THE ROLE OF DEATH RECEPTOR LIGATION AND SIGNAL TRANSDUCTION INHIBITION IN THE KILLING OF LEUKAEMIA CELLS BY CYTOTOXIC DRUGS AND RADIATION.

by
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Abstract

In order to establish effective therapies for leukaemia, it is crucial to understand and exploit mechanisms of apoptosis regulation involving death receptors and other signalling mechanisms.

In some leukaemia cell lines, cytotoxic drugs induce expression of the death receptors (DRs) Fas or DR5, which may contribute to apoptosis via ligation by Fas ligand (Fas-L) or TRAIL respectively. Although Fas mRNA expression in B-CLL cells was elevated following cytotoxic treatments, only γ-irradiation increased Fas protein levels. AML cells expressed Fas protein with no change being observed following treatment. None of the treatments induced Fas-L expression. An agonistic anti-Fas monoclonal antibody (CH-11) induced apoptosis in AML cells only, with no synergistic apoptosis in combination with cytotoxic agents. AML, but not CLL, cells were sensitive to TRAIL, with synergistic apoptosis in combination with cytotoxic drugs. Pro-caspase-8 processing in B-CLL and AML cells was induced by all of the cytotoxic stimuli even in the absence of ligation of Fas or DRs. The data suggests that the Fas and DR signalling system does not play a major role in the induction of apoptosis in B-CLL cells treated with cytotoxic agents. However, the apoptotic synergy observed with TRAIL and daunorubicin (DNR) in AML cells may have clinical significance.

Despite the extended survival of B-CLL cells in-vivo, cells cultured in-vitro die rapidly by spontaneous apoptosis. The data here show that components of autologous plasma contribute to basal survival of B-CLL cells, and protect these cells from killing by the ansamycin antibiotic herbimycin A (HMA), the PI3-K inhibitor LY29400 or chlorambucil (Chl). Co-treatment of B-CLL cells with Chl and either HMA or LY294002 reduced the Chl LD_{50} (lethal dose, 50% viability) significantly. The data suggest that plasma activation of the PI3-K/Akt pathway may abrogate p53 upregulation after Chl mediated DNA damage. Therefore, strategies designed to inhibit plasma-induced anti-apoptotic signalling are likely to be of value in the design of novel therapeutic protocols.
**Declaration**

The work contained in this thesis is the result of original research carried out by myself under the supervision of Dr. R.G. Wickremasinghe and Dr. K. Ganeshaguru. All sources of information have been specifically acknowledged by means of reference. None of the work contained in this thesis has been used in any previous application for a degree.
Acknowledgements

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<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>ALL</td>
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<td>AML</td>
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<td>Apaf-1</td>
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<tr>
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<td>French American British</td>
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<td>FACS</td>
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</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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<td>Fas-L</td>
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<td>Abbreviation</td>
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<td>FCS</td>
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<td>HEPES</td>
<td>N-2-hydroxyethylpipеразин-N'-2-этилсульфоничный кислота</td>
</tr>
<tr>
<td>HMA</td>
<td>Herbimycin A</td>
</tr>
<tr>
<td>hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleuki-1-β converting enzyme</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin growth factor-1</td>
</tr>
<tr>
<td>IGF-BP3</td>
<td>Insulin growth factor-protein binding 3</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of κB kinases</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinases</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>JNKK</td>
<td>JNK kinase</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LD_{50}</td>
<td>Lethal dose, 50% viability</td>
</tr>
<tr>
<td>LRP</td>
<td>Lung resistance protein</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Multiple murine double minute gene 2</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multidrug resistance-1</td>
</tr>
<tr>
<td>ME4o</td>
<td>Myelomonocytic with eosinophilia</td>
</tr>
<tr>
<td>MedCF</td>
<td>Median cell fluorescence</td>
</tr>
<tr>
<td>MEKK1</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukaemia virus reverse transcriptase</td>
</tr>
<tr>
<td>MOPS</td>
<td>N-morpholino propanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NAIP</td>
<td>Neuronal apoptosis inhibitory protein</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear-export signal</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase complex</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OKA</td>
<td>Okadaic acid</td>
</tr>
<tr>
<td>P1, P2</td>
<td>Promotor 1, promotor 2</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-ADP-ribose-polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDK</td>
<td>Phosphatidylinositol-dependent kinase</td>
</tr>
<tr>
<td>PEST</td>
<td>proline (P), glutamic acid (E), serine (S) and threonine (T) polypeptide sequence</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>3'-PIP</td>
<td>3'-phosphatidylinositol phosphates</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB/Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLAD</td>
<td>Pre-ligand-binding domain</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukaemia</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PP</td>
<td>Phosphoprotein phosphatase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted from chromosome 10</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTP</td>
<td>Permeability transition pore</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RARα</td>
<td>Retinoic acid receptor α</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SADS</td>
<td>Small accelerator for death signalling</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>SHR</td>
<td>Steroid hormone receptors</td>
</tr>
<tr>
<td>SMA</td>
<td>Spinal muscular atrophy</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspase</td>
</tr>
<tr>
<td>SMMHC</td>
<td>Smooth muscle myosin heavy chain</td>
</tr>
<tr>
<td>SODD</td>
<td>Silencer of DD</td>
</tr>
<tr>
<td>Sp</td>
<td>Specific</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>SUMO1</td>
<td>Small Ubiquitin-related Modifier 1</td>
</tr>
<tr>
<td>TAF</td>
<td>TATA box binding protein associated factors</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td>tBid</td>
<td>Truncated Bid</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotide transferase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor- α</td>
</tr>
<tr>
<td>TNF-R</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TPCK</td>
<td>N-tosyl-l-phenylalanyl chloromethyl ketone</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF-R associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-R associated factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF related apoptosis inducing ligand</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquitin-conjugating enzymes</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v-Src</td>
<td>pp60&lt;sup&gt;v-src&lt;/sup&gt; kinase</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependent anion channel</td>
</tr>
<tr>
<td>wt</td>
<td>Wildtype</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
</tbody>
</table>
Chapter 1

General introduction
Apoptosis, a conserved mechanism of cell suicide, is responsible for the killing of cells by a wide variety of physiological and toxic stimuli and plays a central role in development, homeostasis and chemical or hormone induced cell death of metazoan cells. Apoptosis is critical to the health of many organisms, is needed to sculpt the nervous system during development and to maintain the normal functioning of the immune system. Because the physiological role of apoptosis is crucial, aberration of this process can be detrimental.

Failure of dividing cells to initiate apoptosis after sustaining DNA damage can contribute to cancer. Consequently, control of apoptosis has been recognised as an important target for therapeutic intervention, making elucidation of the molecular mechanisms regulating this process of primary interest. In contrast to apoptosis, necrotic cell death results from damage to cell membranes. This process involves a dramatic increase in cell volume, rupture of the plasma membrane and consequent release of denatured proteins and nucleic acids into the intracellular space, resulting in local inflammatory responses.

1.1 Mechanisms and cellular physiology of apoptotic cell death
Apoptosis can be triggered by a variety of mechanisms e.g. DNA damage, growth factor deprivation and ligation of the tumour necrosis factor receptor (TNF-R) family, and can be modulated by pro- and anti apoptotic proteins e.g. p53, Bcl-2, FADD and IAP homologues (Fig 1.1). Modulators are checkpoints in the apoptotic pathway that determine if the cell will continue towards apoptosis or be repaired depending on the extent of the damage and cell type.

Although different apoptotic stimuli initially activate distinct pathways leading to cell death, these pathways converge by activating a common execution mechanism involving the activation of a set of cysteine proteases, the caspases 3, 6 and 7, which are the effectors in apoptosis. The cleavage targets of effectors are restricted set of cellular substrates, some of which will lose their function whereas others acquire novel activities leading to
the demise of the cell. The net result is the morphologically distinct apoptotic cell, commencing with rearrangements of phospholipids in the cell membrane. In the early stages of apoptosis phosphatidylserine residues become exposed on the outer surface of the membrane. This change in pattern of phospholipids is important for recognition and removal of apoptotic cells by macrophages. The endoplasmic reticulum dilates and superficial cisternae fuse with the plasma membrane. The next stage of apoptosis involves loss of cell volume due to emission of water and ions through the endoplasmic reticulum and plasma membrane, and an increase in cell density due to cytoskeletal rearrangement. Apoptosis leads to nucleolytic cleavage of the chromosomal DNA into fragments of 50-300 kilobases (kb). The nucleus condenses and chromatin marginates to produce dense nuclear bodies under the intact nuclear membrane. Violent blebbing of the plasma membrane, further degradation of the chromosomal DNA and fragmentation of the nucleus results in generation of apoptotic bodies which are removed by phagocytosis by neighbouring cells without causing any inflammatory response in contrast to cells dying by necrosis.

Figure 1.1 A generalised description of common apoptotic pathway.
1.1.1 Proteins involved in apoptotic regulation in *Caenorhabditis elegans*

The study of the nematode *Caenorhabditis elegans* (C. elegans) has provided a valuable model for studying the core components of cell death machinery and how programmed cell death occurs during development. Apoptotic deaths in *C. elegans* are controlled by three gene products; CED-3 (cell death - 3) and CED-4 which are both pro-apoptotic proteins, and CED-9 which inhibit apoptosis. Mutations of the ced-3 and ced-4 genes block apoptosis in these cells. CED-3 exists as an inactive caspase zymogen, which becomes activated through self-cleavage. CED-4 binds to and promotes CED-3 activation, whereas CED-9 binds to CED-4 and inhibits it from activating CED-3. CED-9 is normally complexed with CED-4 and CED-3, keeping CED-3 inactive. Apoptosis stimulation induces CED-9 dissociation, allowing CED-3 activation and commitment of cell to apoptosis.

Vertebrates have evolved entire gene families that resemble *C. elegans* cell death genes. Mammalian caspases are similar to CED-3, and CED-4 has homology to mammalian Apaf-1 (apoptosis protease activating factor 1). The Bcl-2 family are related to CED-9, but include three sub groups of proteins that either inhibit or promote apoptosis.

1.1.2 Cysteine protease family-Caspases

Caspases (Cysteine Aspartate specific Proteases) are a family of cysteine containing proteases. So far at least 14 caspase family members have been identified. Caspases can be classified according to their amino acid sequence homology into caspase-1 and caspase-3 subfamilies. The caspase-1 subfamily, which includes caspases 1, 4, 5, 11, 12, and 13, are predominantly involved in activation of proinflammatory cytokines. However, recently caspases 1, 11 and 12 have also been shown to be involved in apoptosis. The caspase-3 subfamily, which includes caspases 2, 3, 6, 7, 8, 9 and 10, are predominantly involved in promoting apoptotic cell death. Apoptotic caspases can be divided further into two groups. Caspases that are directly responsible for proteolytic cleavages that lead to cell disassembly are called effectors, for example caspase-3, 6, 7 and 10. Caspases involved in upstream regulatory events are called initiators for example caspase-8 and 9 (Fig. 1.2).
Figure 1.2 Proposed caspase function and apoptotic caspase structure.

The functions of caspases have been tentatively assigned based on various studies. Pro-caspases are cleaved at specific Asp residues (Dn, where n is the position in the protein).

\textsuperscript{a}Exact cleavage site is not known; \textsuperscript{b}the cleavage site of caspase-3 may be at Asp-9 or Asp-28; \textsuperscript{c}caspase 9 can be cleaved at Asp-330 or Asp-315.\textsuperscript{19}

\textsuperscript{19}
1.1.2.1 Caspase structure

All caspases are expressed as pro-enzymes (30 to 55kDa) that contain three domains: an NH2 terminal pro-domain, a large subunit (approx. 20kDa), and a small subunit (approx. 10kDa) (Fig. 1.2). Activation requires a minimum of two proteolytic cleavages at the C-terminal side of aspartate residues, one separating the pro-domain from the large subunit and another separating the large and small subunits. Two large and two small subunits associate to form active heterotetramers. 

Two features of pro-caspase structure are central to the mechanism of activation. First, the NH2 terminal pro domain is involved in regulation of activation, and is highly variable in sequence and range in length from 23 amino acids for caspase-6 and 7 to 219 amino acids for caspase-10. Second, all domains are derived from the pro-enzymes by cleavage at caspase consensus sites. Upstream caspases (initiators) possess large N-terminal pro-domains, allowing them to interact with various proteins that trigger caspase activation. In contrast, downstream effector caspases contain only short N-terminal pro-domains. Although the pro-domains tend to be much more divergent than the catalytic domain, two related motifs have been found in this region. The death effector domain (DED) is found in caspase-8 and 10 and appears to be involved in interaction with DEDs of signalling adapter proteins such as FADD and TRADD. The caspase recruitment domain (CARD) is found in caspase-1, 2, 4, and 9 and appears to be important in promoting interactions of these caspases with one another and with a range of other regulatory and adapter proteins.

1.1.2.2 Mechanisms of caspase activation.

Three general mechanisms of caspase activation have been described. First, caspases can be activated by induced proximity of pro-caspases. Pro-caspases are not entirely inactive but possess weak protease activity. Caspase 8 is activated by localisation and increase in pro-enzyme concentration at the death receptor e.g. when Fas is activated (see section 1.2.1). The low intrinsic protease activity of pro-caspase-8 is sufficient to allow the pro-enzyme to mutually cleave and activate each other. The second and most complex activation mechanism of caspases involves association with a regulatory subunit. Activation of pro-caspase-9 involves its association with a dedicated protein cofactor, Apaf 1 in the presence of ATP or dATP, and release of cytochrome-c from the
mitochondria (see section 1.2.2). In the third mechanism, caspases can be activated by upstream caspases (caspase cascade) which is used extensively by cells for the activation of the three short pro-domain effector caspases 3, 6 and 7. These three downstream effector caspases are directly responsible for proteolytic cleavages that lead to cell disassembly. Studies in cell free systems and in transformed yeast cells have demonstrated that certain caspases are capable of efficient activation of other caspases. For example, caspase-8 cleaves pro-caspase-3, 4, 7, and 9 in-vitro. Likewise caspase-10 cleaves pro-caspase-3, 7, and 8 in-vitro. Finally caspase-9 can activate caspase-3 and 7, while caspase-3 can also activate caspase-6.

### 1.1.2.3 Regulation of caspases

Due to the potent effect of caspases, caspase production, processing and activity can be regulated by several different mechanisms.

Caspases can also be modified post-translationally. Protein phosphorylation is the most common cellular mechanism for post-translational regulation of protein function. Protein tyrosine kinases can either stimulate or inhibit apoptosis and promote cell survival. Protein kinase B (PKB, also called Akt) has been reported to be able to phosphorylate pro-caspase-9 when cells were exposed to an agonists that stimulate PI3-K (Phosphatidylinositol-3 kinase) (see section 1.5.1). Phosphorylation of pro-caspase-9 prevents its cleavage and activation. Other kinases that interact with caspases include RIP (receptor interacting protein) and RIP2, which are serine/threonine kinases. RIP plays a role in recruiting pro-caspase-2 to the TNF-R1 signalling complex, while RIP2/CARDIAK is a RIP-related protein that can associate with intracellular domains of Fas and TNF-R1, which results in recruitment and activation of pro-caspase-1 in-vitro. However, the association of RIP1 and RIP2 with caspases involves the CARD domain rather than their kinase domain, and whether RIP and RIP2 can actually phosphorylate caspases is unclear.

Complex proteolytic system involves a combination of regulatory proteases, cofactors, feedback, and thresholds that converge to control the activity of caspases. Activation of initiator caspases e.g. caspase-8 by death receptors can be regulated by levels of FADD and c-FLIP, while caspase-9 activation can be regulated by Apaf-1 levels.
apoptotic Bcl-2 family can also regulate caspase-9 activation by inhibiting cytochrome-c release from the mitochondria \(^46\). Downstream effector caspases can be inhibited by interaction with inhibitors of apoptosis proteins (IAPs) \(^47,48\).

### 1.1.2.4 Caspase substrates

Apoptotic events include DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly into membrane-enclosed fragments but the overall contribution of caspases to these processes is not fully understood. Close to 100 caspase substrates have been identified \(^18,19,49\). The strict specificity of caspases is consistent with the observation that apoptosis is not accompanied by indiscriminate protein cleavage, rather a select set of proteins is cleaved in a co-ordinated manner, usually at a single site, resulting in a loss or change of function \(^7\). Recognition of at least four amino acids NH2 terminal to the cleavage site is also necessary requirement for efficient catalysis. The preferred tetrapeptide recognition motif differs significantly among caspases and explains the diversity of their biological functions \(^50\). Tertiary structure of substrates may also influence recognition.

One of the first recognised and commonly cleaved substrates is the enzyme poly-ADP-ribose-polymerase (PARP, 116kDa) whose proteolytic cleavage to 24kDa and 89kDa fragments during apoptosis is one of the first detectable events \(^51\). PARP activity plays a role in DNA repair and possibly also in DNA replication, cell proliferation, and differentiation, so the cleavage of this enzyme interferes with its function. PARP is cleaved by caspase-3 and caspase-7 \(^52-54\). The nuclease responsible for DNA fragmentation is activated by caspases - caspase activated DNase (CAD), a DNA ladder nuclease pre-exists in living cells as an inactive complex with an inhibitory subunit called ICAD. Activation of CAD occurs by cleavage of ICAD by caspase-3, resulting in the release and activation of the catalytic subunit \(^55,56\). Lamin, the major structural proteins of the nuclear envelope is cleaved by caspase-6, and may be responsible for some of the observed nuclear changes \(^57,58\).

### 1.1.3 The Bcl-2 protein family

Bcl-2, which was the first Bcl-2 family of proteins to be recognised, was identified as a proto-oncogene in follicular B-cell lymphoma. The Bcl-2 gene was found at the
breakpoint of the translocation between chromosome 18 and chromosome 14, where the Bcl-2 gene is placed under the control of the immunoglobulin (Ig) heavy chain enhancer. Genetic analysis revealed that Bcl-2 is a mammalian homologue to the apoptosis repressor ced-9 of *C. elegans*.

Bcl-2 was initially shown to inhibit cell death induced by IL-3 deprivation and subsequently shown to inhibit cell death induced by various other stimuli including chemotherapeutic agents and heat shock. It is now established that Bcl-2 prevents most forms of apoptotic cell death.

The growing Bcl-2 family comprises over a dozen proteins identified in both mammalian cells and viruses. Sequence homology within the family is confined to four specific regions called the Bcl-2 homology (BH) domains. The Bcl-2 family has been divided into three functional groups (Fig. 1.3). The first group consists of anti-apoptotic Bcl-2 members such as Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1. They share sequence homology in the BH1 through BH4 domains, although some members lack an apparent BH4 domain. In contrast, group II consists of Bcl-2 family members with pro-apoptotic properties and includes Bax (Bcl-2 associated protein X), the first pro-apoptotic member of the family to be identified, Bak and Bok, which share sequence homology in BH1, BH2 and BH3 but not in BH4. Finally, pro-apoptotic group III Bcl-2 proteins include Bad, Bik, Bid, Bim, Hrk, Blk and Bnip3. These have only a 9-16 amino acid BH3 domain. Some of the Bcl-2 members contain a C-terminal hydrophobic tail, which localises the proteins to the outer surface of the mitochondria membrane (and occasionally the endoplasmic reticulum) with the bulk of the protein facing the cytosol.

The properties of some key Bcl-2 family members will now be described.

### 1.1.3.1 Bcl-2

The Bcl-2 protein is a 26 kDa membrane-associated protein with a hydrophobic carboxyl terminal transmembrane domain. Bcl-2 has been reported to be exclusively localised to membranes such as the endoplasmic reticulum, mitochondrial membranes, and the nuclear envelope.
Figure 1.3 The three Bcl-2 subfamilies from Adams et al. All Bcl-2 proteins shown are mammalian, except for NR-13 (chicken), CED-9, and EGL-1 (C. elegans), and the viral proteins BHRF1, LMW5-HL, ORF16, KS-Bcl-2 and E1B-19K.
Unlike previously described oncogenes, Bcl-2 protected cells from death induced by growth factor deprivation. In haemopoietic cells, Bcl-2 protects against cell death induced by withdrawal of IL-2, IL-3, IL-4, IL-7 or GM-CSF (granulocyte macrophage colony stimulating factor). Over-expression of c-myc in serum starved fibroblasts leads to apoptosis which can be prevented by Bcl-2. Bcl-2 can also protect cells from apoptosis induced by γ-irradiation, the tumour suppressor p53 or cytotoxic drugs such as etoposide and camptothecin.

However, Bcl-2 does not always block apoptosis. Bcl-2 does not protect against cell death induced by cytotoxic T lymphocytes and does not block apoptosis during negative selection of thymocytes. Since Bcl-2 does not block every form of apoptosis, alternative apoptosis inhibitors such as other members of the Bcl-2 family are involved.

To date the Bcl-2 protein has been shown to interact with at least 13 different proteins including Bax, Bak, Bid, Bad, Raf-1, calcineurin, ced-4, Bag-1, R-Ras, H-Ras, p53-BP2, SMN and the prion protein pr-1.

1.1.3.2 Bcl-X

The Bcl-X gene was discovered using low stringency hybridisation with a Bcl-2 complementary DNA (cDNA) probe. The Bcl-X protein shows 44% amino acid homology with Bcl-2. The Bcl-X primary gene transcript is processed to produce two mRNA. The larger form, Bcl-XL, encodes a 233 amino acid polypeptide. The smaller cDNA, Bcl-XS lacks the domains of high homology to Bcl-2. The difference between these two cDNAs is a consequence of differential usage of two 5' splicing sites within the first coding exon. Like Bcl-2, Bcl-X has a hydrophobic carboxy terminal domain and shows a similar subcellular distribution. Bcl-X is strongly expressed in lymphoid tissues and in the nervous system. Over expression of Bcl-XL in an IL-3 dependent murine pro B-lymphocytic FL5.12 cell line inhibited apoptosis on removal of growth factor. In contrast, transfection of Bcl-XS into the same cell line prevented the block of apoptosis induced by Bcl-XL over expression. Thymocytes from mice expressing a Bcl-XL transgene targeted to T-cells show enhanced survival and are protected against γ-irradiation, glucocorticoid and anti-CD3 induced apoptosis. In addition, introduction of a Bcl-XL transgene rescued mature T-cells in Bcl-2 null mice.
1.1.3.3 Bax

Bax is a 21kDa protein, which was identified by its co-immunoprecipitation with Bcl-2. Like Bcl-2, Bax protein contains a hydrophobic carboxy terminus and shares homology with Bcl-2 in two conserved regions, BH1 and BH2. Bax can heterodimerize with Bcl-2 or homodimerize with itself. Site specific mutagenesis of Bcl-2 indicates that Gly 145 in the BH1 domain and Trp 188 in the BH2 domain are important for dimerization with Bax. When these residues were mutated and binding of Bax was disrupted, Bcl-2 was unable to inhibit apoptosis suggesting that Bcl-2 must bind to Bax to repress apoptosis. Bax is an apoptosis promoter since over-expression accelerates apoptosis in response to a death signal. The ratio of Bcl-2 to Bax determines the amount of Bcl-2/Bax heterodimers versus Bax/Bax homodimers and is important in determining the susceptibility of a cell to apoptotic stimuli. Bax shows a wide pattern of expression and is frequently highly expressed in tissues characterised by high apoptotic death rates during maturation, such as germinal centre lymphocytes.

1.1.3.4 Bad

Bad was identified by yeast two-hybrid screening and expression cloning. Unlike Bcl-2, Bad lacks the characteristic carboxy terminal hydrophobic tail and that its homologous regions are limited to a few highly conserved amino acids in BH1, BH2 and BH3 domain. Bad dimerizes selectively with Bcl-XL as well as Bcl-2, but does not interact with Bax, Bcl-XS, Mcl-1, A1 or itself. In the FL5.12 cell line, Bad was shown to bind strongly to Bcl-XL, displacing Bax and reducing the death repressing activity of Bcl-XL. It did not however affect the ability of Bcl-2 to inhibit apoptosis.

1.1.3.5 Bid

Bid, a 22kDa BH3 domain pro-apoptotic Bcl-2 family member, was identified using interactive cloning techniques. Bid is a specific proximal substrate of caspase-8 in the Fas type II apoptotic signalling pathway that is cleaved into an active pro-apoptotic 15kDa truncated Bid (tBid). While full-length Bid is localised in cytosol, tBid translocates to the mitochondria and induces mitochondria induced apoptosis. Co-expression of Bcl-XL inhibits all the apoptotic changes induced by tBid. Most Bid-deficient mice were shown to be resistant against injection with an antibody directed against Fas and survived, whereas wild-type mice died from hepatocellular apoptosis and haemorrhagic necrosis.
In half of the Bid-deficient mice, no liver injury and activation of effector caspases 3 and 7 was observed. However, the initiator caspase-8 was activated. Therefore, Bid is a critical substrate for signalling by death-receptor agonists, which mediates a mitochondrial damage induced by caspase-8.

1.1.3.6 Bak
Bak (Bcl-2 homologous antagonist killer) was discovered using degenerate polymerase chain reaction (PCR) cloning or through interaction with the adenovirus E1B 19K protein. Bak is expressed in a wide variety of tissues and was shown to bind strongly to Bcl-XL in yeast. Over-expression of Bak by microinjection into growth factor-deprived sympathetic neurons promotes cell death and inhibits the anti-apoptotic effect of co-injected E1B 19K. Transfection of serum-deprived fibroblast with Bak induces extensive apoptosis.

1.1.3.7 Other Bcl-2 family proteins
Mcl-1 has homology to Bcl-2 at their carboxy terminal, BH1 and BH2 domains. However Mcl-1 also contains a sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST) in its amino terminal portion which allows it to be degraded via the ubiquitin/proteasome pathway. Mcl-1 can bind to Bax, and protect cells from apoptosis. A1 is expressed in the T lineage but not in B-cell or erythroid lineages, and over-expression of A1 has been shown to inhibit apoptosis. Bag-1 has no significant homology to Bcl-2 family members, but binds Bcl-2 in-vitro. Bag-1 increases the anti-apoptotic effect of Bcl-2 in response to a variety of apoptotic stimuli in Jurkat human lymphoid cells. Bik is a pro-apoptotic protein that has very little homology to Bcl-2. Bik interacts with Bcl-2, Bcl-XL, or BHFR1. Viral proteins BHFR1, E1A and E1B are also Bcl-2 like proteins. BHFR1 protein is encoded by Epstein-Barr virus, a human herpes virus that persists in the B-lymphoid system. BHFR1 over-expression protects cells from apoptosis. E1A and E1B proteins are encoded by adenovirus. The E1A proteins have been shown to induce apoptosis, while the E1B proteins can suppress it. The 19kDa E1B protein interacts with Bak and Bik, while the 55kDa E1B protein suppresses apoptosis via interaction with the p53 a tumour suppressor protein.
1.1.3.8 Apoptosis regulation by the Bcl-2 family

Despite the extensive structural data on Bcl-2 family members, little is known about their mechanism of action. Pro and anti-apoptotic Bcl-2 proteins can heterodimerize and seemingly titrate one another’s function, suggesting that their relative concentration may act as a “rheostat” controlling the suicide program. Heterodimerization is not required for the pro-survival function but is required for pro-apoptotic function. Heterodimerization is mediated by the insertion of an α-helix within the BH3 region of a pro-apoptotic Bcl-2 protein into a elongated hydrophobic cleft composed of α-helices BH1, BH2 and BH3 of an anti-apoptotic Bcl-2 protein. BH3 cleft coupling, may account for all dimerization within the family. The BH1 and BH2 domains are required for Bcl-2 and Bcl-X(L) to dimerize with Bax to suppress apoptosis. However, mutations in Bcl-X(L) have been described which prevent heterodimerization with Bax or Bak but still maintain anti-apoptotic activity, suggesting that the anti-apoptotic proteins can also function independently to regulate cell survival. In addition to BH1 and BH2, the BH4 domain is required for anti-apoptotic activity. This domain has been shown to bind to several proteins including Raf-1, ced-4, and calcineurin. Mutants of Bcl-2 lacking the BH4 domain not only lose their anti-apoptotic activity but behave like killer proteins. In contrast, BH3 is both essential and sufficient for pro-apoptotic activity.

Both phosphorylation and proteolysis regulate the activity of some Bcl-2 family members. Bcl-2 can undergo phosphorylation in the immature B-cell line WEHI-231 when exposed to anti-IgM. In these cells Bcl-2 was ineffective at suppressing anti-IgM mediated apoptosis. However, deletion of the flexible loop in the Bcl-2 protein blocked phosphorylation and apoptosis. Bcl-2 phosphorylation has also been shown in several tumour cell lines following exposure to Taxol. The kinase responsible for Bcl-2 phosphorylation has not been identified. However, JNK (c-Jun N-terminal kinase) is a good candidate as it is activated by several stimuli known to lead to cell death. Mutation of phosphorylation sites Ser70 and 87, and Thr 56 and 74 in the predicted loop region of Bcl-2 to alanine residues decreased JNK phosphorylation. Bcl-2 can also be proteolytically cleaved to a Bax like death effector. Inhibitor studies show that cleavage of Bcl-2 may contribute to amplification of the caspase cascade by further activating downstream caspases.
The Bad protein can be phosphorylated by Akt both in-vitro and in-vivo when cells were exposed to agonists that stimulate PI3-K \(^{106-108}\). Exposure of cells to cell survival factors like IL-3, PDGF (platelet-derived growth factor), or NGF (nerve growth factor) resulted in a rapid phosphorylation of Bad at site Serine 136, which was reversed upon growth factor withdrawal. Phosphorylation of Bad renders it unable to bind to Bcl-X\(_L\). Instead, phosphorylated Bad associates with 14-3-3, through a binding pocket for serine phosphorylated sequences \(^{109}\). The Bad 14-3-3 complex is found in the cytosol thus preventing Bad from interacting with Bcl-X\(_L\) at the mitochondria, therefore inhibiting apoptosis. Transfecting cells with mutant Bad whose serine 136 residues were substituted resulted in failure to bind to 14-3-3 protein, and increased apoptosis upon IL-3 withdrawal. This suggests that one mechanism by which growth factors promote survival is through phosphorylation of Bad, resulting in sequestration by 14-3-3 and the consequent freeing of Bcl-X\(_L\) to exert its anti-apoptotic effect. In contrast, growth factor withdrawal may promote apoptosis by dephosphorylating Bad, either through natural decay or via the action of a phosphatase e.g. calcineurin, allowing it to dimerize with Bcl-X\(_L\) \(^{106}\).

In addition to Bid and Bad, Bim, Bcl-X\(_L\) and Bax, are mainly localised in the cytoplasm in living cells even though they contain the C-terminal hydrophobic domain \(^{110-113}\). When apoptosis is induced, Bax can translocate to the outer mitochondria membrane to exert pro-apoptotic activity during apoptosis. Bax translocation to the mitochondria involves homodimerization. The full-length Bax protein is more efficient than the C-terminal truncated protein in triggering mitochondrial apoptosis. Therefore the hydrophobic C-terminal might facilitate mitochondrial localisation \(^{114}\).

### 1.2 Apoptotic pathways.

There are two main pathways that lead to activation of effector caspases and apoptosis. The first apoptotic pathway involves death receptors - cell surface receptors that transmit apoptotic signals initiated by specific “death ligands” – and plays a central role in instructive apoptosis and is especially important in the immune system \(^{115-117}\). These receptors can activate death via caspases within seconds of ligand binding, causing the apoptotic demise of the cell within hours. Death inducing receptors belong to the tumour
necrosis factor receptor (TNF-R) family\(^{115\text{-}117}\). The second pathway is triggered in response to stress conditions such as ultraviolet (UV) irradiation, cytotoxic drugs or growth factor deprivation, and proceeds through a poorly understood pathway involving mitochondrial cytochrome-c release, resulting in the activation of caspase-9 by a complex containing cytochrome-c and Apaf-1\(^{118\text{-}125}\).

