancies. They have also provided some new insights which might be harnessed to help further define anti-apoptotic strategies in cancer therapy.

Chapter 8 References

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Li Wang 2007

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Appendix I CLL patients data (from Birmingham Heartlands Hospital)

Patient	CD38	ZAP70	Mutat status	1st Treat	Chlor	Pred	Flud	Cyclo	CHOP	Alem	Ritux	Nº Treat
	(% of	(% of	0 Unmut	0 CLB	0 No	0 No	0 No	0 No	0 No	0 No	0 No	
	positive	positive	1 Mut	1 Flud	1 Yes	1 Yes	1 Yes	1 Yes	1 Yes	1 Yes	1 Yes	
	cells)*	cells)*		2 FludCy								
				3 CHOP								
Fig3.5-1**	11.3	26.9	1									
Fig3.5-2	25.5	18.0	1	0	1	0	0	0	0	0	0	2
Fig3.5-3	11.3	26.9	1									
Fig3.5-4	25.5	18.0	1	0	1	0	0	0	0	0	0	2
Fig3.5-5	64.9	68.2	0	0	1	1	0	0	0	0	0	6
Fig3.5-6	82.6	14.2	1	0	1	1	0	0	0	0	0	2
Fig3.5-7	8.2	23.0		0	1	1	0	0	0	0	0	1
Fig3.5-8	41.1	50.9	0	1	0	0	1	1	0	1	0	3
Fig3.7-1	11.3	26.9	1									
Fig3.7-2	25.5	18.0	1	0	1	0	0	0	0	0	0	2
Fig3.7-3	11.3	26.9	1									
Fig3.7-4	25.5	18.0	1	0	1	0	0	0	0	0	0	2
Fig3.7-5	64.9	68.2	0	0	1	1	0	0	0	0	0	6
Fig4.2-1	3.7	2.6	1	0	1	0	0	0	0	0	0	1
Fig4.2-2	47.1	76.3	0	0	1	0	0	0	0	0	0	3
Fig4.2-3	3	29.3	0	0	1	0	0	0	0	0	0	2
Fig4.2-4	2.7	5.5	1									
Fig4.2-5	5	14.0	0	0	1	1	1	0	0	0	0	3
Fig4.2-6	3.1	6.9	1	0	1	0	0	0	0	0	0	2
Fig4.2-7	21.5	10.8	1	0	1	1	1	1	0	0	0	6
Fig4.2-8	49.4	69.7	1	0	1	1	1	1	1	1	0	6
Fig5.2	51.2	28.1	1	0	1	1	0	0	0	0	0	3

* Cytoplasmic ZAP-70 expression was determined by using a modification of a previously published flow cytometry method. Crespo, M., F. Bosch, *et al*. (2003). “ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia.” N Engl J Med **348**(18): 1764-75.

**Figures refer to samples from individual patients. Patients’ identities are omitted to preserve anonymity .