1.2.1 The TNF and TNF-R families of ligands, receptors and regulators.

There are at least 21 and 17 known members of the TNF-R and TNF family respectively (Fig. 1.4). The TNF and TNF-R families are mainly involved in the development and function of the immune system and of cells of lymphoid origin\(^{126}\). For example, TNF plays a role in inflammation, and the lymphotoxin system controls the development of peripheral lymphoid organs and splenic architecture. OX40 and 4-1BB play a co-stimulatory role in T-cell proliferation and immunoglobulin isotype switching. A subset of TNF-R family members namely TNF-R1, Fas, TRAMP/DR3, TRAIL-R1/DR4, TRAIL-R2/DR5 and DR6 are involved in transducing death signals and are therefore referred as the ‘death receptors’. Death receptors (DR), contain one to five cysteine rich homologue repeats in their extracellular domain, and an intracellular sequence termed the “death domain” (DD)\(^{115}\). The DD is a protein interaction module consisting of compact bundle of six α-helices\(^{127}\). DDs bind each other, probably forming oligomers of unknown stoichiometry. The DD is essential for transducing apoptotic signal.

1.2.1.1 Fas and Fas-L

The best-characterised death receptor is Fas (also known as CD95 or Apo-1). Fas is a widely expressed glycosylated cell-surface molecule of relative molecular mass of 45,000-52,000 (335 amino acid residues). It is a type I transmembrane receptor, but alternative splicing can result in a soluble form, the function of which is unclear\(^{128}\). Fas mediated apoptosis is triggered by its natural ligand, Fas-L, which is a TNF-related type II transmembrane molecule\(^{129}\). Although Fas is expressed in many tissues, including liver, lung, thymus and heart, Fas-L expression is restricted to activated T-lymphocytes and certain cells of the testis and eye\(^{130\text{-}133}\). Fas and Fas-L play an important role mainly in three types of physiological apoptosis\(^{134}\): (i) Peripheral deletion of activated mature T-cells at the end of the immune response; (ii) killing of targets such as virus-infected cells or cancer cells by cytotoxic T and natural killer cells; and (iii) killing of inflammatory...
Figure 1.4 TNF and TNFR family members from Schneider P et al.\textsuperscript{115}.

Trimeric ligands are at the top of the figure, and receptors at the bottom with interactions indicated by arrows. Cysteine residues are represented as horizontal bars and basic structure modules (e.g. A1, A2, B1, B2) are shown as shaded areas in the extracellular domain of the TNFR family members, while death domains when present in the intracellular domain are represented by black boxes. The length of the intracellular domain is indicated by numbers. Some of ligands and receptors have different names: TRAIL/Apo-2L, TRAILR1/DR4, TRAILR2/DR5, TRAILR3/DcR1, TRAILR4/DcR2.
cells at “immune privileged” sites such as the eye. Evidence for the biological role of Fas comes from certain mouse models and from human patients who have defective genes for Fas or Fas-L. Such mutations can lead to accumulation of peripheral lymphoid cells and to a fatal autoimmune syndrome characterised by massive enlargement of lymph nodes.

Fas-L is a homotrimeric molecule that binds to three Fas molecules, resulting in trimerization and clustering of the receptor DDs. An adapter protein called Fas-associated protein with death domain (FADD, also known as Mort1) then binds through its own DD to the clustered receptor DD to form the DISC (Death inducing signalling complex). This DISC forms within seconds of receptor binding. FADD also contains a DED (death effector domain) that binds and recruits DED containing pro-caspase-8 (also called FLICE) into the DISC. Upon recruitment into the DISC, pro-caspase-8 oligomerization drives its activation through proteolytic self-cleavage and release of active sub-units (p18 and p10) into the cytosol. Caspase 8 activation can be enhanced by a new component of the Fas-mediated apoptosis, a novel 150-amino-acid protein called SADS (small accelerator for death signalling). SADS does not interact directly with Fas, but it interacts with the DED of FADD and pro-caspase-8, and enhances their interaction. Active caspase-8 cleaves various proteins in the cell including pro-caspase-3, which results in its activation. Caspase 3 cleaves more proteins within the cell, which results in the completion of apoptosis. Studies with FADD gene knockout mice and transgenic mice expressing a dominant negative mutant of FADD (FADD-DN) in T-cells have shown that FADD is essential for apoptosis induction by Fas.

Recently the Fas signalling pathways have been subdivided into two types. In type I cells, Fas induced cell death signal involves the caspase cascade initiated by activation of large amounts of caspase-8 at the DISC, which bypass the mitochondria leading to rapid direct activation of caspase-3 and other caspases. By contrast in type II cells, very little DISC is formed after Fas-L ligation, so the caspase cascade cannot be initiated directly but has to be amplified via the mitochondria. Small amounts of caspase-8 are activated in type II cells and cleave the Bcl-2 family member Bid. Cleaved Bid
inserts into the mitochondrial outer membrane and activates the release of pro-apoptotic molecules such as cytochrome-c and Smac/DIABLO from the mitochondrial intermembrane space. Cytochrome-c in the cytoplasm forms a complex called the apoptosome with Apaf-1 and pro-caspase-9, resulting in activation of large amounts of caspase-9. Caspase-9 activates downstream caspase-3 and other caspases, which in turn cleave critical substrates resulting in apoptosis. Over-expression of anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-X\textsubscript{L} can inhibit Fas induced apoptosis in type II cells but not in type I cells, suggesting that mitochondria are involved in type II cells but not essential in type I cells. A number of apoptosis-inhibiting or -inducing stimuli have been shown to only affect apoptosis in type II cells. These stimuli include activation of protein kinase C, which inhibits Fas-mediated apoptosis resulting in a delayed cleavage of BID, and the induction of apoptosis by the ceramide analog C(2)-ceramide. In addition, it has been shown that high Fas expressing cell line Boe(R) (Fas apoptosis-resistant type II cell line) can be sensitised by treatment with cycloheximide without affecting formation of the DISC. This places the effects of cycloheximide in the mitochondrial branch of the type II CD95 pathway, which suggests that strategies that interfere with Fas apoptosis signalling will depend on the cell type. In contrast the only cellular molecule that has been shown to inhibit both type I and type II cells and all receptor mediated apoptosis is c-FLIP (cellular FLICE inhibitor protein), because it acts directly at the DISC.

Recently, a new model of Fas signalling has been proposed involving extracellular pre-ligand-binding domains (PLADs) for Fas and TNF-R, which are supposed to aggregate the receptors before ligand binding. To prevent premature signalling of pre-associated receptors, which will result in premature apoptosis, intracellular receptor associated apoptosis blockers were postulated. It’s not entirely clear how ligand binding interferes with PLAD association and leads to receptor association, which initiates apoptosis. It is also unclear whether the DISC model and the PLAD model complement each other. More structural and biochemical work is needed to resolve these issues.

1.2.1.2 TNF-α and the TNF-R complex
Most of the TNF-α is produced by activated T-cells and macrophages in response to infection and inflammation. TNF-α is synthesised as a membrane-bound protein that
Figure 1.5 Model of the two Fas signalling pathways.
Figure 1.6 Proapoptotic and antiapoptotic signalling by TNF-R1
acts locally through cell to cell contact. Soluble TNF-α is released from the cell surface as a result of metalloproteinase cleavage. Like Fas-L, TNF-α is a homotrimeric molecule that binds to two types of TNF receptors to induce receptor trimerization, a 60kDa receptor TNF-R1 and a 80 kDa receptor TNF-R2. TNF-R2 does not have the intracellular DD to mediate apoptosis and participates only in a limited number of TNF responses, while TNF-R1 does have a DD and is therefore the major receptor responsible for mediating the apoptotic signals in some cell types. Paradoxically, ligation of TNF-R1 by TNF-α can simultaneously activate both pro- and anti-apoptotic signalling pathway (Fig. 1.6). TNF-R1 is rarely involved in mediating apoptosis unless protein synthesis is blocked, since many cell types are triggered to express cellular factors that can suppress the apoptotic stimulus generated by TNF-α. Instead, TNF-R1 is usually involved in activating the transcription factors Nuclear factor-kappa B (NF-κB) and JNK/AP-1, leading to induction of proinflammatory and immunomodulatory genes. Expression of apoptosis-suppressing proteins is also controlled through NF-κB and JNK/AP-1, as inhibition of either pathway sensitises cells to apoptosis induction by TNF-α.

For apoptosis to take place upon TNF-α activation, TNF-R1 recruits a 34 kDa intracellular adapter protein called TRADD (TNF-R associated death domain protein). TRADD functions as a platform adapter that recruits several signalling molecules to the activated receptor. TRADD contains a C-terminal DD that further recruits the DD containing protein FADD to form a TNF-R1 apoptotic signalling complex. The N-terminal DED of FADD recruits pro-caspase-8 or 10 via interaction with their DED. Upon recruitment, pro-caspase-8 (or 10) oligomerization drives its activation through proteolytic self-cleavage and release of active sub-units into the cytosol. Once activated the caspases further activate downstream executioner caspases such as caspase-3, 6 and 7, which finally leads to cleavage of death substrates and eventual apoptosis. However, TNF-R1 is not as potent as Fas in inducing apoptosis. Cells from FADD knockout mice are resistant to TNF-α induced apoptosis, demonstrating the importance of FADD in this role.
TNRF1 activation also initiates anti-apoptotic signalling via TRADD. TRADD recruits the adapter molecule TRAF2 (TNF-R associated factor 2, 56kDa) which results in JNK and NF-κB activation, while TNF-R2 activation recruits both TRAF1 and TRAF2 (45kDa)\(^{116}\). The critical role of TRAF2 in JNK activation has been shown in TRAF2 deficient cells from gene knockout mice where they demonstrated lack of JNK activation in response to TNF-α\(^{152}\). However in NF-κB activation, only a slight defect in NF-κB activation was demonstrated in response to TNF-α\(^{152}\). Therefore, TRAF2 may not be essential for NF-κB activation by TNF-α or there may be another TRAF family member that binds to TRADD\(^{152}\).

Recruitment of TRADD and FADD at the receptor site also promotes interaction with another DD containing protein called RIP (receptor interacting protein) through its C-terminal DD\(^{116}\). RIP contains serine-threonine kinase activity. However the role for RIP kinase in the activation of NF-κB or JNK/AP-1 is not yet known. In RIP deficient mice, NF-κB activation is absent in response to TNF-α activation, whereas JNK activation is intact\(^{153}\). Therefore, RIP is required to couple TNF-R1 to NF-κB, but is not crucial for coupling TNF-R1 to JNK. Both TRAF2 and RIP knockout mice have pathologies that cannot be ascribed to defects in TNF signalling, which suggests that these proteins have additional functions\(^{116}\). RIP has been shown to interact with other adapter proteins such as RAIDD\(^{154}\). RAIDD binds through a DD to the DD of RIP and through a CARD (caspase recruitment domain) motif to a similar sequence in caspase-2 in order to mediate apoptotic signals\(^{154}\). TRAF2 binds to cIAP1 (cellular inhibitor of apoptosis 1) and cIAP2 after recruitment to TRADD and TNF-α stimulation, which could help to delay or protect cells from apoptosis. However, the exact function of cIAPs in TNF-R1 signalling is still unresolved\(^{155}\).

TNF-R2 and RIP stimulate the pathway leading to activation of NF-κB by first activating NF-κB inducing kinase (NIK), which in turn activates the inhibitor of κB (I-κB) kinase complex (IKK)\(^{116}\). IKK phosphorylates IκB on serine 32 and 36, leading to IκB ubiquitination and degradation by the 26S proteosome thus removing its inhibitory effect on NF-κB. This allows NF-κB to migrate to the nucleus to activate transcription. NF-κB can upregulate TRAF1, TRAF2, cIAP1 and cIAP2 gene expression and thus block
caspase-8 and 3 activation\textsuperscript{156}. TRAFs can bind to TNF-R1 and prevent TNF-\(\alpha\) induced activation of caspase-8 at the receptor site, while cIAP1 and cIAP2 can inhibit the activation of effector caspase-3. Thus, NF-\(\kappa B\) upregulates a group of gene products that function co-operatively at the earliest checkpoint to suppress TNF-\(\alpha\)-mediated apoptosis. Thus, transcription and protein synthesis inhibitors appear to block NF-\(\kappa B\) induced survival signals and therefore shifts the balance towards TNF-R1 induced apoptosis.

JNK activation via TRAF2 and RIP involves a cascade of signals that includes the mitogen activated protein (MAP) kinases MEKK1 (AMP/Erk kinase kinase 1), JNKK (JNK kinase), and JNK\textsuperscript{116}. The importance of MEKK1 in JNK activation has been shown in kinase inactive MEKK1 mutants. In MEKK1 mutants JNK activation is blocked after TNF-\(\alpha\) binding to TNF-R1. However, MEKK1 does not bind to TRAF2 directly, which suggests that another TRAF2 binding kinase acts upstream of MEKK1\textsuperscript{116}.

Recently, a novel protein called SODD (silencer of DD) has been identified, and is believed to interact with the DD of TNF-R1 thus keeping it in a monomeric form\textsuperscript{157}. After TNF-\(\alpha\) binding, SODD disassociates, thus allowing TNF-R1 to trimerize and induce intracellular signalling.

Thus, TNF-R1 activation and signalling involves a complex series of interactions with intracellular proteins in order to induce apoptosis or cell survival.

\section*{1.2.1.3 Apoptotic signalling by TRAIL and death receptor 4 and 5}

TNF related apoptosis inducing ligand (TRAIL) or Apo2L, is a member of the TNF family and shows similarity to Fas-L\textsuperscript{158}. Like Fas-L, TRAIL triggers rapid apoptosis in many tumour cell lines\textsuperscript{159-162}. Unlike the expression of Fas-L, which is restricted mainly to activated T-cells and NK cells, and to immune privileged sites\textsuperscript{134}, TRAIL messenger RNA expression is constitutive in many tissues\textsuperscript{158,163}. However, like Fas-L, TRAIL transcription is elevated upon stimulation of peripheral blood T-cells\textsuperscript{160}. A subset of mature T-cells acquires sensitivity to TRAIL induced apoptosis after stimulation by IL-2, suggesting that TRAIL may play some role in peripheral T-cell deletion\textsuperscript{164}. In addition T-cells from human immunodeficiency virus-infected individuals show increased sensitivity to TRAIL, implicating this ligand in the killing of virus infected cells\textsuperscript{160}.
TRAIL can interact with two death receptors, DR4 (Death receptor 4, TRAIL-R1) and DR5 (death receptor 5, TRAIL-R2/TRICK2/KILLER), which contain cytoplasmic DDs, thereby triggering apoptotic signals via caspase-8 and the caspase cascade.\textsuperscript{165,166}

Surprisingly, ectopic expression of FADD-DN in amounts sufficient to block Fas induced apoptosis did not block apoptosis induction by TRAIL, which suggests that a FADD independent pathway links TRAIL to caspases.\textsuperscript{164} However, there are conflicting reports as to the involvement of FADD or other adapter proteins in TRAIL induced apoptosis. Some investigators have observed that FADD is essential for TRAIL induced apoptosis through both DR4 and DR5 \textsuperscript{166-170}. It has also been reported that both receptors can interact with TRADD and RIP, and activate NF-κB using TRADD-dependent pathway. Thus, both DR4 and DR5 may use FADD, TRADD, and RIP in their signal transduction pathways. However, other investigators have detected no such interactions and activation of the NF-κB pathway.\textsuperscript{165,171}

Unlike type II Fas induced apoptosis, TRAIL-induced apoptosis has been shown not to involve the mitochondrial pathway.\textsuperscript{172} Various Bcl-2- or Bcl-X\textsubscript{L} overexpressing tumour cell lines are sensitive to TRAIL-induced apoptosis but are resistant to chemotherapy induced apoptosis, which involves the mitochondria pathway.\textsuperscript{172} This indicates that TRAIL-induced apoptosis depends on caspase-8 activation rather than on the disruption of mitochondrial integrity. Thus, TRAIL may still kill tumours that have acquired resistance to chemotherapeutic drugs by over-expression of Bcl-2 or Bcl-X\textsubscript{L}. However, in a more recent publication, TRAIL induced apoptosis of human acute leukaemia cells was associated with the processing of caspase-8, Bid, followed by cytosolic accumulation of cytochrome-c and processing of caspase-9 and caspase-3.\textsuperscript{173} TRAIL induced apoptosis was significantly inhibited in HL60 cells that overexpressed Bcl-2 or Bcl-X\textsubscript{L}.\textsuperscript{173}

These disagreements could be due to differences in experimental methods and cell types. Because the interactions were observed in transfected cell lines it is possible that the abnormally high amounts of receptor, adapters and modulators led to promiscuous homophilic association between domains that do not normally interact. However, the data will influence the design of treatment strategies involving TRAIL.
Like the TRAIL mRNA, DR4 and DR5 transcripts are expressed in several tissues, suggesting that there may be a mechanism that protect cells from TRAIL induced apoptosis. Like in the Fas pathway, c-FLIP can inhibit TRAIL induced apoptosis. A second and novel type of protection involves decoy receptors (DcR), which compete with DR4 and DR5 for binding to TRAIL. Two DcRs have been characterised; DcR1 (also called TRID, TRAIL-R3, or LIT) and DcR2 (also called TRAIL-R4 or TRUNDD). DcR1 is a 299 amino acid protein with 58 and 54% overall homology to DR4 and DR5, respectively. In contrast to DR4 and DR5, this receptor shows restricted expression, with transcripts detectable only in peripheral blood lymphocytes and spleen. The structure of DcR1 is unique when compared to the other TRAIL receptors in that it lacks a cytoplasmic domain and appears to be glycosylphosphatidylinositol (GPI)-linked. Moreover, unlike DR4 and DR5, DcR1 does not induce apoptosis in a transient overexpression system. DcR1 transfections in TRAIL sensitive cell lines substantially reduces responsiveness to TRAIL induced apoptosis. Therefore, DcR1 functions as a decoy that prevents TRAIL from binding to DR4 or DR5 thus inhibiting apoptosis. Treatment of DcR1 bearing cells with a phospholipase that cleaves the GPI anchor results in marked sensitisation to TRAIL induced apoptosis.

DcR2 is a 386-amino acid protein with an extracellular domain with 58-70% homology to those of DR4, DR5, and DcR1. However, DcR2 has a substantially truncated cytoplasmic DD containing one third of a consensus DD motif. Four out of six amino acid positions that are critical for apoptosis induction are absent in the DcR2 death domain. DcR2 transfections in TRAIL sensitive cell lines substantially reduces responsiveness to TRAIL induced apoptosis, indicating that, like DcR1, this receptor acts as a decoy that prevents TRAIL from binding to DR4 or DR5. However, DcR2 has been shown in one study to activate NF-κB. Whether TRAIL itself stimulates NF-κB through DcR2 has not yet been investigated.

The genes encoding DR4, DR5, DcR1, and DcR2 map together to human chromosome 8p22-21, suggesting that they arose from a common ancestral gene.
Although the expression of DcR occasionally correlates with resistance to TRAIL induced apoptosis, resistance most frequently correlates with the levels of c-FLIP. A fifth receptor for TRAIL has recently been characterised called osteoprotegerin. Osteoprotegerin is a secreted soluble TNF-R homologue, which maps to the chromosome 8q23-24 and is not closely related to DR4, DR5, DcR1 or DcR2. Like the decoy receptors, osteoprotegerin binds to TRAIL and inhibits TRAIL induced apoptosis, but its binding affinity for TRAIL is slightly weaker than that of DR4 or DR5.

Several differences between TRAIL and - or Fas-L suggest that TRAIL may be a safer therapeutic agent. First, while the therapeutic usefulness of Fas-L or antagonistic antibody against Fas has been shown to be limited by hepatotoxicity in mice. By contrast, TRAIL is tumouricidal and non-toxic in mice. Second, NF-κB activation by TNF-R1 with high TNF-α doses has been shown to induce proinflammatory genes in macrophages and endothelial cells which causes a severe inflammatory response syndrome. Although DR4 and DR5 can activate NF-κB upon over-expression, TRAIL itself induces this response only weakly. Finally, normal cells express both DR and DcR at similar levels, while many tumour cell lines express more DR than DcR, rendering them more susceptible to killing by TRAIL while normal cells are protected. Unlike chemotherapeutic agents or radiation therapy, death receptors initiate apoptosis independently of the p53 tumour suppressor gene, which is inactivated by mutation in more than 50% of human cancers. Therefore, the idea of targeting specific death receptors to induce apoptosis in cancer is attractive.

1.2.1.4 FLIPs (FLICE- Inhibitory proteins)

Viruses have evolved many distinct strategies to protect host cells from the apoptotic response. Certain classes of γ-herpes virus (including Kaposi’s sarcoma-associated human herpes virus-8) and the tumourogenic human molluscipoxvirus encode an apoptotic-inhibitory protein designated vFLIP. These proteins contain two DEDs, a structure resembling the N-terminal half of caspase-8 that bind to the Fas-FADD complex (DISC). In transfected cells, vFLIP inhibits the recruitment and activation of caspase-8 at the DISC and therefore inhibits apoptosis induced by several death-inducing
receptors (Fas, TNF-R1, DR3, DR4). Recently a cellular homologue of v-FLIP has been identified by several groups and termed c-FLIP (also called CASH, Casper, CLARP, FLAME, I-FLICE, MRIT, and Usurpin). At the mRNA level, c-FLIP seems to exist as multiple splice variants, but on the protein level only two endogenous forms, c-FLIP$_{long}$ (55kDa) and c-FLIP$_{short}$ (27/28 kDa) could be detected. These proteins are predominantly expressed in muscle and lymphoid tissues. c-FLIP$_{short}$ contains two death effector domains and is structurally related to the vFLIP, whereas the c-FLIP$_{long}$ contains an additional caspase-8-like domain in which the active-centre cysteine residue, which is important for caspase-8 catalytic activity, is substituted by a tyrosine residue.

Although it is widely assumed that c-FLIPs block apoptosis, some reports have controversially suggested that overexpressed c-FLIP can either inhibit or activate apoptosis. High expression of DED containing molecules has been shown to induce apoptosis by aggregation and formation of non-physiological death effector filaments, leading to activation of apoptosis. This may be the reason why several groups found a pro-apoptotic function of c-FLIP in transient over-expression experiments. No stable expression clone has been described in which c-FLIP has a pro-apoptotic effect. Recent experiments with cells from c-FLIP deficient mice support the role of c-FLIP as an anti-apoptotic molecule. In addition, whether c-FLIP interacts with FADD and/or caspase-8 is not clear. Some groups have reported that c-FLIP interacts with both FADD and caspase-8, while others could only detect an interaction between c-FLIP and caspase-8. A recent study has shown that the mechanism in which c-FLIP inhibits apoptosis involves blocking the recruitment of caspase-8 to the DISC. After initial cleavage of both c-FLIP to a p43 fragment and caspase-8 to its intermediate cleavage product p43/p41, the cleavage intermediates remain bound to the receptor and can no longer be replaced by pro-caspase-8. This leads to inhibition of caspase-8 activation and renders the cell resistant to Fas induced apoptosis. Another recent study has demonstrated activation of caspase-8 downstream of mitochondria induced by cytotoxic agents is not inhibited by over-expression of c-FLIP, therefore c-FLIP preferentially suppress apoptosis mediated by death receptors by blocking recruitment and activation of pro-caspase-8 at the DISC.
1.2.1.5 Modulation of Death Receptors and c-FLIP.

As insight is gained into the molecular regulation of DR signalling, strategies to sensitise TRAIL- or Fas-L-resistant tumour cells may emerge. One such approach may be to upregulate the expression or function of Fas, Fas-L, DR or TRAIL. Chemotherapeutic agents or γ-irradiation activate p53, and the genes shown to be transcriptionally induced by p53 in tumour cell lines include DR5, Fas and Fas-L\(^{198-200}\). This raises the possibility that administration of cytotoxic agents that activate p53 in combination with Fas, Fas-L, DR or TRAIL may lead to more efficient regression of cancers with a normal p53 pathway. A second strategy may be to modulate the function or expression of c-FLIP, which inhibits caspase-8 and caspase-10 activation in Fas-L and TRAIL induced apoptosis\(^ {197;201;202}\). Fas- and c-FLIP-expressing monocyte-derived dendritic cells resistant to Fas-induced apoptosis became Fas sensitive after treatment with bisindolylmaleimide, a protein kinase C inhibitor (PKC), by downregulating c-FLIP expression\(^ {202}\).

1.2.2 The mitochondrial apoptotic pathway

1.2.2.1 Regulated release of cytochrome-c from the mitochondria

Mitochondria are not only involved in production of ATP for cell survival, but are also involved in the regulation of apoptosis. Mitochondria contain a potent cocktail of pro-apoptotic proteins that are released during cellular stress. One of these pro-apoptotic proteins is cytochrome-c, which is localised in the intermembrane space of the mitochondria. In addition to its involvement in mitochondrial electron transport, oxidative phosphorylation, and ATP production, cytochrome-c is one of the components required for the activation of caspase-9 in the cytosol\(^ {27;46;118;125;145;203-206}\). Exactly how cytochrome-c crosses the mitochondrial outer membrane is not yet known, but regulation of cytochrome-c release is thought to involve the Bcl-2 family. The addition of pro-apoptotic Bcl-2 family members to isolated mitochondria is sufficient to induce cytochrome-c release, whereas over-expression of anti-apoptotic Bcl-2 family members will prevent it\(^ {14;121;125;207-209}\). There are several hypotheses on how the Bcl-2 family regulate cytochrome-c release from the mitochondria\(^ {14;145;210;211}\). The three main models include channel formation by the Bcl-2 family, formation of channels by interaction of Bcl-2 members with mitochondrial proteins and physical rupturing of the mitochondrial membrane as a consequence of Bcl-2 family – mitochondria regulated metabolic control, which results in mitochondrial swelling.
The first model, channel formation by the Bcl-2 family members, is based on the structural similarity of Bcl-X_L to the pore forming domains of certain bacterial toxins, in particular diphtheria toxin and the colcins A and E1\textsuperscript{212}. The supposed function of the pore domain of diphtheria toxin is to allow the ADP-ribosylating subunit of the toxin to transit from the interior of the lysosomes into the cytosol. Based on this structural similarity, it has been suggested that following conformational change, Bcl-2 proteins might insert into the outer mitochondrial membrane where they could form channels or even large pores to allow cytochrome-c release into the cytosol.\textsuperscript{72} Some Bcl-2 family members such as Bcl-2, Bcl-X_L, and Bax, can oligomerize, and form ion channels with discrete conductance states in synthetic lipid membranes.\textsuperscript{145,213,214} It has also been shown that Bid which has similar structure to Bcl-X_L in solution, creates an ion channel in synthetic lipid membranes\textsuperscript{212,215-217}. Moreover, when Bax is expressed in yeast, cytochrome-c is released from mitochondria inducing apoptosis\textsuperscript{218}. However, it still remains to be determined whether Bcl-2 family proteins actually form ion channels \textit{in-vivo} and whether such channels would ever be big enough for proteins to pass through.

According to the second model, Bcl-2 family members interact with several mitochondrial proteins to form channels\textsuperscript{145}. It is possible that pro-apoptotic Bcl-2 family members recruit mitochondrial outer membrane proteins into forming a large pore channel. An example of such a protein is the voltage dependent anion channel (VDAC), as several Bcl-2 family members have been shown to bind to it and regulate its channel activity\textsuperscript{219}. The VDAC channel is too small to allow proteins to pass through, therefore it is possible that VDAC undergoes a significant conformational change upon binding to a Bcl-2 family member.

The final model is the rupturing of the outer mitochondrial membrane by Bcl-2 family members. In this model, apoptotic signals alter mitochondrial physiology, for example, ion exchange or oxidative phosphorylation, resulting in the swelling and rupturing of the outer membrane thus releasing intermembrane proteins such as cytochrome-c into the cytosol. Alteration in mitochondrial physiology can be induced directly by the Bcl-2 family members or indirectly, through modulation of other mitochondrial proteins such as VDAC. The VDAC protein is a subunit of the mitochondrial permeability transition pore (PTP), a large channel whose opening results in rapid loss of membrane potential and
organelle swelling. Opening the PTP quickly leads to cytochrome-c release and apoptosis. However, release of cytochrome-c also occurs in the absence of loss in membrane potential, therefore PTP cannot be the sole target of the Bcl-2 family proteins.

1.2.2.2 Cytochrome-c and regulation of Apaf-1 activity

The Apaf-1 protein, a mammalian counterpart of C. elegens CED-4, is normally localised in an inactive conformation in the cytosol. Apaf-1 contain a caspase recruitment domain (CARD) at the N-terminus and a 12WD40 repeat on the C-terminus. Apaf-1 can only bind to pro-caspase-9 by homophilic interaction involving CARD motifs after cytochrome-c binds to the WD repeats to induce a conformational change in the presence of dATP or ATP. Therefore, Apaf-1 is dormant until cytochrome-c prompts it into action. The large complex formed by cytochrome-c and ATP dependent oligomerization of Apaf-1 and recruitment of pro-caspase-9 is called the apoptosome. The apoptosome is a very large complex that might contain several additional proteins. Once assembled, pro-caspase-9 is autocatalysed from a single polypeptide zymogen to an active dimeric protease, which leads to pro-caspase-3 cleavage and apoptosis.

It has also been reported that unlike other caspases, proteolytic processing of pro-caspase-9 has only a minor effect on the enzyme's catalytic activity. Rather the key requirement for caspase-9 activation is its association with its dedicated protein cofactor, Apaf-1 in the apoptosome. Activation of caspase-9 is primarily mediated by means of conformational change rather than by proteolysis. Reconstitution studies with recombinant proteins have indicated that the size of the apoptosome is very large, in the order of approximately 1.4 MDa. Addition of ATP to cell lysates results in the formation of two large Apaf-1-containing apoptosome complexes with molecular weight of approximately 1.4 MDa and 700 kDa. Kinetic analysis demonstrates that in-vitro the small complex exhibits a much greater ability to activate effector caspases. Treatment of human tumour monocytic cells with either etoposide or N-tosyl-l-phenylalanyl chloromethyl ketone (TPCK) resulted in producing predominantly the small apoptosome complex, which processed effector caspases. Thus, the approximately 700 kDa apoptosome complex appears to be the correctly formed and biologically active apoptosome complex, which is assembled during apoptosis. Therefore Apaf-1 is not
simply a caspase-9 activator, but it is an essential regulatory subunit of caspase-9 apoptosome.

In summary, mitochondrial apoptosis leads to cytochrome-c release from the intermembrane space into the cytosol. In the presence of dATP/ATP, cytochrome-c associates with Apaf 1 and then pro-caspase-9 (and possibly other proteins) to form the apoptosome. In the apoptosome the dimerization of pro-caspase-9 leads to its activation either by conformational change or by autocatalysis to form an active dimeric protease. Active caspase-9 subsequently activates caspase-3 resulting in apoptosis.

1.2.2.3 Other pro-apoptotic proteins released from mitochondria

Cytochrome-c is not the only mitochondrial pro-apoptotic protein. Also present in the mitochondria are apoptosis inducing factor (AIF), Smac/DIABLO (second mitochondria-derived activator of caspase, also called direct IAP binding protein with low pI) and several pro-caspases e.g. pro-caspase-2,3 and 9 \(^{45;146;146;220;225}\).

AIF is a death effector, which is synthesised as a precursor in the cytosol and imported into the intermembrane space of the mitochondria. The precursor consist of three domains (i) an amino-terminal presequence which is removed upon import into the mitochondria; (ii) a spacer sequence of approximately 27 amino acids; and (iii) a carboxy-terminal 484 amino acid oxidoreductase domain. The function of AIF in the intermembrane cavity of the mitochondria is not yet known but it is presumed to be involved in redox reactions. Upon apoptosis induction, AIF translocates from the mitochondria to the nucleus before the release of cytochrome-c. AIF induces caspase-independent chromatin condensation and large-scale DNA fragmentation when added to purified nuclei \textit{in-vitro}. However, caspases are still required for cells to die by apoptosis. The early stages of chromatin condensation involve AIF, whereas later stages rely on caspase activation by cytochrome-c \(^{226;227}\).

Smac/DIABLO, is also released from the mitochondria into the cytosol during mitochondrial apoptosis \(^{146;228}\). Smac/DIABLO eliminates the inhibitory effects of IAPs (inhibitors of apoptosis) on caspases. Smac interacts with all IAPs that have been examined, including XIAP, c-IAP1, c-IAP2 and survivin \(^{146;228}\). In summary, release of multiple death-promoting molecules may facilitate swift and certain death of cells.
1.3 Inhibitors of Apoptosis (IAPs)

IAPs are a family of anti-apoptotic proteins that are conserved across evolution (Fig. 1.7), with homologues found in both vertebrate and invertebrate animal species. The baculovirus IAPs, Cp-IAP and Op-IAP, were the first members of this family to be identified based on their ability to functionally complement defects in the cell death inhibitor, p35, a baculovirus protein that binds to and inhibits caspases. The first human IAP to be identified was NAIP (neuronal apoptosis inhibitory protein). Deletions in naip are observed in many individuals with spinal muscular atrophy (SMA), consistent with the hypothesis that the motor neuron depletion characteristic of this disorder occurs by the failure to inhibit apoptosis. Subsequently, five human IAPs have been identified, XIAP, cIAP1, cIAP2, survivin, and BRUCE, and two Drosophila IAP homologues, all of which have been shown to inhibit cell death by inhibiting caspase cleavage or activity.

1.4 Life or death

In multicellular organisms, mutations in cells affecting critical genes that regulate cell survival and proliferation could lead to fatal cancers. Cells can repair damaged genes or undergo apoptosis to prevent formation of cancer cells. However, corruption of the cell machinery which is involved in regulating cell survival or repair greatly predisposes the cell to cancer.

In eukaryotic cells the strategy for dealing with damaged DNA can be divided into three phases. The first phase involves recognising the damaged DNA, the second phase involves assessing the damage by checkpoint proteins, and finally the third phase results in implementation of the appropriate response that will lead to upregulation and activation of DNA repair proteins. If the damage is too extensive for repair, pro-apoptotic proteins are upregulated, leading to cell death. These pathways are not linear, but pro-apoptotic or survival paths are activated simultaneously via multiple synchronous signals and are regulated by checkpoint proteins such as ATM (ataxia telangiectasia mutated) and p53, which decide the fate of cells.
BRUCE has not been demonstrated to block apoptosis and therefore does not yet qualify to be a member of the IAP family. Amino acid length is indicated to the right of each protein. The IAP proteins are based on human cDNA cloning results.  

**Figure 1.7** Structures of mammalian BIR domain-containing proteins.
1.4.1 The ATM protein kinase family

ATM is a protein kinase that belongs to a group of PI3-K related kinases that also includes DNA-PK (DNA dependent protein kinase) and ATR (ataxia telangiectasia Rad3 related). \(^{236}\) ATM encodes a 350 kDa protein with a C-terminal domain similar to the catalytic subunit of PI3-K and a DNA binding domain. ATM and DNA-PK can bind directly to free DNA ends, which then triggers kinase phosphorylation cascades to transmit damage signals to checkpoint proteins and repair proteins. Thus, ATM, ATR and DNA-PK act as checkpoint sensors and can trigger a plethora of cellular responses that include activation of cell cycle checkpoints and growth arrest, repair, and apoptosis. One of the target proteins of ATM, ATR and DNA-PK is the tumour suppressor p53 protein.

1.4.2 p53

p53 is a tumour suppressor protein that is normally maintained at low levels within the cell through its interaction with Mdm2, a protein that escorts p53 from the nucleus and targets it for protein degradation by 26S proteosome in the cytoplasm, thus ensuring that the p53 signal is carefully controlled. Like p53, Mdm2 can also be regulated by DNA-PK or ATM \(^{237}\). Phosphorylation of either p53 or Mdm2 by ATM family of proteins after DNA damage prevents the two proteins from interacting, thus stabilising and activating p53 \(^{237}\). The importance of p53 in tumour suppression has been shown in the high frequency of human cancer cells that lack p53 activity due to mutation, deletion, or inactivation by elevated levels of Mdm2. Some examples of haemopoietic malignancies with p53 mutations include erythroleukaemias, burkitts lymphoma, myeloid leukaemias and Hodgkin's disease \(^{238-241}\). Mdm2 is elevated in certain tumours, and is a target for p19\(^{ARF}\) an oncogene protein encoded by the alternative reading frame within the \textit{Ink4a} tumour suppressor gene locus—a site frequently deleted in human malignancies \(^{242}\).

1.4.2.1 p53 structure

The p53 gene is localised on the short arm of chromosome 17 (17p13), and has been conserved through evolution \(^{243-245}\). The human p53 protein consists of 393 amino acids and contains four major domains (Fig. 1.8). The N-terminus contains an acidic transcriptional activation domain (amino acids 1-42), a central sequence specific DNA
binding domain (amino acids 102-292), oligomerization domain (amino acids 323-356) and a C-terminal regulatory domain (amino acids 360-393). The N-terminal region also contains a proline rich region (amino acid 63-97) with striking similarity to SH3 binding proteins and has been shown to be required for p53 mediated apoptosis in some experimental systems, suppressing tumour cell growth \cite{246,247}.

N-terminal transcriptional activation domain allows p53 to recruit the basal transcriptional machinery that includes TATA box binding protein (TBP) and TBP associated factors (TAF), components of TFIIID \cite{237,248}. The transcriptional activating domain also interacts with DNA binding protein RP-A and transcription repair p62 subunit of TFIIH \cite{237,248}. Other proteins such as Mdm2, adenovirus E1B 55Kd, and hepatitis B virus X protein bind the N-terminal transcriptional activating domain and inhibit its transactivation function \cite{237,248}. The Mdm2 gene itself is activated by active p53 and serves as a negative feedback control for p53 activity \cite{248}. Amino acid residues 22 and 23 are involved in the binding of p53 to E1B and Mdm2 \cite{237,248}. The same amino acid residues plus amino acid residue 19 are required for transcriptional activation by the p53 protein \textit{in-vivo} and \textit{in-vitro} binding of the TATA-associated factors TAFII60 and TAFII40 \cite{237,248}.

The DNA binding domain of p53 binds a consensus target sequence \cite{237,248}. The structure of the core DNA binding domain consists of a large \(\beta\)-sandwich which is made up of two antiparallel \(\beta\)-sheets containing four and five \(\beta\)-strands, respectively. The large \(\beta\)-sandwich acts like a scaffold for three loop based elements. The scaffold anchors the loops and participates in head to tail dimerization. The first loop L1 (LSH – loop sheet helix) binds DNA within the major groove, L2 binds to DNA within the minor groove and finally L3 stabilises L1. A striking observation is that 80-90\% of p53 mutations are located in the DNA binding domain, and the majority of the mutations are found within the three loops.

The C-terminal region includes a tetramerization domain (amino acid 323-356) and regulatory domain (amino acid 363-392). Tetramers are the functional form of p53, and appear to be required for efficient transactivation \textit{in-vivo} and for p53 mediated suppression of growth in carcinoma cell lines \cite{237,248}. The regulatory domain acts as a
negative regulator of p53 sequence specific binding. In an allosteric model for regulation of p53 activity, an interaction between the C-terminal domain and another region in a p53 tetramer locks the tetramer in a DNA binding incompetent state. The activation of the p53 C-terminal domain via phosphorylation, acetylation, glycosylation or proteolytic removal are thought to activate p53 by inducing a conformational change in the protein.

1.4.2.2 N-terminal modification of p53

At the protein level the regulation of p53 is complex, and involves protein association, turnover and post-translational modification. p53 activation involves phosphorylation and acetylation of specific sites (Fig. 1.8). The first phosphorylation of human p53 to be demonstrated occurred at serine 15 in response to ionising radiation, DNA damaging drugs and UV. It has now been confirmed that in addition to serine 15, serines 20, 33, 37 and threonine 18 can also be phosphorylated. The serine 15 phosphorylation site is juxtaposed to the Mdm2 binding site (residues 18-23) and it has been proposed that phosphorylation of serines 15 and 37 can block p53 interaction with Mdm2 in-vitro, thus releasing p53 from the p53-Mdm2 complex that consequently block proteasome mediated p53 degradation. However other groups have found phosphorylation of serine 15 and 37 in-vitro has only a weak effect on Mdm2 binding, and the dependence of dissociation of the p53-Mdm2 complex in-vivo is uncertain.

The N-terminus of p53 can be phosphorylated by ATM, ATR or DNA-PK after DNA damage. ATM can only phosphorylate Ser15, whereas DNA-PK and ATR can phosphorylate both Ser15 and Ser37. The kinase responsible for phosphorylating serine 20 has not been identified yet, but this modification significantly weakens the interaction of p53 with Mdm2 in-vitro. Other proposed functions for N-terminal phosphorylation include Mdm2 independent stimulation of the transactivation function of p53, binding to the transcription factor p300/CBP (CREB-binding protein) and regulation of p53 interaction with the TFIID transcription factor.
Figure 1.8 Schematic representation of the structure of p53 protein. At the top are shown the viral and cellular proteins known to interact with particular region of p53. The middle represents the p53 functional domains. And the bottom shows the sites of phosphorylation and acetylation together with kinases and acetylases that have been identified.
Figure 1.9 Schematic representation of induction of p53 in response to DNA damage or dominant oncogene action.

Arrows at end of lines indicate stimulatory effects whereas bars indicate inhibitory effects.
1.4.2.3 C-terminal modification of p53

The C-terminus of p53 is also subject to multiple-regulation modifications (Fig. 1.8). Serines 376 and 378 are normally phosphorylated in unstimulated cells and that irradiation induces rapid dephosphorylation of serine 376. ATM protein kinase has been implicated in the pathway that leads to dephosphorylation of serine 376 through stimulation of a p53 phosphatase following irradiation. This dephosphorylation allows 14-3-3 adapter proteins to bind to p53, leading to stimulation of the site-specific DNA binding function of p53. Thus, ATM plays a dual role in activating p53 through phosphorylation of serine 15 and dissociation of Mdm2 at the N-terminus coupled with dephosphorylation and 14-3-3 binding at the C-terminus. Other forms of modification involve acetylation at lysine residues 320 by PCAF (p300/CBP-associated factor) and at 373 and/or 382 by p300/CBP, in response to both irradiation and UV. This leads to activation of the DNA binding function of p53 by conformational change or blocking non-specific DNA binding function of p53. Phosphorylation of serine 15 or 33 and 37 may enhance recruitment of p300 and PCAF acetylates to p53, leading to enhanced acetylation of the C-terminus.

In summary, DNA damage induced modification of p53 is a carefully co-ordinated and sequential series of changes which favour dissociation of the p53-Mdm2 complex, recruitment of transcriptional components to p53, and activation of the site specific DNA binding function.

1.4.2.4 Activation of p53 by tumour suppressor proteins

The products of the *ink4a* tumour suppressor gene p16INK4A and p19ARF (murine protein) or p14 ARF (human protein) can regulate p53 activation (Fig. 1.9). Mutations in this gene are common in human cancer. p16INK4A restrains growth by inhibiting the activity of cyclin D/CDK4 and CDK6, thus blocking downstream phosphorylation of retinoblastoma protein (RB), which results in inhibition of the S phase entry in the cell cycle. p19 ARF binds the C-terminus Mdm2 when linked to p53. This interaction neutralises the ability of Mdm2 to block p53 activation and degradation. Therefore, p19 ARF expression increases p53 levels and induces p53 dependent transcription, growth arrest, apoptosis and suppression of oncogenic transformation.
Mutation or deletion of the gene encoding p19 ARF compromises an important cell cycle check point, and simultaneously removes a pro-apoptotic signalling pathway. Myc can also selectively induce p19ARF, which results in 10 fold rise in p53 protein levels\textsuperscript{249}. Both p16INK4A and p19 ARF are essential for activating p53, and p53 dependent premature senescence in response to activated Ras\textsuperscript{249}. Ras promotes uncontrolled proliferation and transformation in cells lacking either p53 or p16INK4A, which confirm the importance of p53 and \textit{ink4a} gene products.

Oncogenes induce p53 activity in the absence of DNA damage, similarly DNA damage will induce p53 activity without any detectable changes in oncogene expression\textsuperscript{249}. Therefore, oncogenes provide a mechanism of inducing p53 activation in the absence of DNA damage resulting in apoptosis or premature senescence. However, if an oncogene activation of p53 is inoperative, for example if p19 ARF was mutated, oncogene driven growth transformation will persist unchecked.

1.4.2.5 Downstream targets of p53

Activation of p53 can lead to two different cellular responses, growth arrest in cell cycle stages G\textsubscript{1} and G\textsubscript{2}, or apoptosis (Fig. 1.9). The pathway which the cell will follow depends on cell type, cell environment and cellular factors such as oncogene expression.

In some cell types, cell growth arrest after cell exposure to irradiation leads to p53 activation followed by transient arrest in the G1 phase in the cell cycle with prolonged induction of p21WAF1\textsuperscript{248,252,253}. p21WAF1 is a critical mediator of the p53 mediated G1 arrest response, and is a potent inhibitor of several G1 cyclin dependent kinases (CDKs) which include cyclin D-CDK4/6, cyclin E-CDK2 and cyclin A-CDK2. A potential downstream target of p21WAF1 inhibitory activity in G1 cell arrest is the cell cycle dependent phosphorylation of the RB protein. In the G1 stage, RB is in a hypophorylated form and binds to and sequesters the S phase promoting E2F family of transcription factors\textsuperscript{248,249,252,253}. Sequential phosphorylation of RB by CDKs, results in cell cycle progression through the release of active E2F followed by transcriptional activation of genes required for S phase progression. p53-dependent upregulation of p21 WAF1 inhibits cyclin D-CDK4/6 complexes, which leads to accumulation of the
unphosphorylated form of RB and cell cycle arrest in G1 stage, thus preventing the cell from replicating damaged DNA and allows for DNA repair before entry into S phase. However, embryonic fibroblasts from p21/-/- mice and Rb/-/- mice are only partially defective in their ability to undergo G1 cell cycle arrest compared with p53 +/- mice following exposure to irradiation. This suggests that other gene products are also transactivated by p53 and that RB is not the sole downstream target of p53 in cell cycle arrest. The RB related p107 and/or p130 also form complexes with E2F family protein members, and may play a part in p53 mediated growth arrest.

In certain cell types including haemopoietic cells, p53 activation leads to apoptosis. Functional p53 is necessary for DNA damage induced apoptosis in cortical thymocytes, myeloid progenitor cells, marrow pre B-cells and quiescent peripheral B and T lymphocytes. Stimuli such as DNA damage, growth factor withdrawal, and expression of the oncogenes c-myc, E2F, adenovirus E1A, SV40 large T or HPV E7 has been shown to stimulate p53 dependent apoptosis. p53 can induce the expression of death receptors Fas and anti apoptotic decoy receptors DcR1, death receptor ligand Fas-L, pro-apoptotic Bcl-2 family members such as BAX, Noxa or IGF-BP3 (Insulin growth factor-protein binding 3) which sequesters the cell survival factor insulin like growth factor 1. More recently, p53 has been shown to directly upregulate Apaf-1, thus making cells more sensitive to apoptosis after cytochrome-c release from the mitochondria. p53 can also downregulate anti-apoptotic proteins such as Bcl-2. Thus, cells become more sensitive to apoptosis by upregulating pro- and downregulating anti-apoptotic proteins. However, it is not clear why p53 upregulates DcR1.

Recently a series of p53 induced PIG genes (p53 induced genes) have been identified that are predicted to encode proteins that could generate or respond to oxidative stress. Several studies have shown that p53 protein-protein interaction can also be involved in apoptosis.

How p53 might regulate growth arrest versus apoptosis has not been elucidated, but deletion of p21WAF1 can result in cells that would otherwise undergo cell cycle arrest undergoing apoptosis instead. Several factors such as cell type, the presence or absence
of survival factors, the extent of DNA damage and the levels of p53 may be involved in the choice between cell cycle arrest and apoptosis.\textsuperscript{237,243,244}

1.4.3 Mdm2 (Multiple murine double minute gene 2)

Mdm2 was initially identified by the fact that it is amplified in mouse tumourgenic cell line 3T3DM.\textsuperscript{274} Subsequently, Mdm2 has been found to be amplified in a large proportion of human sarcomas.\textsuperscript{275} The wild-type mdm2 gene encodes p90Mdm2, which is capable of interacting and regulating p53 \textit{in-vivo}.\textsuperscript{276} The N-terminal region of Mdm2 binds to the N-terminus of p53, within the trans-activation domain of p53. The binding of Mdm2 alone inhibits transcriptional properties of p53, but this is not the only way in which Mdm2 controls p53. Direct association of p53 with Mdm2 results in ubiquitination and subsequent degradation of p53 by 26S proteosome in the cytoplasm. More specifically, Mdm2 is believed to translocate p53 from the nucleus of the cell into the cytoplasm, where it undergoes degradation.\textsuperscript{237,248,249,252,254} This activity requires the nuclear-export signal (NES) of p53, and the C-terminal RING domain of Mdm2.\textsuperscript{277} The RING domain is also required for p53 ubiquitination.\textsuperscript{278} Mdm2 also has auto-ubiquitination activity and is therefore likely to regulate its own stability.\textsuperscript{279} Mutations in the RING finger which prevent ubiquitination and degradation of p53 result in an Mdm2 protein which is also unusually stable and expressed at high levels.\textsuperscript{280}

In addition to interaction with p53, Mdm2 has been shown to bind to another tumour suppressor protein, p19 ARF.\textsuperscript{249,281} The ability of Mdm2 to degrade p53 is inhibited by p19ARF. Mdm2 can also bind and alter the function of other proteins that regulate cell cycle. Over-expression of Mdm2 stimulates the activity of the E2F transcription factor and reverses cell cycle arrest mediated by the RB protein or a related protein, p107.\textsuperscript{282-284}

1.5 Phosphorylation events in the regulation of apoptosis

The action of both protein kinases and protein phosphatases are crucial to the modulation of the phosphorylation status and hence the activation of target proteins. Many of these proteins are involved in both induction and inhibition of apoptosis. The PI3-K/Akt pathway and tyrosine protein kinases have been implicated in the regulation of survival and apoptosis in cells.\textsuperscript{285,286}
1.5.1 PI3-K and Akt signalling pathway

PI3-K is an enzyme implicated in growth factor signal transduction by associating with receptor and non-receptor tyrosine kinases. Many receptors, including those for cytokines IL-3, IL-2, and growth factors (insulin-like growth factor-1) transmit survival signals through the PI3-K pathway.\(^{285;287-289}\) Induction of tyrosine phosphorylation results in the activation of PI3-K, which catalyses the transfer of a phosphate group from ATP to the D3 position of phosphatidylinositol, thus generating 3'-phosphatidylinositol phosphates (3'-PIPs) (Fig. 1.10). 3'-PIPs have been termed lipid messengers because they serve as binding sites for proteins that possess a pleckstrin homology (PH) domain. One such protein is Akt.\(^{285}\) Binding of the Akt PH domain to the phospholipids results in its translocation to the plasma membrane and phosphorylation at two critical residues, threonine 308 and serine 473. Phosphorylation at threonine 308 is achieved through additional kinases such as phosphatidylinositol-dependent kinase 1 (PDK-1), which also contains a PH domain and requires PI3-K activity for membrane localisation. The identity of the serine 473 kinase is still unknown, but is referred to as PDK-2 as it is expected that this kinase will also be dependent upon 3'-PIPs.

Akt function is controlled both by localisation to the membrane, which is dependent on available 3'-PIPs, and by the level of its phosphorylation. The generation of 3'-PIPs is counter-balanced by lipid phosphatases that dephosphorylate PIPs. The tumour suppressor phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is one such lipid phosphatase with specificity for 3'-phosphorylated PIPs.\(^{290}\)

Inhibition of PI3-K activity is sufficient to induce death even in the presence of survival factors.\(^{291;292}\) This death can be overcome by constitutive Akt activity. Although it is thought that Akt is a major if not the sole effector of PI3-K-induced survival, the mechanism by which Akt suppresses death is not known. Several Akt targets have been identified that may promote cell survival; however, no one substrate or model has emerged as the clear candidate.\(^{293}\)
Figure 1.10 PI3-K and Akt pathway.
1.5.1.1 Akt signal transduction

Akt targets directly involved in signal transduction include Bad and pro-caspase-9 (see 1.1.3.4, 1.1.3.8 and 1.1.2.3). Other proteins that Akt has been reported to phosphorylate include Forkhead proteins such as FKHRL1, which are phosphorylated and retained in the cytosol by 14-3-3 proteins, suggesting a general role of 14-3-3 proteins in Akt-induced inhibition of apoptosis. Survival factor withdrawal leads to FKHRL1 dephosphorylation, nuclear translocation, and target gene activation. Within the nucleus, FKHRL1 triggers apoptosis most likely by inducing the expression of genes that are critical for cell death, such as the Fas-L gene. The promoter for Fas-L contains a binding site for FKHRL1. Forkhead activity leads to Fas-L expression, which then migrates to the cell surface and activates the Fas-mediated cell death cascade. Another cell survival pathway involves activation of NF-κB by Akt. The ability of NF-κB to block apoptosis has been shown in a variety of cell types including myeloid cells and chronic lymphocytic leukaemia. Several NF-κB target genes that may play a role in blocking apoptosis have been identified. These include cIAP1, cIAP2, c-FLIP, TRAF1, and TRAF2.

1.5.2 Tyrosine protein kinases

Many lines of evidence support the involvement of tyrosine phosphorylation in both apoptosis and survival of cells. Presently, there are more than 90 known tyrosine protein kinase in the human genome, classified as either receptor or non-receptor enzymes; 58 encode transmembrane receptors distributed into 20 subfamilies, and 32 encode cytoplasmic, non-receptor kinases in 10 subfamilies. Signalling by tyrosine kinase receptors requires ligand-induced receptor oligomerization, which results in tyrosine autophosphorylation of receptor subunits. This both activates catalytic activity and generates phosphorylated tyrosine residues that mediate the specific binding of cytoplasmic signalling proteins containing protein tyrosine-binding (PTB) and Src homology-2 (SH2) domains. Proteins that can be activated by tyrosine kinase receptors include Src and PI3-K. Tyrosine kinase activity is normally tightly controlled and regulated. However, perturbation of tyrosine kinase signalling by mutations and other genetic alterations results in deregulated kinase activity and malignant transformation. A large fraction of oncogenes are comprised of cellular tyrosine kinases.
Cellular tyrosine kinases which have transforming potential when overexpressed or freed from normal regulatory restraints include c-Src, c-Abl, JAKs and STATs. The resultant inappropriate phosphorylation of important cellular substrates gives rise to either increased proliferation or resistance to apoptosis thus leading to malignant transformation.

1.6 Biological roles of apoptosis and its involvement in haemopoietic system
Multicellular animals need to dispose of excess cells or damaged cells for survival and development. As important as cell division and cell migration, apoptosis allows organisms to tightly control cell numbers and cell function. Haemopoietic system provides many examples of apoptotic cell death.

Haemopoiesis takes place in the bone marrow, which contains self-renewing, pleuripotent stem cells. Stem cells undergo a series of differentiation and proliferation that involves cytokines and physical interactions with other cells and extracellular matrix molecules to produce mature cells of the haemopoietic system. Stem cells are relatively few in number, comprising between 0.01% and 0.05% of the total marrow population. They possess two characteristic features: first is their capacity for self-renewal to produce more stem cells; second is their potential to undergo differentiation to produce highly specialised mature cell types which allows for enormous amplification.

All the cells in the immune system arise from stem cells through two main lines of differentiation, the lymphoid and the myeloid lineage. These lineages generate committed progenitor cells (CFC = colony-forming cells), which are irreversibly determined to produce only one or a few types of blood cells. The progenitor cells are stimulated to proliferate by specific colony-stimulating factors (CSFs) but progressively lose their capacity for division and develop into terminally differentiated blood cells, which usually live for only a few days or weeks. CSFs not only influence proliferation and differentiation but also have an effect in allowing cell survival. When primitive cells are deprived of growth factors in-vitro, they die by apoptosis. Studies done with progenitor cell lines show that in the absence of IL-3, G-CSF (granulocyte colony-stimulating factor) or GM-CSF (granulocyte-macrophage CSF), cells die by apoptosis. This suggests that growth factors are important in regulating the size of the progenitor cell pools. One view suggests that the default pathway of cells leads to cell death and that cells
need to be constantly stimulated to survive\textsuperscript{305}. The integration of all these signals triggers intracellular cascades that inhibit cell death by default and support cell survival. The balance between life and death that is normally regulated by a network of proto-oncogenes and tumour-suppressor genes needs to be strictly controlled to avoid uncontrolled proliferation of cell clones. Both hyper-proliferation and the inability to die give rise to benign tumours that eventually result in malignancies after acquiring additional mutations in single cells.

1.6.1 B-cell Chronic Lymphocytic Leukaemia (B-CLL)

B-CLL is the most common leukaemia in the Western Hemisphere. B-CLL is characterised by relentless accumulation of long-lived, mature, monoclonal CD5 + B-cells in the blood, secondary lymphoid tissues, and marrow\textsuperscript{306}. Circulating cells are in the G0/G1 phase of the cell cycle and are resistant to apoptosis\textit{in-vivo}\textsuperscript{307,308}. p53 inactivation has been reported in only 10-15% of B-CLL cases which is associated with tumour progression\textsuperscript{309}.

Several chromosomal abnormalities have been documented in B-CLL cells, with the deletion of 13q14 and 11q22-23 being the most common followed by trisomy of chromosome 12\textsuperscript{310,311}. Other chromosomal abnormalities include deletion of 6q (9%) and 17p13 (8%). Homozygous or hemizygous deletions at 13q14 occur in 53% of B-CLL\textsuperscript{310,311}. Deletions at 13q14 may result in inactivation of a tumour suppressor gene following the loss of one allele and mutation of the remaining allele, but the gene or genes which may be important in the pathogenesis of B-CLL have yet to be discovered.

Deletions at 11q22-23 occur in 13 to 19% of B-CLL cases and are associated with disease progression and reduced survival\textsuperscript{310,312-314}. The region of loss includes the ATM gene, and recent data has shown reduced expression of ATM protein in some cases of B-CLL due to mutation in both alleles of the ATM gene or mutation of the remaining allele\textsuperscript{315-317}. Trisomy 12 occurs in 20% of B-CLL and is associated with aggressive disease and atypical morphology, but the mechanism by which it contributes to the pathogenesis in B-CLL is still unknown\textsuperscript{310,318,319}. Candidate genes found on chromosome 12 include\textit{CDK2, CDK4, HMG1-C} and\textit{MDM2}.\textit{MDM2} has been shown to be over expressed in B-
CLL, but no abnormalities have been found at the DNA level, and no association has been found between trisomy 12 and any of these genes\textsuperscript{320,321}.

Immunoglobulins (Ig) play a central role in B-cell physiology. B-CLL cells express surface IgM often together with surface IgD, but the levels of expression are lower than on normal B-lymphocytes, and rarely can IgG or IgA be detected\textsuperscript{322}. Several groups have examined the Ig heavy chain variable region genes (Ig VH genes) expressed by leukaemia cells that express IgA or IgG\textsuperscript{323-325}. Although these cells expressed a surface phenotype similar to IgM B-CLL cells, they generally expressed Ig VH genes that had undergone somatic mutation, which suggests that these cells may result from malignant transformation of mature B-cells at a stage(s) of B-cell differentiation that is distinct from IgM B-CLL cells. It has now been demonstrated that about 60\% of B-CLL cells have somatic mutation of their Ig variable region genes\textsuperscript{326}. They are likely to be in stage A with stable disease and typical morphology with median survival of 25 years, while IgM B-CLL cells are more likely to have an advanced stage, atypical morphology, progressive disease, trisomy 12 and a median survival of about 8 years\textsuperscript{326}.

B-CLL are resistant to apoptosis \textit{in-vivo}, partly due to high level expression of Bcl-2 protein. Although \textit{BCL2} gene rearrangements are rare in B-CLL, more than 85\% of B-CLL express high levels of Bcl-2 protein\textsuperscript{327}. The mechanism involved in Bcl-2 overexpression is currently unclear. However, \textit{in-vitro} B-CLL cells undergo spontaneous apoptosis\textsuperscript{328}, which suggests that in B-CLL cells, apoptotic mechanism is influenced by interaction with the microenvironment\textsuperscript{329-333}. \textit{In-vitro} data suggest that several cytokines including IL-4, IFN\textgreek{a} and IFN\textgreek{y}, and cell to cell interaction with stroma and blood derived nurse-like cells inhibit B-CLL cell apoptosis and may be accompanied by preservation or upregulation of Bcl-2 protein\textsuperscript{329,330,331,333-335}.

\textbf{1.6.2 Acute Myeloid Leukaemia (AML)}

AML is a clonal disorder that is the consequence of acquired somatic mutation in haemopoietic myeloid precursor cells, which confer a proliferative advantage. AML is divided into 8 groups based on morphology according to the French American British
(FAB) scheme, M0 to M7. In AML the most frequent genetic changes result from chromosomal translocation involving core binding factor (CBF), retinoic acid receptor α gene, MLL gene and transcriptional co-activators CBP/p300 (Table 1.1).

CBF is a heterodimeric factor, which has been shown to play a critical role in the transcriptional activation of genes important in haemopoietic development, including IL-3, GM-CSF and M-CSF receptor. Both heterodimeric components of CBF, CBFα (AML1) and CBFβ, are now known to be involved in chromosomal translocation associated with AML, which include t(8;21), inv(16) and the t(12;21) (Table 1.1a). The t(8;21) translocation is the fusion of the AML1 (CBFα) gene on chromosome 21 to the ETO (eight twenty one) gene on chromosome 8, and is present in 15% of adult AML patients and defines AML FAB M2 type. The AML1-ETO fusion protein is thought to inhibit the ability of the wild type AML1 gene to direct normal maturation and development of haemopoietic cells. Both inv(16)(p13;q22) and the t(16;16)(p13;q22) translocation result in the fusion of the CBFβ subunit at 16q22 to the smooth muscle myosin heavy chain (SMMHC) at 16p13 defines AML1 FAB M4 type. The fusion protein is thought to act as a dominant negative inhibitor of wildtype AML1 function. The t(12;21) translocation result in the fusion of TEL to AML1.

Translocation involving retinoic acid receptor α gene t(15;17)(q22;q11.2) is associated with acute promyelocytic leukaemia (FAB subtype M3) and accounts for ~5% of adult AML (Table 1.1b). t(15;17) fuses the promyelocytic leukaemia (PML) gene on chromosome 15 to the retinoic acid receptor α (RARα) gene on chromosome 17. RARα is a transcriptionally active protein that contains two active zinc finger DNA binding domains, and a ligand-binding domain that interacts with retinoic acid derivatives. The function of PML is not well known, but it also contains a zinc finger domain. The resultant PML/RARα fusion protein appears to act as a dominant negative inhibitor of wtRARα function.
Table 1.1 Selected chromosomal translocations in AML.

a) Translocation involving core binding factor (CBF/AML1)

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<td>ETO  (8q22)</td>
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<td></td>
<td>EVII (3q26)</td>
<td>Transcription factor</td>
<td>MDS</td>
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<td>t(3;21)(q26;q22)</td>
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<td>Transcription factor</td>
<td>CML-BC</td>
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<tr>
<td></td>
<td>EAP  (3q26)</td>
<td>Ribosomal protein</td>
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<td>t(3;21)(q26;q22)</td>
<td>AML 1(21q22)</td>
<td>Transcription factor</td>
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<tr>
<td></td>
<td>MDS1 (3q26)</td>
<td>RIZ-related</td>
<td></td>
</tr>
<tr>
<td>inv(16)(q13;q22)</td>
<td>CBFβ(18q22)</td>
<td>Heterodimeric Partner of AML1</td>
<td>M4Eo</td>
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<tr>
<td></td>
<td>MYH11(16p13)</td>
<td>Smooth muscle myosin heavy chain</td>
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<td>t(12;21)(q13;q22)</td>
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<td>Transcription factor</td>
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b) Translocation involving retinoic acid receptor α (RARα)

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<td>T(11;17)(q13;q11)</td>
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<td>RARα (17q11)</td>
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c) Translocations involving the MLL/ALL1/HTRX/HRX gene on chromosome 11q23

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<td>No homology to known proteins; GLGF motif</td>
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<td>T(10;11)(p12;q23)</td>
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<td>Leucine zipper; zinc finger</td>
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<tr>
<td>T(11;17)(q23;21)</td>
<td>AF17 (17q21)</td>
<td>Leucine zipper; zinc finger</td>
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<td>T(11;19)(q23;p13)</td>
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d) Translocations involving transcriptional co-activators/histone acetylases

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<tr>
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<td>Inv(8)(p11;p13)</td>
<td>MOZ TIF2</td>
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</table>

CML-BC = chronic myelogenous leukaemia in blast crisis; M4Eo = AML, FAB subtype ME4o (myelomonocytic with eosinophilia); ALL = acute lymphoblastic leukaemia; APML = acute promyelocytic leukaemia; ETO = eight twenty one
The \textit{MLL} gene (\textit{ALLl, HTRX, HRX}) spans 100Kb of genomic DNA and encodes a 3972aa protein, which is thought to be involved regulating the transcription of genes \textsuperscript{340-342}. The \textit{MLL} gene has been found to have a wide variety of fusion partners, which now number more than 15 (Table 1.1c). The most common of these translocations is the t(9;11)(p22;q23). Other common translocations involving \textit{MLL} in AML include t(6;11)(q27;23), t(10;11)(p12;q23), t(11;17)(q23;21) and t(11;19)(q23;p13) \textsuperscript{343}.

Several chromosomal translocations involving transcriptional co-activators CBP and p300 have been discovered (Table 1.1d). These include the t(11;16)(q23;p13.3), t(11;22)(q23;q13) and t(8;16)(p11;p13) translocations that result in MLL/CBP, MLL/p300 and MOZ/CBP fusion proteins respectively \textsuperscript{344-346}. Although the mechanism of transformation of these fusion proteins is unknown, all involve CBP/p300 transcriptional co-activators, which are capable of interacting with a broad spectrum of transcription factors. CBP/p300 has been implicated in pathogenesis of human cancer as a loss of functional allele.

Anti apoptotic Bcl-2 levels and Bcl-2/Bax ratios are higher in AML cells than in normal cells \textsuperscript{347}. Over-expression of Bcl-2 in AML cells is linked with CD34 positivity, resistance to chemotherapy, and short survival \textsuperscript{348,349}. AML patients with high Bax expression at diagnosis have significantly better prognosis for disease free survival, event free survival and overall survival, and reduction in the Bcl-2/Bax ratio in AML patients correlates with remission \textsuperscript{347,350}. However, there is no correlation between caspase or Apaf-1 levels and sensitivity of AML cells towards apoptosis \textsuperscript{351}. This could be due to the expression of IAPs or post-translational modification of caspases in AML cells. Constitutive Fas expression and function in AML has also been correlated with response to induction chemotherapy, and CD34 positive AML cells show low Fas expression and function\textsuperscript{352,353,353}. Studies have also revealed high Fas expression in differentiated FAB subtypes M2-5 and low Fas expression in immature FAB M1\textsuperscript{352,353}.

Several cellular drug resistance mechanisms in AML cells have been described, such as P-gp (P-glycoprotein), LRP (vault-transporter lung resistance protein) and MRP (ABC transporter multidrug resistance protein) \textsuperscript{354}. P-gp, which is a multidrug resistant protein
and a product of the \textit{mdrl} gene (multidrug resistance-1), has been shown to be involved in AML resistance against spontaneous and drug induced apoptosis\textsuperscript{353,355}. The constitutive expression of Fas and Bcl-2, as well as Fas sensitivity and P-gp function depend on the maturation stage of leukaemic cells in adult AML\textsuperscript{353}. P-gp function, the extent of spontaneous apoptosis \textit{in-vitro} and Fas sensitivity have been suggested to be more predictive for response to induction chemotherapy in adult AML than the constitutive expression levels of the apoptosis related molecules Fas, Bcl-2 and Bax\textsuperscript{353}. Mutation of p53 expression in AML also gives a bad prognosis for response to chemotherapy and survival\textsuperscript{356}. 
1.7 Aims

Although the process of apoptosis and cell survival is well documented, our knowledge of signalling pathways is still limited. In order to establish effective therapies for leukaemia, it is crucial to exploit the inherent apoptotic pathways, which operate in these cells. A comprehensive understanding of death receptor mediated apoptosis, caspase cascade and phosphorylation and dephosphorylation mechanisms, which regulate apoptotic cell death, are therefore fundamental. The aims of this thesis were to address two main issues:

A. The role of death receptor ligation in the killing of leukaemia cells by cytotoxic drugs and radiation.

This was addressed by analysing the potential involvement of Fas, Fas-L, DR4, DR5, DcR1, DcR2, c-FLIP, p53, caspase-8 and caspase-3 in the killing of B-CLL and AML cells by cytotoxic agents with or without agonistic anti-Fas IgM or TRAIL. It is anticipated that these studies may contribute to the understanding of apoptosis in B-CLL and AML cells and the development of novel strategies for treating these diseases with anti-Fas IgM or TRAIL in combination with conventional therapy.

B. Cytoprotection of B-CLL cells by plasma and synergistic apoptosis induction by cytotoxic drugs and signal transduction inhibitors.

This was addressed by analysing the ability of autologous plasma to promote both basal survival as well as resistance to DNA damaging agents in B-CLL cells. The ability of the PI3-K inhibitor LY294002 and the tyrosine kinase inhibitor ansamycin antibiotic herbimycin A (HMA) to synergize with cytotoxic drugs in inducing apoptosis of B-CLL cells cultured in FCS or plasma were examined. Since PI3-K appears to be centrally involved in survival factor signal transduction, specific inhibitors against the kinase may be beneficial in the treatment of leukaemia as well as in elucidating the biological role of PI3-K in survival signalling in B-CLL cells.
Chapter 2

Materials and methods

2.1 Cell culture

2.1.1 Isolation of B-cells

B-CLL cells were obtained from 39 patients (89 multiple samples listed in table 2.1) with informed consent. White blood cell counts ranged from 22 - 476 x 10^6 ml^{-1}. None of the patients had received treatment for at least three months prior to this study. Malignant B-cells were obtained from 30-50ml peripheral blood and clotting was prevented by the addition of preservative free heparin (10U/ml). Plasma was withdrawn after sedimentation at 700xg and clarified by a further high-speed sedimentation step. Patient blood was diluted three to four fold in Hanks Balanced Salts Solution (HBSS; Gibco, Life Technologies, UK) and carefully layered onto lymphoprep (Nyegaard, Oslo, Norway). Tubes were centrifuged at 600xg for 30 minutes. The top layer of the medium was removed and the interface, consisting mononuclear cells, was transferred to another universal tube and washed twice in HBSS and 300µl of sample was taken for cell counting using a COULTER cell counter. The cells were resuspended at 5 x 10^6ml^{-1} in complete medium - RPMI1640 (Gibco, Life technologies, UK) supplemented with 100units/ml penicillin and 100µg/ml streptomycin, 2mM L-glutamine and 10% heat inactivated foetal calf serum. The cells were cultured in a tissue culture flask (Nunc, UK) at 37°C for 60 minutes to permit monocytes to adhere to the plastic. The lymphocytes were removed gently and 300µl was removed for cell counting. One million cells per test were used to evaluate cell purity. Samples with more than 10% T-lymphocytes were diluted to 4x10^6ml^{-1}, and 30ml of sample were combined with 10ml of neuraminidase-treated sheep red cells. Samples were left at 4°C overnight to form rosettes. Rosetteled T-cells were removed from enriched B-cells by sedimentation on a lymphoprep gradient and centrifuged at 600xg for 30min at 4°C. B-cells were recovered from the medium/lymphoprep interface while T-cell/sheep red cell rosettes sedimented to the bottom of the tube. The B-cells were washed twice in HBSS, resuspended in complete medium and 300µl was removed for cell counting and for measuring cell viability by trypan blue exclusion method.
Table 2.1 B-CLL patient details

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2.1.2 Preparation of sheep red blood cells (SRBC)

Twenty ml of sheep blood cells (Tissue Culture Services, UK) were washed three times in HBSS for 5 minutes at room temperature and resuspended in 60 ml HBSS with neuraminadase (0.017 units/ml, Sigma). The cells were incubated at 37°C for 45 minutes, washed 3 times, resuspended in 150 ml HBSS and stored at 4°C.

2.1.3 Evaluation of cell purity

One million purified B-cells from patients were washed twice in HBSS and incubated with 10 μl of primary antibody in 50 μl of PBS. Antibodies used were CD 19 (B-cell marker; Becton Dickinson, San Jose, CA, USA), CD2 (T-cell marker; Becton Dickinson, San Jose, CA, USA) and mouse isotype control. After 30 minutes incubation on ice the cells were washed once in PBS, resuspended in 0.5 ml of PBS and then analysed by flow cytometry (FACScan, Becton Dickinson) with Cell Quest software (Becton Dickinson). All samples studied contained less than 10% CD2-positive T-cells or more than 90% CD19 positive B-cells.

2.1.4 Isolation of AML cells

Peripheral AML blood was obtained from 20 patients (Table 2.2) at presentation with informed consent, and clotting was prevented by the addition of preservative free heparin (10U/ml). White blood cell counts ranged from 3.3 - 242 x 10^6 ml^-1. Ficoll-Hypaque lymphoprep density gradient centrifugation at 600xg for 30 minutes. The top layer of the medium was removed and the interface, consisting mononuclear cells, was transferred to another universal tube and washed twice in HBSS and 300 μl of sample taken for cell counting using a COULTER cell counter. The cells were resuspended at 10 x 10^6 ml^-1 in complete medium - RPMI1640 (Gibco, Life technologies, UK) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine and 10% heat inactivated foetal calf serum. All samples studied contained more than 70% blast cells. The diagnosis of AML and FAB classification was established following morphological, immunological, and cytochemical tests. One patient had FAB type M0, 7 M1, 1 M2, 5 M4, 1 M5, and 1 FAB type M7.
**Table 2.2 AML patient details**

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<th>Source</th>
<th>FAB type</th>
<th>Sex/Age</th>
<th>WBC</th>
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<tr>
<td>BM</td>
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<td>F 68</td>
<td>3.3</td>
</tr>
<tr>
<td>PB</td>
<td>M0</td>
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<td>N/A</td>
</tr>
<tr>
<td>PB</td>
<td>M4</td>
<td>F 37</td>
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<td>F 61</td>
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<td>F 71</td>
<td>50</td>
</tr>
<tr>
<td>BM</td>
<td>M4</td>
<td>F 74</td>
<td>59</td>
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<td>PB</td>
<td>M1</td>
<td>M76</td>
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<td>PB</td>
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<tr>
<td>PB/BM</td>
<td>M5</td>
<td>M76</td>
<td>242/151</td>
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</tbody>
</table>

**2.1.5 Cell culture**

B-CLL cells were cultured for 24h following addition of 33-165 μM chlorambucil (Chl), 17.5 μM fludarabine (Flu) or exposure to 10Gy γ-irradiation. AML cells were cultured for 24 to 48h following addition of 17.5 μM Flu, 0.1 μM daunorubicin (DNR) or 41 μM cytosine arabinoside (Ara-C), or exposure to 10Gy γ-irradiation. Concentration of drugs used were selected in accordance with LD$_{50}$ (lethal dose, 50% viability) values previously observed by Silber et al. for B-CLL cells.$^{357}$ Concentration of drugs used on AML cells were below previously observed *in-vivo* plasma drug concentrations by other groups.$^{358,359}$ The anti-human Fas blocking monoclonal IgG1 antibody (ZB4, Upstate Biotechnology, Lake Placid, NY) was added at 250ng/ml 1h prior to cytotoxic treatments. The agonistic anti-Fas IgM monoclonal (CH-11, Upstate) was added at 50 – 500ng/ml
and agonistic TRAIL (Perpro Tech EC LTD) at 500ng/ml. The Jurkat cell line was used as a control for the actions of these antibodies.

To study plasma effects on B-CLL cells, cells were cultured in complete medium - RPMI1640 supplemented with 100units/ml penicillin, 100μg/ml streptomycin and 50% autologous plasma or FCS (control). Cells were incubated for 24h following addition of 33-165μM Chl, 17.5μM Flu, or exposure to 10Gy γ-irradiation, with or without 20-50μM PI3-K inhibitor LY294002 (Calbiochem) or 0.3-1μM tyrosine kinase inhibitor herbimycin A (HMA) (Calbiochem).

2.2 Protein studies
2.2.1 Protein extraction procedure
Five to twenty million cells were washed once in cold HBSS, transferred to microcentrifuge tubes and resuspended in 50 to 200μl of lysis buffer (20mM Hepes-KOH [pH 7.5], 50mM NaCl, 2% nonidet P40, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate (SDS), 1mM sodium vanadate, 10mM sodium fluoride, 1mM ethylene glycol tetra-acetic acid (EGTA), 0.02M dithiothreitol (DTT) and 1.76mg/ml PMSF). The lysates was kept on ice for 15 minutes and centrifuged for 15 minutes at 12,000xg. Finally the supernatant was removed to another microcentrifuge tube and stored at −80°C until use.

2.2.2 Protein concentration measurement
Protein concentration was quantified using Bio-Rad protein assay kit (BIO-RAD). The dye-binding assay is based on the differential colour change of a dye in response to various concentrations of proteins and measured using a spectrophotometer at 595nm. Bovine serum albumin (Sigma) was used as standard.

2.2.3 Western Blotting
Tris Glycine 4-12% or 8 -16% precast gradient gel plates were assembled according to manufacturer’s instructions (NOVEX). Protein concentration was measured, and 20μg of protein samples were made up with 1 x SDS sample buffer (NOVEX), 80mM DTT reducing agent and ultrapure water. Samples were heat denatured for 3-5 minutes at
100°C in boiling water bath, centrifuged briefly and loaded into gels using a Hamilton syringe. NuPAGE (Polyacrylamide gel electrophoresis) Gels (NOVEX) were run at 200 V using 1x MOPS (N-morpholino propanesulfonic acid) running buffer (NOVEX) and NuPAGE running buffer antioxidant (NOVEX). Two Whatman filter papers and one nylon membrane (Hybond C, Amersham) per gel, cut in the same dimension as the gel were soaked individually in blotting buffer (Bicine 25mM, Bis Tris 35mM, Ethylene diamine tetra-acetic acid (EDTA) 1mM, 10-20% methanol, pH7.2 and 200μl of antioxidant per 200ml of transfer buffer). The gel was placed on top of one filter, followed by nylon membrane then another filter paper, with care taken to avoid trapping air bubbles between layers. The gel, membrane and filter papers sandwiched with blotting pads pre-soaked in transfer buffer were placed in a blot module (NOVEX) with transfer buffer. Transfer of proteins was carried out at 25 V. After blotting, the nylon membrane was stained with Ponceau-S stain (Sigma, UK) to ensure equal and satisfactory transfer of proteins. The membrane was carefully agitated at room temperature in polyvinylpyrrolidone (PVP) blocking buffer (0.02M Tris, 0.14M NaCl, 2% new-born calf serum and 5% PVP (Sigma) [pH7.6]) for 1 hour. After blocking, the membrane was placed on top of a plastic sheath, rolled up and placed in a 50ml Falcon tube with 10ml fresh blocking buffer containing primary antibody. Sources of antibodies are listed in Table 2.3. The tube was placed in a rotisserie for 16-18h at room temperature. After primary antibody incubation, the blot was washed in 1x washing buffer (0.02M Tris, 0.14M NaCl and 0.02% Tween 20) for two 5 min washes followed by one 1 hour wash and finally two 5 min washes. The membrane was incubated with horseradish peroxidase linked secondary antibody (DAKO Denmark, 1/5000 dilution) in blocking buffer for at least 1 hour at room temperature. The membrane was finally washed thoroughly in washing buffer for two 5 min washes, a 1 hour wash and two final 5 min washes to reduce background. The enhanced chemiluminescence (ECL) method (Amersham) was used to detect immunoreactive protein bands as detailed in the protocol. For further antibody staining the blots were stripped by incubation in stripping buffer (162mM Tris [pH 6.7], 2% SDS, 100mM β-mercaptoethanol) for 30 minutes at 50°C.
Table 2.3 Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>monoclonal AC15, Sigma.</td>
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<tr>
<td>Caspase-8</td>
<td>monoclonal B9-2, Pharmingen.</td>
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<tr>
<td>Caspase-8 p20 subunit</td>
<td>polyclonal sc-6136, Santa Cruz.</td>
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<tr>
<td>Caspase-3</td>
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<td>Caspase-9</td>
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<td>PARP</td>
<td>monoclonal 7D3-6, Pharmingen.</td>
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<td>p95 PARP</td>
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<td>FADD</td>
<td>monoclonal A66-2, Pharmingen.</td>
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<tr>
<td>I-FLICE (c-FLIP)</td>
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</tr>
<tr>
<td>DR4</td>
<td>polyclonal, Sigma.</td>
</tr>
<tr>
<td>DR5</td>
<td>polyclonal, Sigma.</td>
</tr>
<tr>
<td>DcR1</td>
<td>monoclonal, Alexis.</td>
</tr>
<tr>
<td>DcR2</td>
<td>Polyclonal, Sigma.</td>
</tr>
<tr>
<td>Fas</td>
<td>Polyclonal C-20, Santa Cruz.</td>
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<tr>
<td>Fas-L</td>
<td>monoclonal G247-4, Pharmingen.</td>
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<td>p53</td>
<td>monoclonal IgG2a, Santa Cruz.</td>
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<tr>
<td>p21 WAF1</td>
<td>monoclonal SX118, Pharmingen, UK.</td>
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</table>

2.2.4 Flow cytometric analysis of Fas and Fas-L expression

Cell surface expression of Fas and Fas-L in B-CLL cells was quantified by flow cytometry. The expression of Fas was analysed by incubating 1×10^6 cells/ml with 10μl monoclonal Fas-FITC antibody (UB2, Immunotech) or 10μl control mouse IgG1 FITC antibody (Becton Dickinson) for 40 min at 4°C. Fas-L was quantified by incubation with 10μl monoclonal Fas-L antibody (NOK-1, Pharmingen) or 10μl control mouse IgG1 antibody (Becton Dickinson) for 30 min at 4°C, followed by a secondary antibody using 10μl FITC-labelled rabbit anti-mouse IgG (Dako, Denmark) for 30 min at 4°C. Data were analysed using CellQuest software and are presented as the ratio of Fas-FITC median cell fluorescence (MedCF) / control IgG1 FITC MedCF values and Fas-L-FITC MedCF/ control IgG1 FITC MedCF values.

2.3 RNA techniques

2.3.1 RNA extraction

Total cellular RNA was isolated by the guanidium-isothiocyanate (GITC) method as described by Chomczynski and Sacchi. Ten million cells were pelleted at 800xg for 5 minutes at 4°C and washed with HBSS. Five hundred μl GITC solution D (4M GITC,
0.75M sodium citrate, [pH 7], 10% N-lauroyl sarcosinate, 100mM 2- β mercaptoethanol) was added to the pellet and the mixture homogenised with a 1ml syringe and 0.8 mm x 40 mm needle (Tyco healthcare, UK). Two molar sodium acetate, pH5 (50μl) was added and the solution vortexed for 5 seconds. Five hundred μl phenol, pH 4.3 (Sigma), was added and the mixture vortexed for 10 seconds. One hundred μl chloroform/ isoamylalcohol (49:1) was added to the tube, vortexed for 10 seconds and incubated in ice for 10 minutes. The tube was centrifuged at 4°C for 20 minutes at 12,000xg. The upper aqueous phase was removed and transferred to a fresh microcentrifuge tube. To the aqueous phase 500 μl phenol and chloroform (1:1) was added and the solution vortexed for 10 seconds and incubated on ice for 5 minutes. The tube was centrifuged at 4°C for 15 minutes at 12,000xg. The upper aqueous phase was transferred to a fresh microcentrifuge tube, and 500 μl chloroform was added. The solution was vortexed for 10 seconds, incubated on ice for 5 minutes and centrifuged at 4°C for 10 minutes at 12,000xg. The aqueous phase was transferred to a fresh microcentrifuge tube and 2 volumes of absolute ethanol was added. The tubes were incubated at -20°C overnight and centrifuged at 12,000xg for 20 minutes at 4°C. The pellet was washed in 70% ethanol, and the air-dried pellet dissolved in 50μl diethy pyrocarbonate (DEPC) treated water.

2.3.2 RNA quantification
Five μl of the RNA solution was diluted in 995μl DEPC water. The absorbance was read at 260 and 280nm using DEPC water as a blank. The A260/280 ratio was calculated. A value of less than 1.8 indicated phenol/protein contamination and a value greater than 2.0 indicated that the RNA sample was degraded. The RNA concentration was calculated using the formula: 1 O.D.=40μg/ml RNA. A 1.0% agarose electrophoresis mini-gel was also used to verify RNA purity, yield and integrity.

2.3.3 Reverse Transcription (RT) of RNA
Ten μl of water containing 1μg of RNA was added to a sterile microfuge tube. It was heated to 65°C for 5min and placed on ice. A further 10μl of RT mix was added so that the final volume of 20μl contained 1μg RNA, 1x RT buffer (Life Technologies), 24units RNasin (Promega, USA), 0.5mM each dNTP (Amersham Pharmacia, UK), 2.5μM
random hexadeoxynucleotide primers (Boehringer Mannheim, UK), 0.02M DTT (Stratagene, UK) and 200 units Moloney murine leukaemia virus reverse transcriptase (MMLV, Life Technologies, UK). The RT reaction was incubated at 37°C for 60 minutes and then heated at 94°C for 5 minutes to stop cDNA synthesis. Twenty µl of water was added and the samples were stored at -70°C until required.

2.3.4 Polymerase chain reaction.
In each PCR reaction, 2µl of the cDNA preparations were amplified using 2.5U of Taq polymerase (Promega) and 0.5mM of each dNTP. The primer sequences and amplification conditions are summarised in table 2.4. All primers were used at 10µg/ml. The PCR programme involved an initial denaturation step of 10 minutes at 94°C, followed by cycles of 1 minute at 94°C, 1min at the annealing temperature and 1min at 72°C for primer extension. PCR products were visualised on ethidium bromide stained 3% agarose gels. Bands were quantified by densitometry using the Gel Doc video camera (Bio-Rad, UK) and Quantity One software (Bio-Rad, UK). Fas and Fas-L band intensities were normalised to the density of actin PCR bands generated from the same sample. Primer sequences for DR4, DR5, DcR1 and DcR2 were the same as primers used by Kim et al. 361.

2.4 Cell death assays
2.4.1 MTT assay
Cell viability was evaluated by quantifying their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Company, UK)362. B-CLL cells were cultured (2x10^5/well) in round bottom microtiter plates in 100µl RPMI1640 / 10%FCS in the presence of cytotoxic agents or medium alone. At 24h or 72h, 10µl of MTT (5mg/ml) was added for an additional 3 hours. The precipitated purple MTT formazan was centrifuged for 5 minutes at 600xg. The supernatant was removed and the formazan pellet was dissolved in 100µl DMSO (dimethyl sulfoxide). Viability was quantified using a plate reader (Anthos Labtec Instruments, Austria) using a 540-nm filter for measurement and a 650-nm filter for reference.
Table 2.4 Oligonucleotide primers used in this study.

S = sense, AS = antisense orientation.

<table>
<thead>
<tr>
<th>Targets</th>
<th>Primer sequence</th>
<th>Annealing temp.</th>
<th>Cycle</th>
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<td></td>
<td>AS 5'-TAGGAATTTTGATATTCTGTGGTCG-3'</td>
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<tr>
<td>Fas-L</td>
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<td>AS 5'-TTCTCCCGTGTTATCACAGA-3'</td>
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2.4.2 Assessment of apoptosis by morphology

Cytospin preparations of cells were stained with May-Grunwald-Giemsa. Apoptotic cell death was quantified by estimating the proportion of cells with condensed chromatin or fragmented nuclei. Slides were examined in random order and at least four hundred cells were counted per cytospin preparation.

2.4.3 Propidium iodide (PI) staining and flow cytometric analysis of apoptotic cells

One million cells were washed and fixed in ice cold 70% ethanol overnight. After washing, the cells were resuspended in 200μl of a solution of PBS containing 40μg/ml PI.
and 100µg/ml RNAse A and incubated at 37°C in the dark for 30 minutes. Cells were analysed on a Becton Dickinson flow cytometer using Cell Quest software.

2.4.4 Viability assessment by trypan blue exclusion
Cells were mixed equally with 0.1% Trypan Blue (Sigma) and loaded onto a Neubauer counting chamber. Viable cells were estimated by counting trypan blue negative cells.

2.4.5 Viability assessment by propidium iodide staining
One million cells were washed in PBS and pelleted. Two µl of PI (1mg/ml, Sigma) was added to the 50 µl pellet (PI final concentration 20µg/ml) and the cells were resuspended in 500µl cold PBS. Viable cells were estimated by quantitation of PI positive dead cells against PI negative viable cells on a Becton Dickinson flow cytometer using Cell Quest software.

2.4.6 Calculation of specific apoptosis/loss of viability
Specific apoptosis or loss of viability induced by cytotoxic agents, agonistic anti-Fas IgM or TRAIL was measured using the equation used by Debatin K.M. et al. 364:

\[
\text{Specific apoptosis or Loss of viability} = \frac{\text{Test apoptosis or viability} - \text{Spontaneous apoptosis or viability}}{\text{100} - \text{Spontaneous apoptosis or viability}} \times 100\%
\]

2.4.7 Calculation of super-additive apoptosis/loss of viability
To investigate possible synergistic killing of individual B-CLL or AML samples by the combinations of cytotoxic agents and anti-Fas IgM or TRAIL, the experimental specific apoptosis or loss of viability induced by the cytotoxic agents in combination with anti-Fas IgM/TRAIL was compared with the theoretical specific apoptosis or loss of viability. Theoretical value is the sum of the percentage of specific apoptosis/loss of viability induced by the cytotoxic agent and anti-Fas IgM/TRAIL when applied alone. Super-additive induced apoptosis or loss of viability of individual B-CLL or AML samples was arbitrarily defined as experimental value which exceeded the theoretical value by more than 5%.
2.5 Statistic analysis

The apoptotic and loss of viability data were evaluated using a two-tailed student \( t \)-test for paired samples. Two-tailed test was used because the results were interesting in either direction. A two-tailed test is a hypothesis test in which the null hypothesis is rejected if the observed sample statistic is more extreme than the critical value in either direction (higher than the positive critical value or lower than the negative critical value). Two-tailed Pearson correlation statistical test was used to determine the relationship between two independent variables based on the assumption that both \( X \) and \( Y \) values are sampled from populations that follow a Gaussian distribution, at least approximately. Nonparametric two tailed Spearman correlation statistical test was used to determine the relationship between two independent variables based on ranks, not the actual values.
Chapter 3

The role of Fas signalling pathway and cytotoxic agents in induction of apoptosis of B-CLL and AML cells.

3.1 Introduction

The mechanisms by which anti-neoplastic cytotoxic drugs kill leukaemic cells is not well understood, although the activation of caspase-3 has been implicated\(^{365}\). Recent studies have resulted in the suggestion that interactions between Fas and Fas-L may mediate cytotoxic killing of at least some leukaemia cell lines\(^{366}\). These conclusions are based on the following observations. First, cytotoxic drug treatment of leukaemia cell lines can induce elevated expression of Fas-L or of Fas\(^{262,263,367}\). Drug-induced Fas expression may be dependent on p53-mediated transcriptional upregulation of the Fas gene\(^{199,368}\). Second, killing of target cells by cytotoxic drugs can be blocked by antibodies which interfere with Fas/ Fas-L interactions, antisense oligos against Fas-L mRNA or ectopic expression of dominant-negative FADD\(^{262,369}\). Third, leukaemia cell lines selected for resistance to Fas-dependent killing are also cross-resistant to cytotoxic drugs\(^{262}\). Similar evidence has also suggested a role for the Fas-mediated signalling in the killing of solid tumour cell lines\(^{199,370-372}\).

However, other studies have failed to reveal a role for Fas/Fas-L interactions in cytotoxic killing of leukaemia cell lines. For example, cytotoxic drugs do not always result in elevation of Fas or Fas-L expression\(^{373,374}\). Blocking of Fas/Fas-L interactions by antibodies failed to block cytotoxic killing of leukaemia cell lines in some studies\(^{373-376}\).\(^{377}\). Fas resistant sub-lines of malignant human T-cells or myeloma cells were found to be as susceptible to cytotoxic drugs as their Fas-sensitive parental lines\(^{378,379}\). Therefore, the potential role of Fas signalling in cytotoxic killing of cancer cells remains controversial.

The majority of studies on the role of the Fas pathway in mediating apoptosis induction by cytotoxic drugs have been carried out using leukaemic and solid tumour cell lines. Therefore, this controversial issue was addressed using malignant cells freshly isolated from the peripheral blood of patients with B-CLL and AML.
3.2 Results

3.2.1 B-CLL cells

3.2.1.1 RT-PCR analysis of Fas and Fas-L transcripts

In order to determine whether Fas or Fas-L transcripts were upregulated following treatment with Chi, Flu or γ-irradiation, mRNA levels in B-CLL cells were quantified by RT-PCR. Analysis of transcripts from a representative experiment showed that treatment with all of these cytotoxic agents resulted in augmented expression of Fas transcripts, but not of Fas-L transcripts, following 24h of culture (Fig. 3.1A). RT-PCR data from multiple B-CLL isolates are summarised in Figure 3.1 B and 3.1 C. Relative to 24h controls, mean Fas mRNA levels increased by 2.3 fold (p>0.05) following treatment with 33μM Chi, 11 fold (p<0.001) with 165μM Chi, 4.0 fold (p=0.013) with 17.5μM Flu and 4.7 fold (p<0.001) following exposure to γ-irradiation (Fig. 3.1B). In contrast, Fas-L mRNA levels did not increase significantly following treatment with Chl, Flu or γ -irradiation (Fig. 3.1C).

3.2.1.2 Flow cytometric and western blot analysis of Fas and Fas-L

A representative histogram analysis of cell-surface Fas is shown in Fig. 3.2 A. Twenty-four hours treatment with 165μM Chl or 17.5μM Flu resulted in only marginal increases in Fas expression compared to the control. However, cells exposed to 10Gy γ-irradiation showed enhanced expression of Fas. FACScan analysis of multiple B-CLL samples showed that cell-surface Fas expression, expressed as the ratio Fas FITC MedCF/IgG FITC MedCF, increased 24h following γ-irradiation by a mean of 2.7 fold (p<0.001), with 16 of 22 B-CLL samples showing increases of 1.5 fold or more (Fig. 3.2 B). Flu increased surface Fas at 24h by a mean of 1.1 fold. Although this increase was statistically significant (p=0.007), none of the 16 samples showed an increase greater than 1.5 fold. No significant increase in Fas protein was observed at 24h treatment with 33 or 165μM Chl.

Western blot analysis of cell lysates from a representative B-CLL sample also showed the induction of Fas protein 24h after exposure to 10Gy γ-irradiation but not following treatment with Flu or Chl (Fig. 3.3 A). Analysis of multiple samples showed a mean 5-
fold increase in Fas expression (p=0.028) following γ-irradiation, with 15 of 20 samples showing increases greater than 1.5 fold (Fig. 3.3 B).

A western blot analysis of cell lysates from a B-CLL sample in a time course study at 6h, 15h and 24h revealed p53 upregulation and stabilisation in B-CLL cells treated with Chl, Flu or γ-irradiation (Fig. 3.4). However, Fas protein induction was only observed in B-CLL cells that had been treated with γ-irradiation with peak expression at 24h while the expression of p53 peaked at 15h. In contrast, no significant increase in Fas expression was observed following 24h incubation with 33μM Chl, 165μM Chl, or 17.5μM Flu (Fig. 3.3 B). Fas-L protein was not detected in any untreated or treated B-CLL sample by either western blot or FACScan analysis (data not shown).

3.2.1.3 Fas ligation does not augment killing of B-CLL cells by cytotoxic agents
To determine whether treatment with cytotoxic drugs or exposure to γ-irradiation enhanced the sensitivity of B-CLL cells to Fas-induced killing, treated B-CLL cells were incubated for 24h in the presence or absence of an agonistic anti-Fas IgM antibody. Apoptosis was quantified by morphological criteria. The percentage of apoptotic cells was evaluated by examination of Giesma-stained cytospin preparations. A representative example showed that an agonistic anti-Fas IgM did not induce apoptosis in B-CLL cells and failed to augment apoptosis induced by 165μM Chl or 10Gy γ-irradiation (Fig. 3.5). The induction of apoptotic morphology was further confirmed by electron microscopy (Fig. 3.6 and 3.7).

Morphological evaluation of apoptosis in multiple B-CLL isolates is summarised in Fig. 3.8. Spontaneous apoptosis at 24 h ranged between <0.5-39%. Different isolates showed varying sensitivity to different cytotoxic agents. The increase over spontaneous apoptosis ranged between <0.5-8.6% following treatment with 33μM Chl, 17-74% with 165μM Chl, 1-26% with 17.5μM Flu and 1.5-44% following exposure to 10Gy γ-irradiation. Mean apoptosis was increased by 1.5 fold (p=0.031) following treatment with 33μM Chl, 6 fold (p<0.001) with 165μM Chl, and 1.7 fold (p=0.031) with 17.5μM Flu. A 3.6 fold (p<0.001) increase in mean apoptosis was observed in cells exposed to 10Gy γ-irradiation. All of these increases were statistically significant (p<0.05). However, the
inclusion of anti-Fas IgM in the incubations did not result in a further significant increase in apoptosis under any of the incubation conditions (p>0.05). The inability of anti-Fas IgM to augment Chl-induced killing of B-CLL cells was also evident by inspection of data obtained using individual B-CLL isolates (Fig. 3.8A and B). However, incubation with anti-Fas IgM resulted in small increases in the percentage of apoptotic cells induced by Flu in 2/13 isolates (Fig. 3.8C) and by γ-irradiation in 5/21 isolates (Fig. 3.8D).

Quantitation of overall spontaneous cell death by flow cytometric analysis of PI-stained cells showed that the percentage of dead cells after 24 h incubation varied between 7.5-41% (Fig. 3.9). Additional killing by cytotoxic agents ranged between <0.5-4% with 33μM Chl, 6-39% with 165μM Chl, 2-29% with 17.5μM Flu and 2-37% following exposure to 10Gy irradiation. No increases in mean cell killing were observed under any of the treatment conditions when anti-Fas IgM was included in the incubations. Examination of data obtained for the individual isolates also failed to reveal a synergistic cytotoxic action between anti-Fas IgM and cytotoxic drugs or γ-irradiation. These conclusions were additionally verified in 17 B-CLL samples by manual counting of trypan blue-stained cells (not shown).

Apoptosis induction was also quantified by assessment of light scattering properties (Fig. 3.10) and by determining the proportion of cells with a subdiploid DNA content (Fig. 3.11). Both of these criteria clearly showed the dose-dependent induction of apoptosis by Chl and by γ-irradiation (Fig. 3.12). Anti-Fas IgM did not substantially augment the induction of apoptosis at any of the concentrations of Chl or doses of γ-irradiation tested. Similar results were obtained using two additional B-CLL isolates (not shown).

The Jurkat cell line, which is sensitive to Fas ligation, was used to verify that the anti-Fas IgM antibody induced apoptosis at the concentration routinely used in the experiments described above. Morphological analysis showed that 24h incubation with anti-Fas IgM increased the percentage of apoptotic cells, which was abrogated by 250ng/ml Fas blocking ZB4 antibody (Fig. 3.13A). Western blot analysis showed that 24h incubation with anti-Fas IgM induced extensive processing of pro-caspases 3, 8 as well as generation
of the p85PARP fragment (Fig. 3.13B). Pro-caspase-9 was also completely processed following Fas ligation. This is consistent with recent observations that Fas-induced death signalling via caspase-8 is amplified via cytochrome-c release and Apaf-1-mediated caspase-9 activation. The anti-p85 PARP antibody used here and in subsequent figures is highly specific for the neo-epitope generated following caspase-3 cleavage of PARP and therefore serves as a sensitive molecular criterion for caspase-3 activation. All of these anti-Fas IgM-induced apoptotic events were abrogated by the Fas blocking antibody ZB4 (Fig. 3.13).

3.2.1.4 Processing of pro-caspases and PARP
The processing of pro-caspases 3, 9 and 8 and the generation of the p85 PARP fragment from 116kDa PARP protein was analysed following treatment of B-CLL cells with apoptosis inducing agents.

Processing of caspases and of PARP in a B-CLL isolate is shown in Fig. 3.14. Substantial spontaneous apoptosis was observed in this experiment, as shown by the generation of p85 PARP at 24h incubation. Spontaneous apoptosis was associated with processing of pro-caspases 8 and 3 (relative to the day 0 control). However, only modest processing of pro-caspase-9 was observed. Additional dose-dependent processing of pro-caspases 3, 8 and 9 as well as PARP cleavage was induced by incubation with Chl. Flu and γ-irradiation also induced additional PARP cleavage, although, in this sample, substantial additional processing of any of the pro-caspases was not detected, when compared to the 24h control.

Data from studies on multiple isolates were normalised to pro-caspase or p85 PARP levels in the 24h control sample and are summarised in Fig. 3.15. Mean pro-caspase-8 levels in B-CLL samples decreased by 20% (p = 0.051) following treatment with 33μM Chl, 62% (p=0.003) with 165μM Chl, 39% (p=0.002) with 17.5μM Flu and 45% (p<0.001) following γ-irradiation exposure compared to the 24h control. Pro-caspase-3 levels also decreased by a mean of 3.5% (p>0.05) following treatment with 33μM Chl, 30% (p=0.016) with 165μM Chl, 19% (p>0.05) with 17.5μM Flu and 23 % (p=0.009) following γ-irradiation (Fig. 3.15A). The p85 PARP fragment in B-CLL samples (Fig.
3.15B) increased by a mean of 1.9 fold (p>0.05) following treatment with 33μM Chl, 2.7 fold (p=0.01) with 165μM Chl, 2.6 fold (p=0.03) with 17.5μM Flu and 2.4 fold (p=0.042) following γ-irradiation compared to the 24h control.

The time course study of caspase activation and PARP cleavage shown in Fig. 3.16 confirms that loss of immunoreactive pro-caspase-3 and 8 is accompanied by the generation of active subunits. Following treatment with 165μM Chl, an increase in generation of the subunits of caspases 3 and 8 and of the p85PARP fragment was first evident at 6h but was more pronounced by 12h. In this experiment, the complete processing of pro-caspase-8 by 24h was observed, whereas residual levels of pro-caspase-3 were still present. Consistently, it was observed that a greater proportion of pro-caspase-8 than of pro-caspase-3 was cleaved in response to each of the cytotoxic stimuli studies here (Fig. 3.15A). A second time course study also revealed substantial processing of pro-caspase-8 preceded pro-caspase-3 processing in B-CLL cells treated with Chl or Flu or exposed to γ-irradiation (Fig. 3.17). It was also noted that generation of the p85 PARP fragment apparently preceded detectable pro-caspase-3 processing. It is plausible that processing of a relatively small proportion of pro-caspase-3, undetectable by western blotting, is nevertheless sufficient to result in detectable PARP cleavage.

3.2.1.5 The Fas blocking antibody ZB4 does not abrogate apoptosis of B-CLL cells
Augmented interaction of pre-existing cell-surface Fas and Fas-L may contribute to the induction of apoptosis in some cellular contexts. Although expression of Fas-L on B-CLL cells was not detected by either western blotting or flow cytometry, it was important to eliminate the possibility that interaction of Fas with undetectable levels of Fas-L may play a role in the killing of B-CLL cells by cytotoxic agents. Therefore, the ZB4 monoclonal antibody was used, whose ability to completely abrogate Fas-induced PARP cleavage in Jurkat cells is shown in Fig. 3.13. Fig. 3.18 A shows that the viability of a B-CLL isolate, quantified by the MTT dye reduction assay, was decreased by Chl, Flu or γ-irradiation. Neither the agonistic anti-Fas IgM or the ZB4 blocking antibody altered the cytotoxic action of any of the agents.
The inability of the anti-Fas IgM to augment generation of the p85 PARP fragment by any of the agents used is shown in Fig. 3.18B confirming earlier conclusions that Fas ligation did not impact on B-CLL cell killing by cytotoxic agents. This figure also confirms that the ZB4 blocking antibody did not protect B-CLL cells from apoptosis induction by cytotoxic drugs or by γ-irradiation. The observations shown in Fig. 3.18 were repeated in two additional experiments (not shown).

3.2.1.6 c-FLIP expression

In the attempt to explain the resistance of B-CLL cells to Fas-mediated killing, expression of FADD and c-FLIP were analysed by western blotting. Different levels of c-FLIP were shown to be expressed in the B-CLL samples analysed (Fig. 3.19A). c-FLIP was also shown to be expressed in the Jurkat cell line. In a B-CLL sample treated with cytotoxic agents (Fig. 3.19B), expression of c-FLIP decreased while Fas expression increased when B-CLL cells were treated with 10Gy γ-irradiation. It was also observed that FADD levels decreased after B-CLL cells were treated with 10Gy γ-irradiation. No significant changes in c-FLIP, FADD or Fas expression was observed in this B-CLL sample after treatment with Chl or Flu.
**Figure 3.1** Semi-quantitative RT PCR analysis of B-CLL transcripts. A, analysis of actin (A), Fas (F) or Fas-L (FL) transcripts in B-CLL cells at day 0 (D0) or following 24h incubation with medium alone (control), Chl, Flu or 24h post-γ-irradiation (10Gy). B, C, intensities of Fas, Fas-L and actin PCR bands from multiple B-CLL samples were quantified. Fas/Actin and Fas-L/Actin ratios following treatment were compared to 24h controls and are presented in panels B and C respectively.
Figure 3.2 Flow cytometric analysis of B-CLL cells. A, analysis of cell-surface Fas expression in a representative B-CLL sample. R is the ratio of the MedCF of anti Fas IgG FITC antibody stained cells (open histogram) against IgG FITC isotype control stained cells (shaded histogram). B, analysis of multiple B-CLL samples after 24h incubation with Chl, Flu or post γ-irradiation.
Figure 3.3 Western blot analysis of B-CLL cells. A, Fas protein expression in a B-CLL sample following 24h incubation with Chl, Flu or post γ-irradiation. Numbers above bands are the intensities of individual bands normalised with respect to actin band intensities in the same lane. B, Fas western blot data for multiple B-CLL samples normalised with respect to actin.
Figure 3.4 Western blot analysis of B-CLL cells. Fas and p53 protein expression in a B-CLL sample following 6, 15h and 24h incubation with Chl, Flu or post γ-irradiation.
Figure 3.5 Morphology of Giesma-stained B-CLL cells. A, untreated cells, B, cells treated with 50 ng/ml anti-Fas IgM, C, cells treated with 165μM Chl, D, cells treated with 165μM Chl plus 50 ng/ml anti-Fas IgM, E, cells treated with 10Gy radiation, and F, cells treated with 10Gy radiation plus 50 ng/ml anti-Fas IgM. Cells were incubated for 24 hours with treatments. Cells showing condensed and fragmented nuclei or nuclear condensation are indicated (solid arrow).
Figure 3.6  Electron microscopic morphology of B-CLL cells (Overall magnification = 6,750x). The cells were incubated for 24h with: A, no addition and B, 165μM Chl.
Figure 3.7  Electron microscopic morphology of B-CLL cells (Overall magnification = 18,000x). The cells were incubated for 24h with: A, no addition and B, 165\( \mu \)M Chl.
Figure 3.8 Action of anti-Fas IgM on apoptosis induction in B-CLL cells. B-CLL cells were treated with cytotoxic agents in the presence or absence of 50 ng/ml anti-Fas IgM as indicated. The percentage of apoptotic cells was determined by morphological criteria. The lines connect data points obtained using individual B-CLL isolates. The standard errors of individual determinations were <10% of the mean values reported. Mean data for all of the isolates, shown as a histogram on the inset, were analysed by student’s t test. The percentage of apoptotic cells in drug-treated or irradiated samples were compared to the corresponding controls (** p<0.05). For each cytotoxic treatment, incubations in the presence of anti-Fas were also compared to the corresponding incubations in the absence of anti-Fas (†, p>0.05).
Figure 3.9 Action of anti-Fas IgM on viability of B-CLL cells. B-CLL cells were treated with cytotoxic agents in the presence or absence of 50 ng/ml anti-Fas IgM as indicated. The percentage of non-viable cells was determined by flow cytometric analysis of PI-stained cells. Mean data for all of the isolates, shown as a histogram on the inset, were analysed by student’s t test. The percentage of apoptotic cells in drug-treated or irradiated samples were compared to the corresponding controls (*, p>0.05; **, p<0.05). For each cytotoxic treatment, incubations in the presence of anti-Fas were also compared († test) to the corresponding incubations in the absence of anti-Fas (‡, p>0.05)
Figure 3.10 Quantitation of B-CLL apoptosis by FSC/SSC analysis. The percentage of apoptotic B-CLL cells at 24h was determined by FSC/SSC analysis with the flow cytometer and Cell Quest software. The percentage of apoptotic cells found in the R2 region is indicated.
Figure 3.11 Quantitation of B-CLL apoptosis by flow cytometric analysis. The percentage of apoptotic B-CLL cells at 24h was determined by flow cytometric analysis with Cell Quest software of the proportion of cells with a subdiploid DNA content after propidium iodide staining. The percentage of cells found in the M1 region is indicated.
Figure 3.12 Graphic representation of the data from Figs 3.10 and 3.11. The percentage of apoptotic B-CLL cells was determined by flow cytometric determination of the proportion of cells with a subdiploid DNA content (A) or changes in light scattering properties (B). Incubations were carried out in the absence or presence of 50 ng/ml anti-Fas IgM.
Figure 3.13 Effect of anti-Fas ZB4 antibodies on apoptosis, and caspase and PARP processing in Jurkat cells. Jurkat cells were incubated with 50 ng/ml agonistic anti-Fas IgM or 250 ng/ml Fas blocking antibody ZB4 with anti-Fas IgM as indicated above. A. The percentage of apoptotic cells was determined by morphological criteria. B. Western blot analysis of the processing of pro-caspases and PARP.
Figure 3.14 Western blot analysis of the processing of pro-caspases and PARP in B-CLL cells from a patient following treatment with Chl, Flu or γ-irradiation. Numbers above bands are the intensities of individual bands normalised with respect to actin band intensities in the same lane.
Figure 3.15 Caspase processing in B-CLL cells. A, processing of pro-caspase 8 and pro-caspase 3 in B-CLL samples after 24h treatment, estimated by western blotting. Pro-caspase band intensities were normalised to actin and the ratio was set to 1.0 for the control for each sample. B, processing of PARP to its p85 fragment in B-CLL samples following 24h treatment. PARP p85 was compared to actin levels and the ratio was set to 1.0 for the control in each sample. Student's *t* test was carried out using pro-caspase/actin or p85 PARP/actin ratios (*, p=0.05; **, p>0.05; †, 0.01<p<0.05; ††, p<0.01).
**Figure 3.16** Time course of processing of pro-caspases and PARP in a B-CLL isolate. B-CLL cells were incubated with no additions or with 165 μM Chl. Samples were processed for western blot analysis at the times indicated. Pro-caspase 8 was detected using the Pharmingen antibody. The processed form of caspase 8 was detected using goat polyclonal anti-caspase 8 p20 antibody (Santa Cruz; sc-6136).
Figure 3.17 Time course of processing of pro-caspases and PARP in a B-CLL isolate. Pro-caspase band intensities were normalised with respect to actin and the ratio was set to 100% for day 0. PARP p85 band intensities were normalised to actin levels.
**Figure 3.18** Actions of anti-Fas antibodies on B-CLL killing. A, MTT assay of a B-CLL sample incubated with 250 ng/ml ZB4, 50ng/ml anti Fas IgM or no antibody in addition to cytotoxic agents. The absorbance of MTT reduced by the 24h control was set at 1 and the viability of treated cells were compared to this value. B, western blot of the processing of PARP to PARP p85 in B-CLL cells from same patient following treatment with Chl, Flu or γ-irradiation with or without 50ng/ml anti Fas IgM (F) or 250ng/ml ZB4 (Z).
Figure 3.19 A, Western blot of c-FLIP and FADD expression in B-CLL samples. B, Western blot of Fas, c-FLIP and FADD expression in B-CLL samples at 24h following treatment with 10Gy γ-irradiation. Jurkat cell line was used as control. Values correspond to ratio of c-FLIP or FADD band intensity normalised to actin bands.
3.2.2 AML cells

3.2.2.1 Fas and Fas-L expression and Fas sensitivity.

FACScan analysis for cell-surface Fas expression on multiple AML samples (n=14) revealed expression in all samples (Fig. 3.20). The intensity of Fas expression varied between samples, with a range of Fas-FITC/IgG-FITC median ratio of 1.7-5.8. The highest Fas expression was found on a FAB M5 AML (n=1, ratio=5.8) followed by M4 (n=4, median =3.8, range 3.2-4.1), M0 (n=1, ratio = 3.0), M2 (n=1, ratio=2.0) and M1 (n=7, median=1.9, range 1.8-3.2). No FAB types M3, M6 or M7 were available for this study.

To determine if AML cells were sensitive to anti-Fas IgM, cells were incubated for 24 hours in the presence or absence of 500ng/ml of anti-Fas IgM. Apoptosis was quantified by morphological criteria and loss of cell viability was quantified by flow cytometric analysis of PI-stained cells. Spontaneous apoptosis and loss of viability at 24 h ranged between 1.5-38%, and 1.9-53% respectively. Different isolates showed varying sensitivity towards anti-Fas IgM. Agonistic anti-Fas IgM induced apoptosis in 10 out of 12 AML samples (p=0.046, Student t-test) and induced loss of viability in 7 out of 9 samples (p=0.123) (Fig. 3.21). Specific Fas induced apoptosis and loss of viability ranged from 0.4–62% (n=10) and 0.6-39% (n=7) respectively in Fas sensitive AML samples. However, using Spearman’s rank correlation statistical test, no significant relationship was found between Fas expression or FAB type against specific Fas induced apoptosis (p>0.05) (Fig. 3.22). The same conclusion was obtained by analysis of % loss of viability (data not shown).

Fas-L could not be detected by flow cytometry or by western blot analysis in AML cells (data not shown).

3.2.2.2 Induction of apoptosis by anti-Fas IgM in combination with cytotoxic agents

To determine whether treatment with cytotoxic drugs or exposure to γ-irradiation enhanced the sensitivity of AML cells to Fas-induced killing, treated AML cells were incubated for 24h in the presence or absence of an agonistic 500ng/ml anti-Fas IgM
antibody. Apoptosis was quantified by morphological criteria. Different isolates showed varying sensitivity to different cytotoxic agents (Fig. 3.23). The mean % apoptosis values obtained for all the samples are shown in the inset to Fig. 3.23. Compared to mean spontaneous apoptosis, mean apoptosis increased by 1.8 fold (p=0.010) following treatment with 17.5μM Flu, 1.7 fold (p=0.020) with 41μM Ara-C, and 1.4 fold (p=0.035) with 0.1μM DNR. A 2.4 fold (p=0.002) increase in mean apoptosis was observed in cells exposed to 10Gy γ-irradiation. All of these increases were statistically significant (p<0.05). The specific apoptotic increase in 13 AML samples ranged from 0 - 32% following treatment with 17.5μM Flu, 0 - 24% with 41μM Ara-C, 0 -19% with 0.1μM DNR, and 0 - 42% following treatment with 10Gy γ-irradiation. The inclusion of 500ng/ml anti-Fas IgM with cytotoxic agents or γ-irradiation did increase % apoptosis significantly (p<0.05, n=13) when compared with cytotoxic treatment or γ-irradiation alone. Furthermore, different AML isolates showed different sensitivity towards anti-Fas IgM in combination with cytotoxic agents or γ-irradiation. Inspection of data from individual isolates showed incubation with anti-Fas IgM resulted in additional increases in the percentage of apoptotic cells in 10/12 isolates treated with Flu, 11/12 with Ara-C, 11/12 with DNR and 8/12 isolates following treatment γ-irradiation.

To determine whether the combination of anti-Fas IgM and cytotoxic agents was either additive or synergistic, the experimentally observed apoptosis induced by drugs or γ-irradiation in combination with anti-Fas IgM was compared with the theoretical apoptosis (Fig. 3.24). There was no significant difference (p>0.05, n=13) in apoptosis between experimental apoptosis and theoretical apoptosis with cytotoxic agents in combination with anti-Fas IgM, suggesting that the killing of AML cells by simultaneous incubation with anti-Fas IgM and cytotoxic agents was essentially additive. However, looking at individual AML isolates, different samples responded differently to anti-Fas IgM in combination with cytotoxic agents. Looking at individual cases, super-additive Fas induced apoptosis was observed in 2/12 samples treated with Flu, 2/12 with Ara-C, 3/12 with DNR and 3/12 samples following exposure to γ-irradiation.

Quantitation of cell death assessed by flow cytometric analysis of PI-stained cells confirmed the apoptotic results (data not shown).
3.2.2.3 Effect of cytotoxic agents on Fas and Fas-L expression

FACScan analysis of cell-surface Fas was expressed as the ratio Fas FITC MedCF/IgG FITC MedCF on multiple AML samples (n=15). When the aggregated data were analysed, Fas expression did not increase significantly at 24h following treatment with Flu (p>0.05), Ara-C (p>0.05), DNR (p>0.05) or γ-irradiation (p>0.05) (Fig. 3.25). However, looking at individual cases, greater than 1.5 fold increase in Fas expression was observed in 1 sample after treatment with DNR or γ-irradiation, and 2 other AML samples treated with γ-irradiation.

Western blot analysis of Fas protein from multiple AML samples (n=11), showed no significant increase at 24h following treatment with Flu, Ara-C, DNR or γ-irradiation (Fig. 3.26). However, like FACScan analysis, looking at individual cases, greater than 1.5 fold increase in Fas expression was observed in 1 sample treated with DNR or γ-irradiation, and 1 other sample treated with γ-irradiation. The samples showing increased Fas expression by western blotting were the same samples that showed Fas upregulation by FACScan analysis.

3.2.2.4 Processing of pro-caspases and PARP

The processing of pro-caspases 3, 9 and 8 and the generation of the p85 PARP fragment from 116kDa PARP protein was analysed following treatment of AML cells with cytotoxic agents in presence or absence of agonistic anti-Fas IgM.

Processing of caspases and of PARP in AML isolates is shown in Fig. 3.27 and Fig. 3.28. Processing of pro-caspases 3, 8 and PARP to its p85 fragment was observed in AML samples after treatment with cytotoxic agents, with a modest additional cleavage in the presence of anti-Fas IgM. Pro-caspase-9 cleavage was also observed after treatment with cytotoxic agents (Fig. 3.27).

Data from western blotting studies on additional eight AML isolates also showed similar pro-caspase-8 and pro-caspase-3 cleavage after cytotoxic treatment (data not shown).
3.2.2.5 The Fas blocking antibody ZB4 does not abrogate apoptosis of AML cells

Although expression of Fas-L on AML cells was not detected by either western blotting or flow cytometry, it was important to eliminate the possibility that interaction of Fas with undetectable levels of Fas-L may play a role in the killing of AML cells by cytotoxic agents. Figure 3.29 A shows that the viability of an AML isolate, quantified by the MTT dye reduction assay, was decreased by Flu, DNR or γ-irradiation. Anti-Fas IgM induced a slight reduction in viability in the AML isolate, however the ZB4 Fas-blocking antibody did not alter the cytotoxic action of any of the agents. This was further confirmed by apoptotic analysis quantified by morphological criteria and loss of viability using PI and FACScan analysis (data not shown).

A western blot analysis of the same AML isolate confirmed the inability of the ZB4 blocking antibody to protect AML cells against apoptosis induced by cytotoxic drugs or by γ-irradiation (Fig. 3.29B). Generation of the p85 PARP fragment was induced by all of the cytotoxic agents and was not inhibited when cells were co-incubated with ZB4. This was further confirmed with the generation of active-caspase-3 p20 subunit.
Figure 3.20 Expression of cell-surface Fas on AML cells. Fas expression was detected by FACScan analysis and is represented as the ratio of the MedCF of anti Fas IgG FITC antibody stained cells against IgG FITC isotype control stained cells.
Figure 3.21 Action of Anti-Fas IgM on AML cells. A, Percentage apoptosis (assessed by morphology) and B, percent loss of viability (PI staining) of AML samples after 24h in the presence or absence of 500ng/ml anti-Fas IgM. Data was analysed using the student’s t-test.
Figure 3.22 Relationship between AML FAB type, Fas expression and anti-Fas mediated apoptosis.  

A. Relationship between Fas expression assessed by flow cytometric analysis and specific anti-Fas mediated apoptosis at 24 hours.  

B. Relationship between FAB type and specific anti-Fas mediated apoptosis at 24 hours. Apoptosis was assessed by morphology.
Figure 3.23 Action of anti-Fas IgM on apoptosis induction of AML cells. AML cells were treated with cytotoxic agents in the presence or absence of 500 ng/ml anti-Fas IgM as indicated. The percentage of apoptotic cells was determined by morphological criteria. The lines connect data points obtained using individual B-CLL isolates. Mean data for all of the isolates, shown as histograms on the inset, were analysed by student’s t test. The percentage of apoptotic cells in drug-treated or irradiated samples were compared to the corresponding controls (**, p<0.05). For each incubation condition, incubations in the presence of anti-Fas were also compared to the corresponding treatment in the absence of anti-Fas (††, p<0.05).
Figure 3.24 Specific apoptotic induction in AML samples treated with cytotoxic agents in combination with anti-Fas IgM after 24h. Histograms represent experimental observed % specific apoptosis after treatment with cytotoxic agents and anti-Fas IgM combined versus theoretical sum of cytotoxic agents and Anti-Fas IgM added alone.
**Figure 3.25** Flow cytometric analysis of multiple AML samples after 24h incubation with Flu, Ara-C, DNR or post γ-irradiation. Analysis of cell-surface Fas expression in AML samples is represented as the ratio of the MedCF of anti Fas IgG FITC antibody stained cells against IgG FITC isotype control stained cells.
Figure 3.26 Western blot analysis of Fas expression in multiple AML samples following 24h incubation with Flu, Ara-C, DNR or post γ-irradiation. Fas expression was normalised with respect to actin.
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**Figure 3.27** Western blot analysis of the processing of pro-caspases and PARP in AML (AML229) cells from a patient following treatment with Flu, Ara-C, DNR or γ-irradiation. Numbers below p85-PARP bands are intensities of individual bands normalised with respect to p116 PARP band intensities in the same lane. Numbers below pro-caspase bands are intensities of individual bands normalised with respect to actin band intensities in the same lane. The caspase ratio obtained for the day 0 control bands were arbitrarily set at 1.
Figure 3.28  Western blot analysis of the processing of pro-caspases and PARP in AML (AML223) cells from a patient following treatment with Flu, Ara-C, DNR or γ-irradiation. Numbers below p85-PARP bands are intensities of individual bands normalised with respect to p116 PARP band intensities in the same lane. Numbers below pro-caspase bands are intensities of individual bands normalised with respect to actin band intensities in the same lane. The pro-caspase ratio obtained for the day 0 control bands were arbitrarily set at 1.
Figure 3.29 Actions of anti-Fas antibodies on AML killing. A. MTT assay of a AML sample incubated with 250 ng/ml ZB4, 500ng/ml anti Fas IgM or no antibody in addition to cytotoxic agents. B, western blot of the processing of PARP to PARP p85 and pro-caspase 3 to active-caspase 3 p20 subunit in AML cells from same patient following treatment with Flu, Ara-C, DNR or γ-irradiation with or without 250ng/ml ZB4. Numbers below pro-caspase and PARP p85 bands are intensities of individual bands normalised with respect to actin band intensities in the same lane. The pro-caspase3 ratio obtained for the day 0 control bands were arbitrarily set at 1. Numbers below active-caspase 3 p20 bands are intensities of individual bands normalised with respect to pro-caspase 3 band intensities in the same lane.
3.3 Discussion

The potential role of Fas signalling in cytotoxic killing of cancer cells remains controversial. The majority of studies on the role of the Fas pathway in mediating apoptosis induction by cytotoxic drugs have been carried out using leukaemic and solid tumour cell lines\textsuperscript{262,263,366,367}. Therefore this issue was addressed by studying the potential roles of Fas and Fas-L in the killing of freshly isolated B-CLL and AML cells by cytotoxic agents.

Freshly isolated B-CLL cells expressed low levels of Fas. Treatment with Chl, Flu or $\gamma$-irradiation resulted in augmented expression of Fas transcripts. Killing of B-CLL cells by cytotoxic agents has been shown to be compromised by p53 mutations\textsuperscript{385}. Here, it was observed that the killing of B-CLL cells by cytotoxic drugs or $\gamma$-irradiation was preceded by the upregulation of p53 levels and of p53-mediated transcription. p53-mediated transcription and apoptosis have been demonstrated to be blocked by the p53 inhibitor pifithrin-$\alpha$ suggesting that killing of B-CLL cells by cytotoxic agents is at least partially dependent on p53\textsuperscript{386,387}. Since Fas\textsuperscript{199,260,368} and Fas-L\textsuperscript{388} genes are potential transcriptional targets of p53, it was shown that Fas but not its ligand was induced at the mRNA level following treatment with these agents. However, a substantial increase in cell-surface Fas expression was only observed following $\gamma$-irradiation. This observation suggests that Fas expression may be regulated by a complex mechanism, which permits translation of Fas transcripts following $\gamma$-irradiation, but not in cells treated with Chl or Flu. Fas-L protein expression was not detected in untreated B-CLL cells or following treatment with drugs or $\gamma$-irradiation.

Morphological criteria, PI and trypan blue exclusion, quantitation of cells with a subdiploid DNA content, FACScan analysis of light-scattering, the MTT test and western blotting quantitation of the p85 PARP fragment was used to assess the putative roles of Fas and Fas-L in the killing of B-CLL cells by cytotoxic agents. Analysis of data obtained using multiple B-CLL isolates showed that cell killing by Chl, Flu or $\gamma$-irradiation was not significantly augmented by an agonistic anti-Fas IgM antibody. However, inspection of individual experiments suggested that Fas ligation resulted in the augmentation of
morphologically detectable apoptosis in a minority of samples treated with Flu or \( \gamma \)-irradiation. However, these effects were modest and were not evident when other cell death criteria were employed. Killing by each of the agents was also refractory to inhibition by the ZB4 Fas blocking antibody, ruling out the possibility that interactions between Fas and pre-existing Fas-L may contribute to the killing mechanism. Taken together, the data show that signalling by the Fas/ Fas-L system does not play a significant role in the apoptotic killing of B-CLL cells by cytotoxic agents.

Cytotoxic drugs induce apoptosis by triggering cytochrome-c release with the consequent Apaf-1-dependent activation of caspase-9. Western blot experiments have shown that pro-caspase-9 processing was only observed when B-CLL cells were treated with high dose Chl. However, activation of pro-caspase-9 following binding to Apaf-1 does not require its processing. Therefore, the role of caspase-9 in killing of B-CLL cells by other cytotoxic treatments other than high dose Chl is unclear at present.

It was observed that pro-caspase-8 processing was accompanied by the activation of pro-caspase-3 in response to all of the death stimuli studied here in B-CLL cells. In contrast to an earlier report by King et al., a substantial proportion of pro-caspase-8 was processed in response to cytotoxic treatments in B-CLL cells, which is suggestive of an important role in apoptosis induction. In some cellular contexts, cytotoxic drugs can activate caspase-8 in a Fas-independent manner. Since neither an agonistic nor a blocking anti-Fas IgM antibody significantly affected apoptosis induction, it was concluded that the cytotoxic agents used here also activated caspase-8 via a mechanism independent of Fas signalling. The death receptors DR4 and DR5 also activate caspase-8 as a consequence of binding their cognate ligand, TRAIL. However, it was observed that B-CLL cells were completely refractory to killing by TRAIL, either when added alone or in conjunction with Chl, Flu or \( \gamma \)-irradiation (see chapter 4).

Although Apaf-1 itself does not activate caspase-8, pro-caspase-8 may be processed by a purely intracellular mechanism analogous to the activation of caspase-9 by the apoptosome. For example, recent studies on the Jurkat and NCI-H460 lung cancer cell lines have shown that caspase-8 activation in response to cytotoxic drugs is mediated
by mitochondria rather than by death receptors. In Jurkat cells, caspase-8 may function as a terminal executioner caspase rather than as an apical activator of caspase processing pathways. The close similarity in the time courses of activation of caspases 3 and 8 in Chl-treated B-CLL cells is consistent with the possibility that both of these proteases function as executioners in the apoptotic pathway. The low specificity of peptide-aldehyde caspase-8 inhibitors and the refractory nature of B-CLL cells to transfection with dominant negative inhibitory constructs or with the selective caspase-8 inhibitor crmA have precluded a definitive assessment of relationship between the activation of caspases 3 and 8 in B-CLL cells.

In some studies, B-CLL cells were reported to be resistant to killing consequent to Fas ligation. However, in these studies cell-surface Fas expression was augmented by incubation with anti CD40 antibodies or γ-IFN. Signalling via CD40 or the IFNs provides a potent anti-apoptotic signal to B-CLL cells in addition to Fas upregulation, suggesting that death induction by Fas may have been blocked by the stimulus used to upregulate Fas. Therefore, it is unclear whether Fas ligation in the absence of cytoprotective signals is toxic to these cells. In fact, other reports suggest that B-CLL cells may be susceptible to Fas-mediated killing and that sensitivity may be dependent on the stimulus used to upregulate Fas expression. Therefore, sensitivity of B-CLL cells to apoptosis induction consequent to Fas ligation has remained controversial.

The data here suggest that B-CLL cells even when induced to express substantial levels of cell-surface Fas by γ-irradiation treatment, cannot be induced to activate the apoptotic pathway in response to Fas ligation. The reasons for this defect are unclear. B-CLL cells express functional pro-caspase-8 as well as substantial levels of FADD, the signalling components required for downstream signalling by Fas. The existence of inactivating mutations in the Fas molecule have also been ruled out. c-FLIP, an inhibitor of death signalling by Fas, was shown to be expressed in B-CLL cells. Levels of c-FLIP in B-CLL cells decreased after treatment with 10Gy γ-irradiation, while Fas expression increased. These changes would be expected to sensitise B-CLL cells to anti-Fas IgM. However, FADD levels also decreased after treatment with 10Gy γ-irradiation. Low levels of FADD could impair the formation of the DISC after Fas activation.
Jurkat cells, expression of c-FLIP is lower than in some B-CLL samples, which could explain why the Jurkat cell line is highly sensitive to anti-Fas IgM, while B-CLL cells are resistant. However, some B-CLL samples also express small amounts of c-FLIP, suggesting that c-FLIP expression alone may not account for Fas-resistance. Recent studies have suggested that molecules other than c-FLIP may block Fas signalling. It is plausible that the resistance of B-CLL cells to killing via death receptor ligation may result from elevated expression of an unidentified inhibitor. Elucidation of the basis of Fas resistance in B-CLL may be of importance, since this may contribute to the expansion of malignant cells in this disease.

Unlike B-CLL cells, AML cells expressed relatively high but variable levels of cell surface Fas, with M4 and M5 FAB subtypes expressing higher levels of Fas, confirming previously published studies by Lewis et al., Li et al. and Iijima et al. Fas expression did not change following treatment with Flu, Ara-C, DNR or γ-irradiation. However, individual isolates showed that Fas expression did increase following treatment with γ-irradiation in a minority of AML samples, and DNR in one sample. Fas-L protein expression in untreated AML cells or following treatment with drugs or γ-irradiation was not detected.

Some AML samples were weakly sensitive to anti-Fas IgM mediated apoptosis. However, sensitivity to anti-Fas IgM did not correlate with cell surface Fas expression or FAB type. Analysis of data obtained using multiple AML isolates showed that cell killing by Flu, Ara-C and DNR was often additive with killing by agonistic anti-Fas IgM antibody. However, no super-additive killing was observed. The difference in % apoptosis between the experimental combination of cytotoxic agent in combination with anti-Fas IgM against the theoretical combination was not statistically significant. Therefore, the cytotoxic agents did not sensitise AML cells to anti-Fas IgM. In this study it was observed that anti-Fas IgM induced additive apoptosis in combination with cytotoxic agents, while Lewis et al. did not observe additive or synergistic cytotoxic effects with AML cells treated with DNR in combination with anti-Fas IgM. It was also established that Fas-blocking ZB4 antibody did not inhibit Flu, Ara-C, DNR or γ-
irradiation induced cell death, ruling out the possibility that interactions between Fas and pre-existing Fas-L may contribute to the killing mechanism. Other studies have also shown that treatment of AML cells with DNR or etoposide does not involve the Fas/Fas-L system.

By western blot analysis, it was observed that pro-caspase-8 processing accompanied the activation of pro-caspase-3 in response to all of the death stimuli studied here in AML cells. However the addition of anti-Fas IgM resulted in additional caspase-3 processing and PARP cleavage to its p85 fragment in combination with cytotoxic agents, confirming the additive apoptotic effect of ant-Fas IgM. In addition, the Fas-blocking ZB4 antibody did not inhibit PARP cleavage and pro-caspase-3 processing, further confirming that the Fas/ Fas-L system does not play a significant role in the apoptotic killing of AML cells by cytotoxic agents. Taken together, the data show that signalling by the Fas/ Fas-L system does not play a significant role in the apoptotic killing of AML cells by cytotoxic agents. However, AML cells are sensitive to Fas mediated apoptosis, and Fas and cytotoxic treatments induced apoptosis through two distinct routes which converge downstream to activate caspases and initiate PARP cleavage.

In some studies, a direct correlation between Fas expression and drug sensitivity was observed. Furthermore, a direct correlation between Fas and drug resistance in cell lines have also been demonstrated. However, the study by Lewis et al. has been confirmed by establishing the absence of a correlation between DNR induced apoptosis and levels of Fas expression, and that DNR-induced apoptosis was not related to susceptibility to Fas-mediated apoptosis. In addition, it was observed that their was no correlation between sensitivity towards Flu, Ara-C or γ-irradiation induced apoptosis and levels of Fas expression by AML cells, or the susceptibility of AML cells to anti-Fas IgM mediated apoptosis. Studies on different cell types including human myeloma cell lines have also shown a lack of cross-resistance to Fas and cytotoxic drugs.

In conclusion, the observations here argue against a major role for the Fas/Fas-L signalling pathway in drug- or γ-irradiation-induced apoptosis in B-CLL and AML cells. Nevertheless, the activation of caspase-8, which has been considered to be exclusively...
involved in killing via ligation of death receptors, appears to play a role in the killing of both B-CLL and AML cells by these agents. The refractory nature of B-CLL cells to Fas cytotoxicity precludes the therapeutic use of Fas-L either singly or in combination with cytotoxic drugs in the development of novel treatment protocols in this malignancy. However, although cytotoxic agents could not upregulate Fas expression or induce apoptosis in a Fas-dependent pathway, the additive nature of Fas and drug-induced killing of at least some AML cells suggests that combined use of these agents may be potentially useful in AML therapy.
Chapter 4

In-vitro susceptibility of B-cell chronic lymphocytic leukaemia cells and acute myeloid leukaemia to TRAIL-induced apoptosis.

4.1 Introduction

The best characterised death receptors are Fas and the TNF receptor 1 which bind to the ligands Fas-L and TNF, respectively, thereby directly triggering a suicide signal transduction pathway. However, the therapeutic usefulness of Fas-L and TNF against cancer is limited by their acute toxicity. TRAIL has been reported to induce apoptosis in various tumour cells but not normal cells. Studies in mice and non-human primates have shown that TRAIL can induce apoptosis in human tumours, but is non-toxic to normal organs or tissues, suggesting that TRAIL may be a potentially useful anticancer agent.

Recent in-vitro evidence suggests upregulation of the TRAIL receptor DR5 via transcription factor p53 and/or NF-κB consequent to DNA damage by cytotoxic agents. In human glioma cells, etoposide increased DR5 expression via p53, resulting in augmented TRAIL induced apoptosis. However, other studies have also shown p53 independent upregulation of DR5 in acute leukaemia, glioblastoma, ovarian, and colon cancer cell lines. Acute leukaemia cell lines lacking wild type p53 treated with etoposide, Ara-C, or doxorubicin increased DR5 but not DR4, DcR1, DcR2, Fas-L, or TRAIL protein levels. This change in DR5 expression could be mediated by transcription factor NF-κB. Other studies have shown that treatment of epithelial cell-derived tumour cell lines with etoposide upregulate DR4 and DR5 via NF-κB activation. More importantly, treated cell lines were significantly more sensitive to TRAIL induced apoptosis than untreated cells. Therefore, upregulation of DR5 or DR4 by cytotoxic drugs can lead to a synergistic apoptosis with TRAIL. However, it has also been reported that DcR1 and DcR2 can also be upregulated by p53 thereby making cells resistant to TRAIL induced apoptosis.
TRAIL may have potential in treating human malignancies in combination with cytotoxic drugs\textsuperscript{198}. The majority of studies which have implicated TRAIL and its receptors in the killing of leukaemia cells by cytotoxic agents have been carried out using cell lines. Therefore, this controversial issue was addressed using malignant cells freshly isolated from the peripheral blood of patients with B-CLL and AML, and determined whether additive or super-additive killing could be obtained by simultaneous \textit{in-vitro} treatment with cytotoxic agents and TRAIL.
4.2 Results

4.2.1 B-CLL cells

4.2.1.1 Sensitivity of B-CLL cells to TRAIL.

The TRAIL-sensitive Jurkat cell line was used as a control to verify that TRAIL induced apoptosis (Fig. 4.1A). Jurkat cells were incubated for 24h in the presence of 0, 10, 50, 100 or 500ng/ml TRAIL. Morphological analysis showed that 24h incubation with TRAIL increased the percentage of apoptotic cells in a dose-responsive manner. Similar data were obtained using the viability assay (data not shown).

To determine if B-CLL cells were sensitive to TRAIL alone, nine B-CLL samples were incubated for 24h in the presence or absence of 500ng/ml TRAIL. Apoptosis was quantified by morphological criteria (Fig. 4.1B). Spontaneous apoptosis at 24h ranged between 2-18%. However, B-CLL samples were resistant to 500ng/ml of TRAIL (p>0.05). This was confirmed by quantifying cell death by PI staining followed by flow cytometric analysis (Fig. 4.1C). The data showed that the percentage of dead cells after 24h incubation in the absence of TRAIL varied between 9.6-32% and that B-CLL cells were resistant towards TRAIL induced cell death (p>0.05). Inspection of data for the individual isolates showed incubation with TRAIL resulted in very low increases in the percentage of non-viable cells in 6 out of 9 samples with a specific loss of viability range of 0.1-3.3%.

4.2.1.2 TRAIL does not augment killing of B-CLL cells by cytotoxic agents

To determine whether treatment with cytotoxic drugs or exposure to γ-irradiation enhanced the sensitivity of B-CLL cells to TRAIL-induced killing, B-CLL cells were treated with 33μM Chl, 165μM Chl, 16.5μM Flu or 10Gy γ-irradiation for 24h in the presence or absence 500ng/ml TRAIL. Apoptosis was quantified by morphological criteria and loss of viability by PI staining and FACScan analysis.

Different isolates showed varying sensitivity to different cytotoxic agents (Fig. 4.2). Compared to mean spontaneous apoptosis, mean apoptosis increased by a 5.5 fold (p=0.133) following treatment with 33μM Chl, 11 fold (p<0.001) with 165μM Chl and 4.7 fold (p=0.005) with 17.5μM Flu. A 5.9 fold (p=0.07) increase in mean apoptosis was
observed in cells exposed to 10Gy γ-irradiation. The specific apoptotic increase over spontaneous apoptosis ranged between 2.1 - 38% following treatment with 33μM Chl, 76 - 98% with 165μM Chl, 15 - 48% with 17.5μM Flu, and 13% - 91% following exposure to 10Gy γ-irradiation. One B-CLL sample was resistant to γ-irradiation. The inclusion of 500ng/ml TRAIL in the incubations did not result in a significant increase in apoptosis in combination with cytotoxic agents or γ-irradiation (p>0.05) compared with cytotoxic treatment or γ-irradiation alone. However, individual isolates incubated with TRAIL resulted in very small increases in the percentage of apoptotic cells induced by 33μM Chl in 2/4 samples, 165μM Chl in 2/4 samples, 17.5μM Flu in 3/6 samples and by 10Gy γ-irradiation in 2/6 samples.

To further investigate possible synergistic killing by the combinations of TRAIL and cytotoxic agents, the experimental apoptosis induced by the cytotoxic agents in combination with TRAIL was compared with the theoretical apoptosis (Fig. 4.3). Looking at individual cases, no super-additive TRAIL induced apoptosis was seen. There was no significant difference (p>0.05) between experimental and theoretical apoptosis after treatment with cytotoxic agents in combination with TRAIL.

Similar results were obtained by quantitation of cell death assessed by flow cytometric analysis of PI-stained cells (Fig. 4.4). Compared to mean spontaneous cell death, mean loss of viability increased by 1.1 fold (p=0.007) following treatment with 33μM Chl, 2.2 fold (p=0.093) with 165μM Chl, and 1.3 fold (p=0.132) with 17.5μM Flu. A 1.5 fold (p=0.036) increase in loss of viability was observed in cells exposed to 10Gy γ-irradiation. Not all of these increases were statistically significant. This could be due to the low sample numbers analysed. The specific loss of viability induced by cytotoxic agents ranged between 2.5-4.1% with 33μM Chl, 1-68% with 165μM Chl, 0.2-25% with 17.5μM Flu and 8.8-20% following treatment with 10Gy irradiation. Two B-CLL samples were resistant to γ-irradiation. The inclusion of TRAIL in the incubations did not result in a significant increase in loss of viability in combination with cytotoxic agents or γ-irradiation compared with cytotoxic treatment or γ-irradiation alone (p>0.05). However, individual isolates incubated with TRAIL resulted in very small increases in loss of
viability induced by 33μM Chl in 2/3 samples, 165μM Chl in 5/6 samples, 17.5μM Flu in 4/7 samples and by 10Gy γ-irradiation in 4/7 samples.

To further investigate possible synergistic killing by combinations of TRAIL and cytotoxic agent, the experimental loss of viability induced by the cytotoxic agent in combination with TRAIL was compared with the theoretical loss of viability (Fig. 4.5). Looking at individual cases, no super-additive TRAIL induced loss of viability was seen. There was no significant difference (p>0.05) between experimental and theoretical apoptosis after treatment with cytotoxic agents in combination with TRAIL.

4.2.1.3 RT-PCR analysis of DR4, DR5, DcR1 and DcR2 transcripts

In order to determine whether DR4, DR5, DcR1 or DcR2 transcripts were upregulated following treatment of B-CLL cells with Chl, Flu or γ-irradiation, their mRNA levels were quantified by RT-PCR (Fig. 4.6). Relative to 24h controls, mean DR4 mRNA levels did not increase significantly (p>0.05) following treatment with 33μM Chl, 165μM Chl, 17.5μM Flu or γ-irradiation (Fig. 4.7A). In contrast, mean DR5 mRNA levels at 24h increased by 1.4 fold (p>0.05) following treatment with 33μM Chl, and increased significantly by 2.0 fold (p=0.018) with 165μM Chl, 1.6 fold (p=0.011) with 17.5μM Flu and 1.3 fold (p=0.015) following γ-irradiation (Fig. 4.7B). Compared to the 24h control, mean DcR1 mRNA levels did not increase following treatment with 33μM Chl, but increased by 1.5 fold (p>0.05) with 165μM Chl, 1.4 fold (p>0.05) with 17.5μM Flu and 1.5 (p>0.05) fold following γ-irradiation (Fig. 4.8A). However, increase in DcR1 mRNA was not significant. Mean DcR2 mRNA levels increased by 1.3 fold (p>0.05) following treatment with 33μM Chl, and increased significantly by 3.4 fold (p=0.001) with 165μM Chl, 2.5 fold (p=0.004) with 17.5μM Flu and 2.9 fold (p=0.013) following γ-irradiation (Fig. 4.8B).
Figure 4.1 Action of TRAIL on B-CLL cells. A, TRAIL induces apoptosis in Jurkat cells in a dose-responsive manner at 24h. Similar data was observed using the viability assay (data not shown). B, Percent apoptosis and C, percent loss of viability (PI staining) of B-CLL samples after 24h in the presence or absence of 500ng/ml TRAIL (B-CLL numbers below the histograms do not correspond to numbers in table 2.1).
Figure 4.2 Action of TRAIL on apoptosis induction in B-CLL cells. B-CLL cells were treated with cytotoxic agents in the presence or absence of 500 ng/ml TRAIL as indicated. The percentage of apoptotic cells was determined by morphological criteria. The lines connect data points obtained using individual B-CLL isolates. Mean data for all of the isolates, shown as a histogram in the inset, were analysed by student’s t test. The percentage of apoptotic cells in drug-treated or irradiated samples were compared to the corresponding controls (* p>0.05; **, p<0.05). For each cytotoxic treatment, incubations in the presence of anti-Fas were also compared to the corresponding treatment in the absence of anti-Fas (†, p>0.05).
**Figure 4.3** Specific apoptotic induction in B-CLL samples treated with cytotoxic agents in combination with 500ng/ml TRAIL after 24h. Histograms represent experimental observed % specific apoptosis after treatment with cytotoxic agents and TRAIL combined versus theoretical sum of cytotoxic agents and TRAIL added alone (B-CLL numbers below the histograms do not correspond to numbers in table 2.1).
Figure 4.4 Action of TRAIL on viability of B-CLL cells. B-CLL cells were treated with cytotoxic agents in the presence or absence of 500 ng/ml TRAIL as indicated. The percentage of non-viable cells was determined by flow cytometric analysis of PI-stained cells. Mean data for all of the isolates, shown as a histogram in the inset, were analysed by student’s t test. The percentage loss of viable cells in drug-treated or irradiated samples were compared to the corresponding controls (*, p>0.05; **, p<0.05). For each cytotoxic treatment, incubations in the presence of TRAIL were also compared to the corresponding incubations in the absence of TRAIL (†, p>0.05).
Figure 4.5 Specific percentage loss of viability of B-CLL samples treated with cytotoxic agents in combination with 500ng/ml TRAIL after 24h. Histograms represent experimental observed % loss of viability after treatment with cytotoxic agents and TRAIL combined versus theoretical sum of cytotoxic agents and TRAIL added alone (B-CLL numbers below the histograms do not correspond to numbers in table 2.1).
Figure 4.6 RT-PCR analysis of actin, DR4, DR5, DcR1 and DcR2 transcripts in a representative B-CLL sample.
Figure 4.7 Semi-quantitative RT-PCR analysis of B-CLL transcripts. A, Comparison of DR4/Actin ratio and B, DR5/Actin ratio in multiple B-CLL samples incubated for 24h in medium alone (control) or following treatment with cytotoxic agents.
Figure 4.8 Semi-quantitative RT PCR analysis of B-CLL transcripts. A, Comparison of DcR1/Actin ratio and B, DcR2/Actin ratio in multiple B-CLL samples incubated for 24h in medium alone (control) or following treatment with cytotoxic agents.
4.2.2 AML cells

4.2.2.1 TRAIL killing of AML cells

To determine if AML cells were sensitive to TRAIL alone, fifteen AML isolates were incubated for 24 hours in the presence or absence of 500ng/ml of anti-Fas IgM. Apoptosis was quantified by morphological criteria and loss of cell viability was quantified by flow cytometric analysis of PI-stained cells. Spontaneous apoptosis ranged between 1.5-38% (n=15), and loss of viability between 1.9-53% (n=13) respectively. Different isolates showed varying sensitivity towards TRAIL. TRAIL induced apoptosis in 10 out of 15 AML samples (p=0.035) and loss of viability in 11 out of 13 samples (p=0.002) (Fig. 4.9). Specific TRAIL induced apoptosis ranged from 0.3-28% (n=10) and loss of viability ranged from 1-13% (n=11) in TRAIL sensitive AML samples, with 3 out of 15 samples showing more than 5% - TRAIL induced apoptosis and 7 out of 13 samples showing more than 5% TRAIL induced loss of viability.

Using Spearman’s rank correlation statistical test, no significant relationship was found between FAB type and specific TRAIL induced apoptosis (Fig. 4.10) or TRAIL induced loss of viability (data not shown).

4.2.2.2 TRAIL augments killing of AML cells by cytotoxic agents

To determine whether treatment with cytotoxic drugs or exposure to γ-irradiation enhanced the sensitivity of AML cells to TRAIL-induced killing, AML cells were treated with 16.5μM Flu, 41μM Ara-C, 0.1μM DNR or exposed to 10Gy γ-irradiation and incubated for 24h in the presence or absence 500ng/ml TRAIL. Apoptosis was quantified by morphological criteria. Different isolates showed varying sensitivity to different cytotoxic agents (Fig. 4.11). Compared to mean spontaneous apoptosis, mean apoptosis increased by a 1.7 fold (p=0.009) following treatment with Flu, 1.9 fold (p=0.041) with Ara-C, and 1.4 fold (p=0.035) with DNR. A 2.4 fold (p=0.001) increase in mean apoptosis was observed in cells exposed to 10Gy γ-irradiation. All of these increases were statistically significant (p<0.05). The specific apoptotic increase over spontaneous apoptosis of all the isolates ranged between 0-32% following treatment with 17.5μM Flu, 0-23% with 41μM Ara-C, 0-19% with 0.1μM DNR and 0-42% following exposure to 10Gy γ-irradiation. The inclusion of 500ng/ml TRAIL in the incubations resulted in a
significant increase in apoptosis in combination with cytotoxic agents or \( \gamma \)-irradiation (p<0.05) compared with cytotoxic treatment or \( \gamma \)-irradiation alone (Fig. 4.11). Different AML isolates showed different sensitivity towards TRAIL in combination with cytotoxic agents or \( \gamma \)-irradiation. Data from individual isolates showed that incubation with TRAIL resulted in increases in the percentage of apoptotic cells induced by Flu alone in 12/15 isolates, Ara-C in 9/15 isolates, DNR in 14/15 isolates, and by \( \gamma \)-irradiation in 11/15 isolates.

To quantify possible synergy between the combination of TRAIL and cytotoxic agents, the experimental apoptosis induced by cytotoxic agents in combination with TRAIL was compared with the theoretical sum of apoptosis induced separately by the cytotoxic agents or TRAIL alone (Fig. 4.12). Statistical analysis of the complete data set showed that there was no significant difference between experimental and theoretical apoptosis induced by Flu, Ara-C or \( \gamma \)-irradiation in combination with TRAIL (p>0.05). However there was a significant difference (p=0.025) between the experimental and theoretical apoptosis following treatment with DNR in combination with TRAIL. Looking at individual cases, super-additive TRAIL induced apoptosis was observed in 5 samples treated with Flu (range 6.1-21%), 6 with Ara-C (range 6.8-21%), 7 with DNR (range 7.7-16%) and 6 samples exposed to \( \gamma \)-irradiation (range 5.7-32%) out of 15 AML samples.

Quantitation of cell death assessed by flow cytometric analysis of PI-stained cells (Fig. 4.13) confirmed the above conclusions. Compared to mean spontaneous cell death, mean loss of cell viability increased by 1.4 fold (p=0.028) following treatment with 17.5\( \mu \)M Flu, 1.4 fold (p=0.004) with 41\( \mu \)M Ara-C, and 1.2 fold (p=0.032) with 0.1\( \mu \)M DNR. A 1.8 fold (p=0.001) increase in cell death was observed in cells exposed to 10Gy \( \gamma \)-irradiation. The specific loss of viability induced by cytotoxic agents ranged between 0-25% following treatment with Flu, 0-17% with Ara-C, 0-23% with DNR and 0-51% following exposure to 10Gy \( \gamma \)-irradiation. The inclusion of TRAIL in the incubations resulted in an increase in loss of viability in combination with cytotoxic agents or \( \gamma \)-irradiation compared with cytotoxic treatment or \( \gamma \)-irradiation alone (p<0.05). Different AML isolates showed different sensitivity towards TRAIL in combination with cytotoxic
agents or γ-irradiation. Data from individual isolates showed that incubation with TRAIL resulted in increases in loss of viability induced by Flu alone in 10/13 isolates, Ara-C alone in 11/13 isolates, DNR alone in 12/13 isolates, and by γ-irradiation alone in 11/13 isolates.

To further quantify possible synergy between the combination of TRAIL and cytotoxic agents, the experimental loss of viability induced by cytotoxic agents in combination with TRAIL was compared with the theoretical sum of loss of viability induced separately by the cytotoxic agents or TRAIL alone (Fig. 4.14). Statistical analysis of the complete data set showed that there was no significant difference between experimental or theoretical loss of viability induced by Flu, Ara-C or γ-irradiation in combination with TRAIL (p>0.05). However, similar to the results obtained by analysing apoptosis, there was a significant difference (p=0.044) between experimental and theoretical loss of viability when AML cells were treated with DNR in combination with TRAIL. Looking at individual cases, super-additive TRAIL induced loss of viability was observed in 5 samples treated with Flu (range 7.4-25%), 5 with Ara-C (range 5.1-19%), 6 with DNR (range 5.4-30%) and 6 exposed to γ-irradiation (range 7.1-41%) out of 13 AML samples.

The super-additive apoptosis induced by cytotoxic agents in combination with TRAIL was clearly demonstrated when a TRAIL-resistant AML isolate was treated for up to 48h with different doses of cytotoxic agents (Fig. 4.15). Both γ-irradiation and Ara-C in combination with TRAIL induced super-additive apoptosis when AML cells were analysed at 24h and 48h. This AML sample was relatively resistant to Flu at 24h. However, at 48h Flu in combination with TRAIL induced super-additive apoptosis. Super additive apoptosis was induced by DNR in combination with TRAIL at 24h. At 48h, DNR alone induced 92% specific apoptosis, and the effect of TRAIL in addition with DNR was minimal.

**4.2.2.3 The combination of cytotoxic agents and TRAIL induces caspase-mediated apoptosis**

To obtain molecular evidence for additive or super-additive interactions between TRAIL and cytotoxic agents, the processing of pro-caspase and the generation of the p85 PARP
fragment from 116kDa PARP protein was analysed by western blotting of lysates from AML isolates (Fig. 4.16 and Fig. 4.17).

Processing of pro-caspases and PARP was observed in AML samples after treatment with cytotoxic agents, with additive or super-additive cleavage in the presence of TRAIL. However, levels of pro-caspase and PARP cleavage varied between samples after treatment. In cells from AML 229, which was previously observed to be TRAIL resistant (Fig. 4.12), no cleavage of PARP or processing of caspases was detected at 24h incubation in either the presence or absence of TRAIL (Fig. 4.16). However, TRAIL clearly augmented PARP cleavage induced by Flu, Ara-C, DNR or γ-irradiation. Numerical analysis of the data (Fig. 4.16) was consistent with interpretation that the actions of TRAIL and cytotoxic agents were super-additive. The failure of TRAIL alone to kill this AML sample was reinforced by the observation that TRAIL treatment did not induce pro-caspase-8 processing. However, each cytotoxic treatment used here induced some pro-caspase-8 processing, which in the case of Flu, DNR and γ-irradiation, was further augmented by TRAIL. No cleavage of pro-caspase-3 was observed when AML cells were treated with TRAIL alone, while cytotoxic agents induced some pro-caspase-3 processing, which was moderately augmented by TRAIL.

A low level of PARP cleavage was seen in the control 24h incubation of cells from AML223 (Fig. 4.17). This was slightly augmented by TRAIL treatment. All of the cytotoxic treatments tested here induced PARP cleavage, which was augmented by co-incubation with TRAIL. Numerical analysis of the blot data was consistent with super-additive induction of PARP cleavage by each of the cytotoxic agent and TRAIL. In this isolate, TRAIL alone induced pro-caspase-8 processing. Flu or γ-irradiation also induced pro-caspase-8 and pro-caspase-3 processing, with evidence of increased processing induced by co-incubation with TRAIL. DNR or Ara-C alone induced little pro-caspase-8 and pro-caspase-3 processing, but substantial cleavage was seen in cells treated with DNR and TRAIL, while a modest cleavage was seen with Ara-C plus TRAIL.
Figure 4.18 shows the correlation between observed and theoretical sum of PARP cleavage and percentage specific apoptosis in cells from AML229 after treatment with cytotoxic agents and TRAIL (Pearson correlation test, p<0.0001). The observed PARP cleavage and cell killing by cytotoxic agents in combination with TRAIL was more than the theoretical sum of cytotoxic agents and TRAIL alone. A similar result was observed with AML226 treated with cytotoxic agents, excepting γ-irradiation (Pearson correlation test, p<0.01)(Fig. 4.19).

4.2.2.4 DR4, DR5, DcR1, DcR2 and c-FLIP expression in AML cells

TRAIL has four distinct receptors that can either mediate cell death or block TRAIL induced apoptosis. It was explored whether the difference in AML sensitivity to TRAIL could be explained by the pattern of TRAIL receptor expression. In addition, since expression of c-FLIP has been shown to inhibit TRAIL induced apoptosis, the expression of c-FLIP in AML samples was examined. The TRAIL sensitive Jurkat cell line was used as a comparison. Jurkat cells expressed not only the functional DR4 and DR5 but also DcR2 and c-FLIP (Fig. 4.20). Expression of DcR1 was low in Jurkat cells. Heterogeneous expression of DR4, DR5, DcR2 and c-FLIP was observed in AML cells (Fig. 4.20), while DcR1 was weakly expressed in all AML samples. No statistical correlation was observed between DR4, DR5, DcR1, DcR2 or c-FLIP expression and TRAIL sensitivity (Pearson correlation test, p>0.05).

To assess whether super-additive apoptosis with TRAIL in combination with cytotoxic agents is due to changes in levels of TRAIL receptors, the expression of DR4, DR5, DcR1, DcR2 and c-FLIP in two AML samples which showed evidence of super-additive killing by TRAIL and cytotoxic agents were examined. At 24h after treatment with Flu, Ara-C, DNR or γ-irradiation, no significant changes in TRAIL receptor expression or c-FLIP was seen in either samples studied after treatment with cytotoxic agents in comparison to the 24h control (Fig. 4.21).
Figure 4.9. Action of TRAIL on A, apoptosis and B, viability (PI staining) of AML isolates samples after 24h. Data was analysed using the student’s t-test.
Figure 4.10 Relationship between FAB type and specific TRAIL mediated apoptosis at 24 hours. Apoptosis was assessed by morphology.
Figure 4.11 Action of TRAIL on apoptosis induction of AML cells. AML cells were treated with cytotoxic agents in the presence or absence of 500 ng.mL⁻¹ TRAIL as indicated. The percentage of apoptotic cells was determined by morphological criteria. The lines connect data points obtained using individual AML isolates. Mean data for all of the isolates, shown as a histogram in the insets, were analysed by student’s t test. The percentage of apoptotic cells in drug-treated or irradiated samples were compared to the corresponding controls (**, p<0.05). For each cytotoxic treatment, incubations in the presence of TRAIL were also compared to the corresponding treatment in the absence of TRAIL (††, p<0.05).
Figure 4.12 Specific apoptotic induction in AML samples treated with cytotoxic agents in combination with TRAIL after 24h. Histograms represent experimental observed % specific apoptosis after treatment with cytotoxic agents and TRAIL combined versus theoretical sum of cytotoxic agents and TRAIL added alone.
Figure 4.13 Action of TRAIL on viability of AML cells. AML cells were treated with cytotoxic agents in the presence or absence of 500 ng.mL⁻¹ TRAIL as indicated. The percentage of non-viable cells was determined by flow cytometric analysis of PI-stained cells. Mean data for all of the isolates, shown as a histogram on the insets, were analysed by student’s t test. The percentage loss of viable cells in drug-treated or irradiated samples were compared to the corresponding controls (**, p<0.05). For each cytotoxic treatment, incubations in the presence of TRAIL were also compared to the corresponding incubations in the absence of TRAIL († †, p<0.05).
Figure 4.14 Specific percentage loss of viability in AML samples treated with cytotoxic agents in combination with TRAIL after 24h. Histograms represent experimental observed % specific loss of viability after treatment with cytotoxic agents and TRAIL combined versus theoretical sum of cytotoxic agents and TRAIL added alone.
Figure 4.15  An AML (AML229) sample was incubated with different concentrations or dose of cytotoxic agents (as indicated) with or without 500ng/ml TRAIL for 24-48h. Data point show % specific apoptosis determined by morphological criteria.
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<tr>
<td></td>
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<td>Actin</td>
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**Figure 4.16** Western blot analysis of the processing of pro-caspase 8, pro-caspase 9, pro-caspase 3 and PARP to p85 in protein extracts from AML229 following treatment with Flu, Ara-C, DNR or γ-irradiation in the presence or absence of TRAIL. Numbers below p85-PARP bands are intensities of individual bands normalised with respect to p116 PARP band intensities in the same lane. Numbers below pro-caspase bands are intensities of individual bands normalised with respect to actin band intensities in the same lane. The caspase ratio obtained for the day 0 control bands were arbitrarily set at 1.
Figure 4.17 Western blot analysis of the processing of pro-caspase 8, pro-caspase 3 and PARP to p85 in protein extracts from AML223 following treatment with Flu, Ara-C, DNR or γ-irradiation in the presence or absence of TRAIL. Numbers below p85-PARP bands are intensities of individual bands normalised with respect to p116 PARP band intensities in the same lane. Numbers below pro-caspase bands are intensities of individual bands normalised with respect to actin band intensities in the same lane. The caspase ratio obtained for the day 0 control bands were arbitrarily set at 1.
Figure 4.18 Analysis of AML229. A. Specific p85/PARP ratio at 24hr analysed by western blotting and B. specific apoptosis at 24h determined by morphological criteria. Histograms represent experimental observed values after treatment with cytotoxic agents and TRAIL combined versus theoretical sum of cytotoxic agents and TRAIL added alone. Correlation between PARP cleavage and specific apoptosis (Pearson correlation test, p<0.0001)
**Figure 4.19** Analysis of AML223. A. Specific p85/PARP ratio at 24hr analysed by western blotting and B. specific apoptosis at 24h determined by morphological criteria. Histograms represent experimental observed values after treatment with cytotoxic agents and TRAIL combined versus theoretical sum of cytotoxic agents and TRAIL added alone. Correlation between PARP cleavage and specific apoptosis (Pearson correlation test, p<0.01)
Figure 4.20 Western blot analysis of expression of A. DR4 and DR5, B. c-FLIP, C. DcR1 and D. DcR2 in AML samples.
### Figure 4.21

Western blot analysis of DR4, DR5, DcR2 and e-FLIP protein expression in two AML samples 24h after treatment with cytotoxic agents. Numbers below bands represent ratio against actin. Jurkat cell line was used as a comparison.

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<tr>
<td>Actin</td>
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<tr>
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<tr>
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<td>0.23</td>
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<tr>
<td>Actin</td>
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4.3 Discussion

Analogous to the proposed role of Fas, TRAIL may be involved in cytotoxic killing of cancer cells. Therefore, the potential roles of TRAIL induced killing of freshly isolated B-CLL and AML cells by cytotoxic drugs and γ-irradiation was examined.

In parallel with the results observed with anti-Fas IgM, 500ng/ml of TRAIL had no pro-apoptotic effect on B-CLL cells. Morphological criteria and PI staining with FACS analysis was used to assess the putative role of TRAIL and DRs in the killing of B-CLL cells by cytotoxic agents. Analysis of data obtained using multiple B-CLL isolates showed that cell killing by Chl, Flu or γ-irradiation was not significantly augmented by co-incubation with TRAIL. However, individual experiments suggested that TRAIL ligation resulted in a moderate augmentation of morphologically detectable apoptosis and of viability loss in a minority of samples treated with cytotoxic agents. When comparing the theoretical and the observed combination of each cytotoxic agent with TRAIL, no super-additive induction of apoptosis or loss of viability was observed in any of the B-CLL isolates. Taken together, the data show that signalling by the TRAIL/ DR system does not play a significant role in the apoptotic killing of B-CLL cells by cytotoxic agents. However, DR5 and DcR2 transcripts were shown to be significantly upregulated after treatment with cytotoxic agents. DcR1 transcript also increased but this was shown to be not significant. Previous studies on cell lines has shown that DR5, DcR1 and DcR2 can be upregulated after treatment with cytotoxic agents.

One possible mechanism by which TRAIL induced apoptosis may be inhibited in B-CLL cells is the expression and upregulation of TRAIL decoy receptors following treatment with cytotoxic agents. The TRAIL decoy receptors may be induced by p53 after cytotoxic treatment, thereby attenuating an apoptotic response involving DR5 by competing for TRAIL. Therefore, the p53-dependent induction of TRAIL decoy receptors may provide a mechanism to transiently favour cell survival over cell death. Although the expression of DcR occasionally correlates with resistance to TRAIL induced apoptosis, resistance most frequently correlates with the levels of c-FLIP.
As in the Fas pathway, c-FLIP, an inhibitor of death signalling by Fas\textsuperscript{162} can also inhibit TRAIL induced apoptosis\textsuperscript{174}. Levels of c-FLIP in B-CLL cells decreased after treatment with 10Gy γ-irradiation. However, FADD levels also decreased after treatment with 10Gy γ-irradiation. Low levels of FADD could impair the formation of the DISC after TRAIL activation. However, the role of FADD in TRAIL induced apoptosis is controversial, with some studies showing that FADD plays no part in TRAIL induced apoptosis\textsuperscript{165,416}, while other studies show that TRAIL induced apoptosis is FADD dependent\textsuperscript{166-169}. More recently, a GTP binding protein called DAP3 (death associated protein 3) has been shown to link FADD to DR4 and DR5\textsuperscript{417}. It is possible that expression of c-FLIP, FADD or DAP3 in B-CLL cells may regulate sensitivity towards TRAIL induced apoptosis.

Unlike B-CLL cells, AML cells were shown to be sensitive to TRAIL mediated apoptosis. Morphological criteria, PI staining and generation of the p85 PARP fragment was used to assess the putative role of TRAIL and its receptors in the killing of AML cells by cytotoxic agents. Analysis of AML samples showed that some were sensitive to TRAIL mediated apoptosis. However, sensitivity to TRAIL did not correlate with FAB type, as previously shown by Wuchter et al.\textsuperscript{415}. Analysis of data obtained using multiple AML isolates showed that cell killing by Flu, Ara-C, DNR or γ-irradiation was significantly augmented by TRAIL. Analysis of the complete data set showed that the difference between the observed apoptosis of AML cells treated with DNR in combination with TRAIL was significantly higher than the theoretical sum of apoptosis induced by each agent alone and TRAIL alone, indicative of a super-additive interaction. No super-additive apoptosis was observed with Flu, Ara-C or γ-irradiation. However, treatment with Flu, Ara-C or γ-irradiation in combination with TRAIL induced super-additive apoptosis in some AML samples. Furthermore, when AML cells were incubated with different doses of cytotoxic agents in combination with TRAIL, super-additive apoptosis was induced at certain doses and at specific time points. Therefore, cytotoxic agents appear to sensitise AML cells to TRAIL mediated apoptosis. Western blot analysis for PARP cleavage further confirmed the super-additive apoptosis induced by TRAIL in combination with cytotoxic agents.
To determine whether the expression of TRAIL receptors or c-FLIP may correlate with TRAIL sensitivity, the expression of these receptors in AML samples was investigated. Relative expression levels were heterogeneous, and the expression of c-FLIP, DR4, DR5, DcR1 or DcR2 did not correlate with TRAIL sensitivity. Therefore, the expression pattern of TRAIL receptors does not determine TRAIL sensitivity in AML cells, in agreement with other studies. Interestingly, sensitivity to TRAIL mediated apoptosis correlated with Flu and DNR induced apoptosis, but not with Ara-C or γ-irradiation. These data suggest that in some AML samples the mechanism underlying sensitivity to certain cytotoxic agents could also be involved with sensitivity towards TRAIL mediated apoptosis.

To investigate how super-additive apoptosis is induced by TRAIL in combination with cytotoxic agents, the expression of components of the death receptor pathway was investigated. The enhancement of TRAIL mediated apoptosis by cytotoxic agents by upregulation of DR4 and DR5 receptors in other cell types has been previously published. However, the data show no significant changes in TRAIL receptor proteins after cytotoxic treatment. The expression of anti-apoptotic c-FLIP was also assessed. Again, no significant changes in protein levels were observed. One possible explanation for super-additive apoptosis between TRAIL and cytotoxic agents is a synergistic activation of caspases. It was observed in some AML samples that cytotoxic agents and TRAIL used alone induced relative weak to moderate PARP cleavage, which was augmented in a super-additive manner by co-incubation with at least some cytotoxic stimuli. These observation are consistent with the interpretation that the death pathways induced by TRAIL and by cytotoxic stimuli converge at a point preceding caspase-3 activation, resulting in synergistic activation of this enzyme. The mechanism of this synergy remains to be established.

In summary, the experiments here demonstrate that combination of cytotoxic agents and TRAIL can lead to super-additive apoptosis of AML cells. However, B-CLL cells are resistant to TRAIL mediated apoptosis and synergy with cytotoxic agents was not observed. It is possible that the mechanism that inhibits Fas induced apoptosis in B-CLL
cells (chapter 3) overlap the TRAIL apoptotic pathway. For AML cells, the concentrations of cytotoxic drugs used in these experiments are within the clinically achievable range \(^{358,359}\). Similarly, a TRAIL concentration of 500ng/ml has been achieved in animal studies \(^{184}\). Since super-additive apoptosis was observed with different concentration of cytotoxic drugs, the dose of each agent may be determined by in-vivo studies so as to achieve optimal killing in combination with TRAIL. The data suggest that cytotoxic chemotherapy in conjunction with TRAIL offer a promising means of treating AML. In the experiments, and those reported elsewhere, AML cells were treated simultaneously with TRAIL and cytotoxic agents \(^{361,409,413}\). While this resulted in super-additive apoptosis, it remains to be seen if this is the best strategy to combine cytotoxic agents and TRAIL to induce optimal apoptosis. Indeed, it is plausible that sequential administration of these agents may also be beneficial. The in-vivo use of TRAIL might, however, be associated with severe liver damage, death of normal brain cells and erythroblasts \(^{380,419,422}\). Therefore, the use of TRAIL in the treatment of human malignancies must be considered with caution and further studies would be required for the pre-clinical and clinical evaluation of TRAIL \(^{419,422}\).
Chapter 5

Cytoprotection of B-CLL cells by plasma: synergistic apoptosis induction by cytotoxic drugs and signal transduction inhibitors.

5.1 Introduction

Despite the extended survival of B-CLL cells in-vivo, B-CLL cells cultured in-vitro die rapidly by spontaneous apoptosis, suggesting that humoral or cellular factors play an important part in promoting B-CLL survival in-vivo. These factors may also contribute to signalling apoptotic resistance against standard chemotherapy. Understanding the mechanisms that contribute to the resistance of B-CLL cells to apoptosis in-vivo could lead to new and more effective strategies in treating patients with the disease.

Anti-apoptotic growth factors and cytokines have been shown to activate the PI3-K/Akt pathway, which plays an important part in modulating cell survival. Anti-apoptotic signalling by IL-3 and IGF-1 in-vitro and in-vivo involves phosphorylation of Bad by Akt. Phosphorylation of FKHRL1 by Akt can also inhibit apoptosis. In contrast, growth factor withdrawal may promote apoptosis by dephosphorylating Bad, either through natural decay or via the action of a phosphatase, allowing it to dimerize with Bcl-X\textsubscript{l}. Another cell survival pathway involves activation of NF-\textkappaB by Akt. However, it is likely that anti-apoptotic signalling via the PI3-K/Akt pathway involves phosphorylation of additional, yet undiscovered, substrates.

Several cytokines have been suggested to be important in the proliferation of B-CLL cells in a paracrine or autocrine fashion, while some lymphokines act as 'survival' factors for the leukaemic cells. B-CLL cells, but not normal CD5+ B-cells, can also be protected from spontaneous apoptosis or corticosteroid-induced apoptosis when cultured with human marrow stromal cells. Recently blood-derived nurse like cells have been shown to protect B-CLL cells from spontaneous apoptosis through secretion of stromal cell derived factor-1. Expression of regulatory proteins, such as Bcl-2 has been
shown to be critical in regulating spontaneous apoptosis in B-CLL cells \(^{427}\). Loss of the Bcl-2 protein in B-CLL cells \textit{in-vitro} can be inhibited by culturing the leukaemic cell in \(\alpha\)-IFN or IL-4, or on stroma cells \(^{329,330,426}\). However, it is unclear whether any of these cytokines that promote viability \textit{in-vitro} play a significant role \textit{in-vivo}. In addition, the biochemical pathways important in maintaining B-CLL survival have not yet been characterised.

Therefore, the ability of autologous plasma to promote both basal survival as well as resistance to DNA damaging agents in B-CLL cells was investigated. The ability of the PI3-K inhibitor LY294002 and the protein tyrosine kinase inhibitor herbimycin A (HMA) to synergize with cytotoxic drugs in inducing apoptosis of B-CLL cells cultured in FCS or plasma were also investigated \(^{428}\). HMA, is specific for non-receptor protein tyrosine kinases and does not inhibit receptor type kinases. This ansamycin antibiotic causes irreversible inactivation of tyrosine kinase by reacting with a critical sulphydryl group in the active site \(^{429}\). LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), an analogue of quercetin, completely abolishes PI3-K activity in fMet-Leu-Phe-stimulated human neutrophils, and inhibits proliferation of smooth muscle cells in cultured rabbit aortic segments \(^{292}\). Since PI3-K appears to be centrally involved in survival factor signal transduction, specific inhibitors against the kinase may be beneficial in the treatment of leukaemia as well as in elucidating the biological role of PI3-K in survival signalling in B-CLL cells.
5.2 Results

5.2.1 Plasma protects B-CLL cells from spontaneous apoptosis

To determine if plasma protects B-CLL cells from spontaneous apoptosis, cells were incubated for 24h in RPMI 1640 medium containing 50% Plasma. Medium containing 50% FCS was used as a control. Apoptosis was quantified by morphological criteria (Fig. 5.1A). Spontaneous apoptosis at 24h in FCS ranged between 0-20% (n=12), with a mean spontaneous apoptosis of 5.9%. Incubation in autologous plasma resulted in a significant decrease in apoptosis induction which ranged from 0-15%, with a mean spontaneous apoptosis value of 3% (p=0.047). The protective effect of plasma was also shown to be significant when cell viability was quantified by using the MTT assay (p=0.022) (Fig. 5.1 B).

5.2.2 Plasma retards PARP and pro-caspase-3 cleavage

The processing of pro-caspase-3 and the generation of the p85 PARP fragment from 116kDa PARP protein was analysed by western blotting following 24h incubation with 50% FCS or 50% plasma (Fig. 5.2). Time dependent cleavage of caspase-3 to its active subunits and the generation of p85 PARP was evident in B-CLL cells incubated in 50%FCS for 24h. However, both of these events were suppressed when B-CLL cells were cultured in 50% plasma, confirming the ability of plasma to retard spontaneous apoptosis in B-CLL cells.

5.2.3 Plasma protects B-CLL cells from DNA damaging agents

When cultured in FCS, Chl induced apoptosis in B-CLL cells (Fig. 5.3A). However, when cells were cultured in plasma (n=12), a significant decrease in Chl mediated apoptosis in all B-CLL samples analysed was observed (p=0.001). Different B-CLL isolates responded differently to the protective effect of plasma. The protective effect of plasma against Chl induced apoptosis was further confirmed in B-CLL samples (n=10, p=0.006) when cell viability was analysed by MTT assay (Fig. 5.3 B).

In order to determine whether the protective action of plasma retards PARP and pro-caspase-3 cleavage by DNA damaging agents, B-CLL cell lysates were analysed by western blot (Fig. 5.4). Studies on isolates showed that incubation in plasma did retard
PARP and pro-caspase-3 cleavage when B-CLL cells were treated with Chl. Plasma can also protect B-CLL cells from \( \gamma \)-irradiation induced apoptosis (data not shown).

### 5.2.4 Plasma delays p53 upregulation following Chl treatment

A western blot analysis of cell lysates from a B-CLL sample cultured in 50% FCS in a time course study revealed p53 upregulation at 8h and 24h when cells were treated with 20\( \mu \)g/ml Chl (Fig. 5.5). This was followed by upregulation of p21 WAF1, a transcriptional target of p53. However, when B-CLL cells were cultured in plasma, a delayed p53 upregulation was observed. A longer time course revealed delayed p21 WAF1 upregulation in plasma cultured B-CLL samples following treatment with Chl (data not shown).

### 5.2.5 Effects of LY294002 and Herbimycin A on B-CLL viability cultured in FCS

To determine whether the PI3-K inhibitor LY294002 or the protein tyrosine kinase inhibitor HMA can enhance sensitivity of B-CLL cells to DNA damaging agents, 24 B-CLL samples cultured in FCS were treated with increasing doses (1x10\(^{-3} \) – 100\( \mu \)g/ml) of Chl or Flu in the presence or absence of 0.1 or 0.5\( \mu \)M HMA, or 2 or 10\( \mu \)M LY294002 for 3 days. The MTT dye reduction assay was used to measure B-CLL viability and calculate the Chl or Flu concentration required for 50% inhibition of cell viability (LD\(_{50}\)) in the presence of cytotoxic agent alone or when cells were incubated with cytotoxic agent plus each of the signal transduction inhibitors.

Synergy in cell killing induced by cytotoxic drugs in combination with LY or HMA was clearly demonstrated when a B-CLL isolate was treated for 3 days with increasing doses of Chl or Flu (Fig. 5.6). The LD\(_{50}\) for HMA or LY294002 was 0.10\( \mu \)M and 0.80\( \mu \)M respectively (Fig.5.6A and B), while LD\(_{50}\) for Chl or Flu alone was 2.1\( \mu \)g/ml and 31\( \mu \)g/ml respectively (Fig. 5.5C and D). When B-CLL cells were treated with Chl (Fig. 5.6C) in combination with 0.1\( \mu \)M HMA, Chl LD\(_{50}\) was reduced to 0.59\( \mu \)g/ml. With LY294002 at 2\( \mu \)M, Chl LD\(_{50}\) was reduced to 0.69\( \mu \)g/ml. When B-CLL cells were treated with Flu (Fig. 5.6D) in combination with 0.1\( \mu \)M HMA, Flu LD\(_{50}\) was reduced to 3.0\( \mu \)g/ml. With LY294002 at 2\( \mu \)M, Flu LD\(_{50}\) was reduced to 2.5\( \mu \)g/ml. In 24 B-CLL samples the LD\(_{50}\) for HMA and LY294002 treatment alone was calculated (Table 5.1). A correlation was
observed between LD$_{50}$ of tyrosine kinase inhibitor HMA and PI3-K inhibitor LY394002 in B-CLL cells (p=0.029) (Fig. 5.7). This suggests that the HMA-sensitive tyrosine kinase pathway and PI3-K/Akt pathway are closely associated.

In 24 B-CLL samples the mean LD$_{50}$ for Chi was 40μg/ml, with a range of 2 to 152μg/ml (Fig. 5.8A). When B-CLL cells were treated with Chi in combination with 0.1μM HMA, the mean Chi LD$_{50}$ reduced significantly to 16μg/ml, with a range of 0.6-59μg/ml (p=0.0005, n=24). Treatment of B-CLL cells with Chi in combination with 2μM LY294002 reduced the mean Chi LD$_{50}$ to 16μg/ml, with a range of 0.7-109μg/ml (p=0.004, n=23). For 23 B-CLL samples the mean LD$_{50}$ for Flu was 14μg/ml, with a range of 0.06 to 75μg/ml (Fig. 5.8B). When B-CLL cells were treated with Flu in combination with 0.1μM HMA, the mean Flu LD$_{50}$ was reduced to 10μg/ml, with a range of 0.6-73μg/ml. Treatment of B-CLL cells with Flu in combination with 2μM LY294002 reduced the mean Flu LD$_{50}$ to 10μg/ml, with a range of 0.02-75μg/ml. The ability of HMA or LY294002 to decrease the LD$_{50}$ of Flu was not statistically significant when the total data was analysed (p>0.05).

5.2.6 Combination of cytotoxic agents and signal transduction inhibitors result in super-additive apoptosis of B-CLL cells cultured in plasma

Because of the potent anti-apoptotic action of plasma, it was important to demonstrate that super-additive killing by signal transduction inhibitors in combination with cytotoxic drugs could also be demonstrated when B-CLL cells were cultured in plasma.

By morphological criteria, HMA (Fig 5.9A) or LY294002 (Fig 5.9B) induced super-additive apoptosis of B-CLL cells in combination with Chi. Super-additive killing was observed when cultures were supplemented with FCS or plasma, although the concentration of agents required for optimal killing were different, presumably due to the potent anti-apoptotic action of plasma. HMA at 300nM or 1000nM was super-additive with either 5μg/ml or 20μg/ml Chi in the presence of FCS. However, in the presence of plasma, super-additive apoptosis induction was clearly seen only at the higher dose of HMA (1000nM).
Super-additive killing by 20μM or 50μM LY294002 in combination with Chl was clearly observed when B-CLL cells were cultured in FCS (Fig 5.9B). In the presence of plasma, super-additivity between the signal transduction inhibitor and Chl was clearly seen only with 20μM LY294002.

Morphological analysis of super-additive killing by combinations of signal transduction inhibitors and Chl in plasma cultures of B-CLL cells have been carried out using multiple B-CLL isolates. Synergistic killing by HMA and Chl was observed in 2 out of 4 isolates, and by LY294002 and Chl in 4 out 6 isolates studied.

Processing of PARP was observed in a B-CLL sample after treatment with Chl, with super-additive cleavage in the presence of HMA when cultured in 50% FCS or plasma (Fig. 5.10). Moderate cleavage of PARP was detected at 8h incubation in 50% FCS or plasma alone. Numerical analysis of the data from B-CLL cells cultured in FCS showed that 8h incubation with Chl or HMA alone increased the cleavage of PARP in a dose-responsive manner. When B-CLL cells were treated with Chl in combination with HMA, PARP cleavage was clearly augmented. When B-CLL cells were cultured in plasma, PARP cleavage was retarded in relation to cells cultured in FCS (Fig. 5.10B). Numerical analysis of the data revealed that when B-CLL cells were treated with Chl in combination with HMA in FCS or plasma, the observed cleavage of PARP was greater than the theoretical sum of PARP cleavage in B-CLL cells treated with Chl or HMA alone (Fig. 5.11A, Fig. 5.12A). The conclusion from PARP cleavage analysis was consistent with the interpretation that the actions of HMA and Chl induced super-additive apoptosis as analysed by morphological criteria (Fig. 5.11B, Fig. 5.12B). Analysis of specific apoptosis revealed that when B-CLL cells were treated with Chl in combination with HMA in FCS or plasma, the observed specific apoptosis was more than the theoretical sum of specific apoptosis induced by Chl or HMA alone.

Similar results were observed when B-CLL cells were treated with LY294002 alone or in combination with Chl (Fig. 5.13). Quantitative analysis showed that super-additive PARP cleavage was evident, particularly when 20μg/ml Chl was used in combination with LY294002 in FCS cultures (Fig. 5.14A). This conclusion was essentially confirmed by morphological analysis of apoptosis (Fig. 5.14B). Both PARP cleavage (Fig 4.15A) and
apoptosis induction (Fig. 5.15) induced by either Chl or LY294002 alone was suppressed in cultures supplemented with 50% plasma. By the criteria of PARP cleavage, super-additive action of Chl and LY294002 were only evident when 20μM LY294002 was added together with 20μg/ml Chl (Fig. 5.15A). Super-additive apoptosis induction quantified by morphological criteria was clearly seen with all of the combinations of LY294002 and Chl tested (Fig. 5.15B)

5.2.7 The combination of cytotoxic agents and HMA or LY294002 induces p53 upregulation.

The expression of the tumour suppressor protein p53 in lysates from a B-CLL isolate treated with HMA or LY294002 in combination with Chl, cultured in FCS (Fig. 5.16A) or plasma (Fig. 5.16B) for 8h was analysed by western blotting. Numerical volume analyses of the band intensities from the B-CLL isolate are represented in Figure 5.16 C. Levels of p53 increased after treatment with Chl in a dose dependent manner. However, expression of p53 in the B-CLL isolate treated with Chl was retarded when cultured in plasma in comparison to B-CLL cells cultured in FCS. Elevated expression of p53 was observed when the B-CLL isolate was treated with 5μg/ml Chl in combination with 1000nM HMA, while a lower p53 expression was observed when the B-CLL isolate was treated with 20μg/ml Chl in combination with 1000nM HMA. The difference in p53 expression could be due to kinetics of p53 expression in B-CLL cells, related to incubation time and drug dosage. LY294002 at 20μM in combination with Chl also upregulated p53 levels in B-CLL cells. However, LY294002 at 50μM in combination with Chl induced a low p53 increase in expression, which could also be due to the kinetics of p53 expression at 8h.

The time course study of caspase-3 activation, PARP cleavage and p53 expression shown in Figure 5.17 confirms synergistic cleavage of PARP, the protective effects of plasma and the kinetics of p53 upregulation. When the B-CLL isolate was treated with 20μg/ml Chl alone in FCS, p53 expression was at its highest at 24h, with 62% apoptotic B-CLL cells (Fig. 5.17B). Chl in combination with LY294002 increased p53 expression to its highest at 8h, when cultured in FCS, with 92% apoptotic B-CLL cells at 24h (Fig. 5.17D).
When the B-CLL isolate was cultured in plasma, B-CLL cells were protected from Chl mediated apoptosis, and reduced p53 expression was at 24h was accompanied by lower caspase-3 activation and PARP cleavage (Fig. 5.17B). Treatment of the B-CLL isolate with LY294002 alone induced apoptosis both in B-CLL cells cultured in FCS and to a lesser extent in plasma, but no changes in p53 expression were observed (Fig. 5.17C). However, when the B-CLL isolate was treated with Chl in combination with LY294002, the protective effects of plasma was blocked, resulting in upregulation in p53 levels, activation of caspase-3, PARP cleavage and 70% apoptotic B-CLL cells at 24h (Fig. 5.17D).
Figure 5.1 Action of plasma on A, spontaneous apoptosis and B, cell-death in B-CLL cells. B-CLL cells were incubated in 50% plasma or 50% FCS for 24h. The lines connect data points obtained using individual B-CLL isolates. The standard errors of individual determinations were <10% of the mean values reported. Mean data for all of the isolates (shown as a histogram on the inset) were analysed by student’s t test.
**Figure 5.2** Western blot analysis of the processing of pro-caspases and PARP in B-CLL cells cultured in 50% FCS or plasma.
Figure 5.3 Action of plasma on Chl-induced A, apoptosis and B, cell-death in B-CLL cells. B-CLL cells were incubated in 50% plasma or 50% FCS for 24 h. The lines connect data points obtained using individual B-CLL isolates. Mean data for all of the isolates (shown as a histogram on the inset) were analysed by student’s t test. The percentage of apoptotic cells or MTT absorbance of cells treated with Chl were compared to the corresponding controls (†, p>0.05 ; ††, p<0.05). B-CLL cells incubated in plasma were also compared to the corresponding FCS incubation (**, p<0.05).
Figure 5.4 Western blot analysis of the processing of pro-caspases and PARP in B-CLL cells treated with when cultured in 50% FCS or plasma.
Figure 5.5 Western blot analysis for p21 and p53 expression in a B-CLL sample after treatment with Chl when cultured in 50% FCS or plasma.
Figure 5.6 MTT assay of a B-CLL samples at 3 days after treatment with A, HMA or B, LY294002 alone, or in combination with C, Chl or B, Flu. LD_{50} for HMA, LY294002, Chl and Flu were calculated using Graph Pad Prism software.
Table 5.1 LD$_{50}$ of HMA or LY294002 in 24 B-CLL samples.
(B-CLL numbers do not correspond to numbers in table 2.1)

<table>
<thead>
<tr>
<th>Sample</th>
<th>HMA LC50 μM</th>
<th>LY294002 LC50 μM</th>
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</thead>
<tbody>
<tr>
<td>CLL 1</td>
<td>0.15</td>
<td>1.16</td>
</tr>
<tr>
<td>CLL 2</td>
<td>0.58</td>
<td>3.17</td>
</tr>
<tr>
<td>CLL 3</td>
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<tr>
<td>CLL 5</td>
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</tr>
<tr>
<td>CLL 6</td>
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<tr>
<td>CLL 7</td>
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</tr>
<tr>
<td>CLL 8</td>
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<td>CLL 13</td>
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<td>CLL 16</td>
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<td>CLL 17</td>
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<tr>
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<td>0.80</td>
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<tr>
<td>CLL 24</td>
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<td>39.89</td>
</tr>
<tr>
<td>Mean</td>
<td>1.65</td>
<td>21.51</td>
</tr>
</tbody>
</table>
Slope = 6.3 ± 2.0
95% confidence intervals: slope = 2.1 to 10.5
Pearson $r = 0.46, p = 0.029$.

**Fig 5.7** LD$_{50}$ of HMA versus LD$_{50}$ of LY294002 in 24 B-CLL samples.
Figure 5.8 LD50 for A, Chl and B, Flu with or without HMA or LY294002 in multiple B-CLL isolates cultured in FCS. B-CLL viability was analysed using the MTT assay and LD50 was calculated using Graph Pad Prism software. Mean LD50 data for all of the isolates (shown as insets) were analysed by student’s t test.
Figure 5.9 Specific apoptosis at 24hr of a B-CLL sample cultured in 50% FCS or plasma treated with A, HMA or B, LY294002 in combination with Chl determined by morphological criteria.
A. 50% FCS

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5 µg/ml Chl</th>
<th>20 µg/ml Chl</th>
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</table>

B. 50% Plasma

<table>
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<th>5 µg/ml Chl</th>
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**Figure 5.10** Western blot analysis of the processing of PARP in protein extracts from a B-CLL sample following treatment with Chl in the presence or absence of HMA. Cells were cultured for 8h in A, 50% FCS or B, 50% plasma. Numbers below p85 bands are the intensities of individual bands normalised with respect to PARP band intensities in the same lane. Quantitative analysis of the band intensities are presented in Fig. 5.11A and Fig 5.12A.
Figure 5.11 A, Quantitative analysis of western blot data of Fig. 5.10A (50% FCS). Specific p85/PARP ratios at 8hr analysed by western blotting are shown. B, specific apoptosis at 24h determined by morphological criteria. Histograms represent experimental observed values after treatment with Chl and HMA combined versus the expected theoretical additive action of the two agents.
Figure 5.12 A. Quantitative analysis of western blot data of Fig. 5.10B (50% Plasma). Specific p85/PARP ratios at 8hr analysed by western blotting are shown.

B, specific apoptosis at 24h determined by morphological criteria. Histograms represent experimental observed values after treatment with Chl and HMA combined versus the expected theoretical additive action of the two agents.
Figure 5.13 Western blot analysis of the processing of PARP in protein extracts from a B-CLL sample following treatment with Chl in the presence or absence of LY294002. Cells were cultured for 8h in A, 50% FCS or B, 50% plasma. Numbers below p85 bands are the intensities of individual bands normalised with respect to PARP band intensities in the same lane. Quantitative analysis of the band intensities are presented in Fig. 5.14A and Fig. 5.15A.
Figure 5.14 A, Quantitative analysis of western blot data of Fig 5.13A (50% FCS). Specific p85/PARP ratios at 8hr analysed by western blotting are shown. B, specific apoptosis at 24h determined by morphological criteria. Histograms represent experimental observed values after treatment with Chl and LY294002 together versus the expected theoretical additive actions by the two agents.
Figure 5.15  A, Quantitative analysis of western blot data of Fig 5.13B (50% Plasma). Specific p85/PARP ratios at 8hr analysed by western blotting are shown. B, specific apoptosis at 24h determined by morphological criteria. Histograms represent experimental observed values after treatment with Chl and LY294002 together versus the expected theoretical additive actions by the two agents.
Figure 5.16 Western blot analysis of p53 expression in protein extracts from a B-CLL samples following 8h treatment with Chl in the presence or absence of LY294002 or HMA. Cultures contained A, 50% FCS or B, 50% plasma. Numbers below bands are the intensities of individual p53 bands normalised with respect to Actin band intensities in the same lane, which are summarised as histograms in C.
Figure 5.17 Western blot time course analysis of caspase 3, PARP and p53 expression in protein extractions from a B-CLL isolate following treatment with A, control, B, Chl alone, C, LY294002 alone and D, Chl in combination LY294002 when cultured in 50% FCS or 50% plasma.
5.3 Discussion

The data here have shown that plasma contributes to cell basal survival of B-CLL cells and also protects them from DNA damaging agents Chl or γ-irradiation (data not shown). The PI3-K/Akt cytoprotective-signalling pathway has been shown to be activated by plasma, which may contribute to the anti-apoptotic action of plasma\(^{293}\). The ability of plasma to protect B-CLL cells against spontaneous and Chl induced apoptosis was further shown by analysing pro-caspase-3 and PARP cleavage by western blotting. Plasma was shown to retard pro-caspase-3 activation and PARP cleavage during spontaneous or Chl mediated apoptosis. The ability of plasma to retard Chl induced apoptosis is downstream of DNA damage. The induction of DNA cross-linking by Chl has been shown to be the same in B-CLL cells cultured in FCS or plasma\(^{293}\). In addition, the ability of plasma to protect against radiation-mediated apoptosis is consistent with its ability to activate a pro-survival-signalling pathway, as it is unlikely that plasma blocks radiation-induced DNA damage\(^{293}\). The pathway downstream of DNA damage involves activation of the tumour suppressor protein p53\(^{234,250,430}\). When B-CLL cells cultured in FCS were treated with Chl, p53 upregulation was observed, which was followed by upregulation of p21WAF1. The p21 WAF1 protein is a critical mediator of the p53 mediated G1 arrest response, and is a potent inhibitor of several G1 CDKs\(^{431,432}\). However, when B-CLL cells cultured in plasma were treated with Chl, a delayed p53 upregulation was observed, which was followed by a delayed p21 WAF1 upregulation. Therefore, since there is no difference in DNA damage induced by Chl between B-CLL cells cultured in FCS or plasma, the data suggests that activation of the PI3-K/Akt pathway or another unidentified pathway by plasma may abrogate p53 upregulation, which leads to delayed p53 transactivation and apoptosis.

It was next determined whether LY294002 or HMA could block the anti-apoptotic effect of plasma or FCS on B-CLL cells. First, FCS cultured B-CLL cells were treated with HMA or LY294002 alone for 72 hours in order to ascertain if LY294002 or HMA can induce apoptosis in B-CLL cells. The sensitivity of B-CLL samples to HMA or LY294002 varied. However, a correlation was observed between LD\(_{50}\) of HMA and LY294002 in B-CLL samples. The more resistant the B-CLL samples were to HMA
mediated apoptosis, the more resistant they were to LY294002 mediated apoptosis. The PI3-K and HMA-sensitive steps may be sequential within a single plasma-activated pro-survival pathway. Alternatively, these steps may be part of separate pathways, which converge on a common pro-survival event.

FCS cultured B-CLL cells were treated with Chl or Flu with or without LY294002 or HMA for 72 hours in-order to ascertain if LY294002 or HMA can sensitise B-CLL cells to Chl or Flu mediated apoptosis. The combination of HMA or LY294002 with Chl reduced the Chl LD_{50} significantly in B-CLL samples. However, a non-significant reduction in mean Flu LD_{50} in combination with HMA or LY294002 was observed. This suggests that the mechanism in which Flu induces apoptosis is different from the Chl mechanism in B-CLL cells. The ability of LY294002 and HMA to induce apoptosis and synergize in combination with Chl or to a lesser extent with Flu in killing B-CLL cells cultured in FCS underscores the importance of pro-survival intracellular pathways in apoptotic protection induced by pro-survival constituents in plasma.

Reduced apoptotic synergy with LY294002 or HMA in combination with Chl was observed when B-CLL samples were cultured in plasma. However, different B-CLL samples responded differently to the protective effects of plasma, and the apoptotic effect of HMA or LY294002 in combination with Chl. The variation in the sensitivity of individual B-CLL isolates is likely to be a consequence of cell intrinsic differences in susceptibility to DNA damage induced death, possibly related to differences in expression of apoptosis-regulating proteins. In a B-CLL isolate cultured in plasma, HMA reversed the protective effects of plasma against Chl induced apoptosis. Similar observations have been made using additional isolates.

To obtain molecular evidence for synergy with HMA or LY294002 in combination with Chl, the generation of the p85 PARP fragment from 116kDa PARP protein was analysed. Quantitative analysis of PARP cleavage in B-CLL cells revealed that Chl in combination with LY294002 or HMA induced PARP cleavage when cultured in FCS. However, in plasma culture, reduced synergistic cleavage of PARP was observed in comparison to B-CLL cells cultured in FCS, confirming the potent anti-apoptotic effect of plasma. Specific PARP cleavage and percentage apoptosis observed in some plasma cultured B-CLL
isolates treated with Chl in combination with HMA or LY294002, were equivalent to the levels observed when FCS cultured B-CLL isolates were treated with Chl alone. Therefore, HMA and LY294002 were able to reverse, at least in part the anti-apoptotic effects of plasma in B-CLL cells against Chl mediated apoptosis to the level observed in FCS cultured B-CLL cells treated with Chl alone.

The PI3-K inhibitor LY294002 has been shown to completely abrogate induction of Akt by plasma, which suggests that plasma activation of Akt was mediated by PI3-K. However, in this study, LY294002 at similar concentrations induced synergistic apoptosis with Chl but failed to totally sensitise B-CLL cells cultured in plasma to Chl mediated apoptosis to the levels observed in similarly treated cells cultured in FCS. The protein tyrosine kinase inhibitor HMA, which has been shown not to block plasma activation of Akt, was also capable of sensitising B-CLL cells to Chl. However, the level of apoptosis in a B-CLL isolate treated with HMA in combination with Chl cultured in plasma was closer to those observed when the same similarly treated B-CLL isolate was cultured in FCS. Therefore, in addition to Akt, other intracellular signalling pathways involving HMA-sensitive tyrosine kinases may be activated by plasma when B-CLL cells were cultured in plasma.

Since plasma blocks Chl-mediated p53 upregulation, p53 expression in B-CLL cells treated with LY294402 or HMA in combination with Chl was analysed. It was observed that HMA and LY294002 marginally increased Chl induced p53 levels when B-CLL cells were cultured in FCS. When B-CLL cells were cultured in plasma, Chl induction of p53 levels was retarded relative to cells cultured in FCS. In a time course experiment, LY294002 in combination with Chl upregulated p53 in a plasma cultured B-CLL sample, possibly by inhibiting the PI3-K/Akt pro-survival signalling pathway. Therefore, since there is no difference in DNA damage induced by Chl between B-CLL cells cultured in FCS or plasma, the data suggest that activation of the PI3-K/Akt or tyrosine kinase pathway by plasma may abrogate p53 upregulation after Chl mediated DNA damage, resulting in resistance to spontaneous or DNA damage mediated apoptosis. However, LY294002 or HMA alone failed to increase p53 expression, which suggests that p53 does not play a direct role in the pathway of programmed cell death induced by either of these signal transduction inhibitors. Therefore, strategies designed to abrogate plasma-induced
anti-apoptotic signalling are likely to be of value in the design of novel therapeutic protocols, which involves synergistic application of conventional cytotoxic drugs and signal transduction inhibitors that block anti-apoptotic pathways.

IL-4, IL-6, IFN-α or IFN-γ have all been shown to augment the survival of B-CLL cells in-vitro. In addition, IL-4 has been shown to block Chl-mediated apoptosis in B-CLL cells. However, Wickremasinghe et al. have shown that neutralising antibodies against these cytokines failed to block the protective effect of plasma against spontaneous or Chl-induced apoptosis. Furthermore, plasma failed to activate JAK1, which confirms that IL-2, IL-4, IL-6, IL-7, IFN-α, IFN-γ, IL-9, IL-15, ciliary neurotrophic factor, oncostatin M or leukaemia inhibitory factor do not contribute to the protective effect of plasma on B-CLL cells. In addition, JAK2 is not involved in the protective effects of plasma on B-CLL cells, ruling out protective roles for erythropoietin, GM-CSF, G-CSF, IL-3 or IL-5. Therefore, the constituent in plasma responsible for protecting B-CLL cells from apoptosis is currently unknown.

Inhibition of the PI3-K/Akt pathway has been proposed as a possible therapeutic target in ovarian cancer and CML, malignancies that are characterised, respectively, by over-expression or aberrant activation of PI3-K. In addition, inhibition of tyrosine kinase has been proposed as a possible target for CML and ALL, which overexpress active tyrosine kinases. Here, it was shown that LY294002 and HMA augments the ability of Chl to induce B-CLL apoptosis by synergistically activating caspase mediated PARP cleavage. Second, it was also shown that plasma abrogates Chl mediated p53 upregulation. Third, it was shown that LY294002 and HMA blocks plasma PI3-K/Akt and/or tyrosine kinase signalling that abrogates Chl-mediated apoptosis and upregulation of p53. In summary, inhibition of the PI3-K/Akt or HMA-sensitive tyrosine kinase pathway may be of therapeutic use in combination with conventional cytotoxic agents, and may therefore prove more effective in the treatment of B-CLL than existing strategies.
Final Discussion

6.1 Introduction
It is well known that proliferation of malignant cells is deregulated cell proliferation, which, together with the obligate compensatory suppression of apoptosis needed to support it, provides a platform necessary to support further neoplastic progression. Targeting one of these events should have potent and specific therapeutic consequences. A large number of chemotherapeutic agents, which act by inducing apoptosis physical damage to DNA, the cytoskeleton or other structural components have been identified. These include ionising radiation, Chl, Flu, DNR, Ara-C, etoposide, cis-platinum, vincristine, 5'-fluorouracil, methotrexate and adriamycin. The way in which these agents kill cells have not yet been fully elucidated. Several reports have suggested that anticancer drugs kill susceptible cells by inducing expression of death receptor or their ligands. Other reports have indicated that chemotherapeutic agents trigger apoptosis by inducing release of cytochrome-c from mitochondria.

Another way of killing malignant cells is to interfere with pro-survival signals that help to block the apoptotic machinery and consequently promoting cell expansion. Anti-apoptotic signalling by survival factors involve activation of PI3-K/Akt pathway and protein tyrosine kinase. By blocking these pathways, malignant cells will become more susceptible to chemotherapeutic agents.

There is a great variation in the susceptibility of malignant cells to undergo apoptosis due to cell-intrinsic differences. Therefore it is crucial to understand the differences and parameters which determine the sensitivity of malignant cells to apoptosis to help combine and optimise cancer treatments, which may prove more effective than existing strategies.
6.2 The role of death receptors in killing leukaemia

Some authors have shown direct correlation between resistance to Fas-mediated apoptosis and the level of resistance to chemotherapeutic agents\(^{406,444,445}\). Interactions between Fas and Fas-L, and DR and TRAIL may mediate cytotoxic killing of at least some leukaemia cell lines\(^{198,366}\). These observations support the hypothesis that apoptosis mediated by cytotoxic agents and Fas/TRAIL ligation shared a common downstream effector and that an intact Fas/TRAIL system played a key role in determining sensitivity or resistance towards anticancer therapy by upregulating Fas, Fas-L or DR expression\(^{198,262,263,367,408}\). However, recent publications have shown that drug-induced apoptosis is not necessarily mediated by the Fas signalling pathway\(^{394,446,447}\). Here, it was shown that sensitivity to Fas/TRAIL mediated apoptosis in B-CLL and AML cells did not correlate with drug resistance. However, while B-CLL cells were Fas/TRAIL resistant, AML cells were weakly sensitive, and cytotoxic drugs in combination with TRAIL induced super-additive apoptosis in AML isolates.

B-CLL cells induced to express substantial levels of cell-surface Fas by \(\gamma\)-irradiation cannot be induced to activate the apoptotic pathway in response to Fas ligation. The reasons for this defect are unclear, but could involve the balance of expression between c-FLIP and FADD. It is possible that since Fas protein is not mutated in B-CLL cells and caspase-8 can be activated by some cytotoxic agents, a point mutation may exist in the FADD protein that inhibits the formation of the DISC complex or recruitment and binding of pro-caspase-8. Cytotoxic drugs Chl and Flu had no effect on Fas protein expression. However, in addition to \(\gamma\)-irradiation, Chl and to a lesser extent Flu did induce Fas transcript levels, which was preceded by the upregulation of p53 levels and p53-mediated transcription. This observation suggests that Fas expression may be regulated by a complex mechanism, which permits translation of Fas transcripts following \(\gamma\)-irradiation, but not in cells treated with Chl or Flu. Fas-L protein expression was not detected in untreated B-CLL cells or following treatment with drugs or \(\gamma\)-irradiation.

In parallel with the results observed with Fas, B-CLL cells were resistant to TRAIL mediated apoptosis. Transcripts of DR5 and DcR2 were shown to be significantly upregulated after treatment with cytotoxic agents. However, at the time of the
experiments, DR protein expression was not analysed due to the unavailability of antibodies against the receptors. Future studies may involve analysing DR expression in order to see if like Fas protein expression, DR expression increases after treatment with γ-irradiation. One possible mechanism by which TRAIL induced apoptosis may be inhibited in B-CLL cells is the expression and upregulation of TRAIL decoy receptors following treatment with cytotoxic agents. However, resistance to TRAIL is more likely to be due to the same mechanism that inhibits the Fas apoptotic pathway. Therefore, the data show that signalling by the Fas or TRAIL system does not play a significant role in the killing of B-CLL cells by cytotoxic agents. Elucidation of the basis of Fas and TRAIL resistance in B-CLL may be of importance, since this may contribute to the expansion of malignant cells in this disease.

In contrast to B-CLL cells, AML cells were shown to be weakly sensitive to TRAIL and Fas mediated apoptosis. Expressions of Fas and DRs proteins from AML isolates were variable, and expression did not change following treatment with cytotoxic agents. Fas-L protein expression was not detected in treated or untreated AML cells. Significant super-additive apoptosis was observed in AML cells treated with TRAIL in combination with DNR. Fas and cytotoxic agents did not induce super-additive apoptosis. However, different isolates responded differently to treatment, and super-additive apoptosis was observed in some AML isolates treated with TRAIL or Fas in combination with cytotoxic agents. The differences in sensitivity between AML samples could be due to possible differences in intrinsic expression of apoptosis - regulatory proteins. These observation are consistent with the interpretation that the death pathways induced by TRAIL and by cytotoxic stimuli converge at a point preceding caspase-3 activation, resulting in synergistic activation of this enzyme. It was also observed that AML samples that were sensitive to Fas mediated apoptosis were sensitive to TRAIL, which suggests an overlap in Fas/TRAIL apoptotic mechanism within the cells. However, due to differences in TRAIL and Fas super-additive apoptosis in AML cells, the regulation of the Fas and TRAIL death pathway by cytotoxic agents vary. The mechanism of this synergy induced by TRAIL in combination with cytotoxic agents in AML cells remain to be established.

Elucidation of the mechanisms of resistance to Fas/TRAIL mediated apoptosis in leukaemic cells may be of importance. One potential decoy receptor for the Fas/Fas-L
system, known as DcR3 or TR6, was recently identified by searching expressed sequence
tag databases for novel TNF receptor family members. DcR3 lacks an obvious transmembrane domain, and unlike DcR1 and DcR2, which are membrane-associated proteins, DcR3 is directly secreted into the extracellular space. DcR3 is a receptor for Fas-L, which inhibits apoptosis mediated by this ligand in-vitro. Other death receptor regulatory proteins that have been identified include, DAP3 and SADS, which are involved in regulating the DICS complex and activation of pro-caspase-8. It is possible that expression of these proteins may play an important part in regulating Fas/TRAIL mediated apoptosis in B-CLL or AML cells.

Another mechanism which may potentially be involved in abrogating Fas mediated apoptosis is constitutive expression of PKC and NF-κB. Studies on a panel of murine B lymphoma cell lines, which express different levels of Fas protein, revealed that expression of the Fas receptor did not correlate with their capacity to undergo Fas-mediated apoptosis. WEHI-231 and CH33 murine B lymphoma cell lines express high levels of Fas, and yet were resistant following incubation with anti-Fas Jo2 monoclonal antibody. Furthermore, Fas ligation in Fas expressing murine B lymphoma cell lines resulted in formation of the DISC complex, regardless of whether they were sensitive or resistant to Fas-mediated death. Downstream of Fas in the CH33 cell line, caspase-8 was activated upon Fas receptor activation. However, the cell line was still resistant to Fas mediated apoptosis, suggesting that the block in apoptosis is downstream of the DISC complex. In contrast, in WEHI-231 murine B lymphoma cell line, caspase-8 was not activated by the DISC complex upon Fas activation due to the expression of c-FLIP. The protein synthesis inhibitor, cycloheximide, and PKC inhibitors, such as bisindolylmaleimide, rendered Fas resistant CH33 murine B lymphoma cell line sensitive to Fas induced apoptosis. This suggests that constitutive PKC activation may play a role in Fas resistance by upregulating NF-κB or downregulating anti-apoptotic Bcl-2 family members. WEHI-231 murine B lymphoma cell line remained Fas-resistant after treatment with cycloheximide or bisindolylmaleimide, suggesting that PKC or NF-κB does not regulate c-FLIP expression. However, the expression of c-FLIP has recently been demonstrated to be regulated by NF-κB. Therefore, since Fas is not mutated in the WEHI-231 cell line, it is possible that a mutation exists in FADD or caspase-8 that
prevents the recruitment and activation of caspase-8. Transcription factor NF-κB is a major effector of anti-apoptotic receptor associated proteins. These include cIAP1, cIAP2, TRAF1, and TRAF2. Recently NF-κB activation has been demonstrated to upregulate c-FLIP expression, resulting in increased resistance to Fas, TNF or TRAIL mediated apoptosis. Resistance to either Fas or TRAIL mediated apoptosis can be overcome with cycloheximide or bisindolylmaleimide, which rapidly downregulates c-FLIP.

These findings suggest that c-FLIP is an important mediator of NF-κB-controlled anti-apoptotic signals. Both B-CLL and AML samples were shown to express c-FLIP, and since c-FLIP is an inhibitor of Fas and TRAIL mediated apoptosis, treatment of B-CLL or AML with cycloheximide or bisindolylmaleimide may sensitize the cells to TRAIL or Fas mediated apoptosis. In chapter 5, it was shown that autologous plasma enhances basal survival and resistance to DNA damage-induced apoptosis in B-CLL cells by activating Akt. B-CLL cultured in FCS also activated low levels of Akt. Since Akt activates NF-κB, it is possible that c-FLIP expression is upregulated in B-CLL cells thus inhibiting Fas/TRAIL mediated apoptosis. The protective effect of plasma may also enhance Fas/TRAIL resistance in AML cells. It would be of importance to investigate if plasma protects AML cells from TRAIL or Fas mediated apoptosis.

The activation of caspase-8, which has been considered to be exclusively involved in killing via ligation of death receptors, appears to play a role in the killing of both B-CLL and AML cells by cytotoxic agents. Although Apaf-1 itself does not activate caspase-8, pro-caspase-8 may be processed by active caspase-9 or caspase-3 via a feedback loop. Therefore, both caspase-3 and 8 may function as executioners in the apoptotic pathway. Release of cytochrome-c from mitochondria, with the consequent Apaf-1-dependent activation of caspase-9 has been proposed as the major apoptotic pathway that activates caspase-3 and subsequent apoptosis in response to genotoxic damage by cytotoxic agents. Western blot experiments have shown that pro-caspase-9 processing was only observed when B-CLL cells were treated with high dose Chl. However, activation of pro-caspase-9 following binding to Apaf-1 does not require its processing. Therefore, the role of caspase-9 in killing of B-CLL cells by cytotoxic...
treatments is unclear at present. In AML cells, pro-caspase-9 processing was observed when cells were treated with cytotoxic agents (data not shown), which could also be responsible for processing pro-caspase-8. In order to expand further upon these observations, studies should be undertaken to explore and elucidate the apoptotic pathway induced by different cytotoxic agents.

The role of mitochondria in apoptosis has been further complicated by the report that caspase-3 can be activated in the absence of cytoplasmic cytochrome-c elevation during apoptotic death of B-CLL cells. Release of mitochondrial cytochrome-c to the cytosol was not observed following treatment of B-CLL cells with Chl, radiation or electron transport inhibitor antimycin A. Furthermore, mitochondrial swelling and rupture, which has been suggested as a possible mechanism of cytochrome-c release, were only present in cells which were in advanced stages of apoptosis. One possible mechanism of cytochrome-c independent caspase-3 induced apoptosis involves Smac. Smac is a promoter of caspase activation in the cytochrome-c/Apaf-1/caspase-9 pathway. A recent study showed that multiple myeloma (MM) cells treated with dexamethasone (Dex) triggers the release of Smac from the mitochondria to the cytosol, thus activating caspase-9 without concurrent release of cytochrome-c and Apaf-1 oligomerization. Smac interacts with IAPs, such as XIAP, and eliminates their inhibitory effects on caspase-9. In Myc-XIAP transfected MM cells, Dex treatment induced an interaction between XIAP and Smac, which led to dissociation of XIAP from caspase-9. These findings are in concert with other studies demonstrating that Smac promotes caspase activity of initiator caspase-9 by binding to and inhibiting IAPs. Studies on Smac and cytochrome-c regulation in B-CLL cells and AML cells after cytotoxic treatment would be of interest.

While Fas ligation has been shown to be a potent inducer of apoptosis in different cell types, its usefulness have been limited by its acute toxicity. Recently, the in-vivo use of TRAIL, may be associated with severe liver damage, death of normal brain cells and erythroblasts in normal human cells. Therefore, the use of TRAIL and Fas ligation in the treatment of human malignancies must be considered with caution, and further studies would be required for the pre-clinical and clinical evaluation of TRAIL.
6.3 The role PI3-K/Akt and tyrosine kinase pathway in B-CLL survival and apoptosis.

The studies presented in this thesis show that plasma protects B-CLL cells from spontaneous and DNA damage induced apoptosis, and that selective inhibition of PI3-K or tyrosine kinase by LY294002 or HMA respectively, sensitises B-CLL cells cultured in FCS or plasma to cell killing by cytotoxic agents. Plasma protects B-CLL cells from apoptosis by activating the PI3-K/Akt pathway, which is inhibited by LY294002\(^{293}\). However, the plasma activated tyrosine kinase target for HMA in B-CLL cells has not yet been identified. Plasma has been shown not to be involved in activating JAK1 or JAK2 in B-CLL cells\(^{293,438}\). It is possible that HMA may sensitis B-CLL cells to cytotoxic agents by inhibiting 90-kDa heat-shock protein (hsp90)\(^{452}\). Furthermore, the survival signals via the PI3-K pathway and HMA inhibited tyrosine kinase pathway in B-CLL cells were shown to be closely associated due to the correlation between LD\(_{50}\) for HMA and LY294002.

Apoptosis induced by Chl resulted in DNA damage, which was followed by p53 upregulation and transcription. However, when B-CLL cells were cultured in plasma, p53 upregulation was retarded. The block in Chl mediated p53 upregulation in plasma cultured cells was abrogated when cells were treated with LY294002 or HMA, which suggests that the PI3-K/Akt or tyrosine kinase pathway regulates p53 expression.

Activation of PI3-K and Akt by cytokines or growth factors promote cell survival through multiple mechanisms\(^{285,285,287-289}\). Phosphorylation of pro-apoptotic Bad by Akt prevents Bad from interacting with Bcl-X\(_L\)\(^{107,108}\). Instead, phosphorylated Bad associates with 14-3-3 protein\(^{109}\). The Bad 14-3-3 complex is found in the cytosol thus preventing Bad from interacting with Bcl-X\(_L\) at the mitochondria, therefore inhibiting apoptosis. Akt can also phosphorylate and inhibit caspase-9 activation mediated by cytotoxic agents\(^{40,453}\). In contrast, growth factor withdrawal may promote apoptosis by promoting dephosphorylation of Bad, either through natural decay or via the action of a phosphatase e.g. calcineurin, allowing it to dimerize with Bcl-X\(_L\)\(^{106}\). Phosphorylation and inactivation
of pro-apoptotic FKHRL1 by Akt may also contribute to the inhibition of apoptosis. However, the possibility that anti-apoptotic signalling via the PI3-K/Akt pathway involving phosphorylation of yet undiscovered substrates cannot be ruled out.

The mechanism, through which plasma block p53 upregulation in B-CLL cells is presently unknown, but may involve Mdm2. p53 is normally maintained at low levels within the cell through its interaction with Mdm2, a protein that escorts p53 from the nucleus and targets it for degradation in the cytoplasm, thus ensuring that the p53 signal is carefully controlled. Phosphorylation of either p53 or Mdm2 by ATM family of proteins after DNA damage prevents the two proteins from interacting, thus stabilising and activating p53. Therefore, activation of PI3-K/Akt or tyrosine kinase pathway by plasma may promote direct suppression of p53 activation via Mdm2. Recently, it has been reported that the PI3-K/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus by phosphorylating it near the nuclear localisation site on serine 166 and serine 186, and plays a role in p53 degradation and suppression of p53 function. LY294002 can block nuclear localisation of Mdm2 by directly inhibiting Akt, and increase endogenous levels of p53. However, export of the Mdm2/p53 complex from the nucleus to the cytoplasm is necessary for p53 degradation. This activity requires the nuclear-export signal (NES) of p53, and the C-terminal RING domain of Mdm2. Thus, the PI3-K/Akt pathway appears to be required, but may not be entirely involved in regulating Mdm2 degradation of p53. Therefore, plasma activation of Akt followed by nuclear translocation of Mdm2 may provide a mechanism that targets p53 for degradation consequently delaying apoptosis in B-CLL cells.

The Mdm2 protein has been found to be overexpressed in B-CLL cells compared with normal B-cells, and exist as multiple forms p57, p59, p67, and p90. Since Mdm2 protein analysis from these B-CLL samples were done immediately following isolation from patients or following storage at -70°C, the effect of patient plasma on B-CLL cells may have upregulated Mdm2 levels. Levels of Mdm2 and p53 degradation may play an important part in protecting in-vivo B-CLL cells from cytotoxic agents and in promoting the disease in plasma.
Another cell survival pathway that may inhibit p53 transcription activity involves activation of NF-κB by Akt. NF-κB can be activated by a large variety of stimuli, including inflammatory cytokines such as TNF-α and IL-1, T-cell activation signals, growth factors, and stress inducers. NF-κB and p53 can inhibit each other’s ability to stimulate gene expression by competing for relative levels of transcription co-activator proteins p300 and CBP. This mechanism of transcription decision making governs cellular process such as survival or apoptosis, and provides another mechanism for plasma to protect B-CLL cells from apoptosis and to contribute to tumourigenesis and disease. Akt activation has also been reported to inhibit p53 transcriptional activity directly by phosphorylating co-activators.

The mechanism of the pro-apoptotic action of HMA on B-CLL is unclear. This agent was originally postulated to inhibit tyrosine kinases, but recent studies have demonstrated that hsp90 represents the major known direct target for HMA. Hsp90 is one of the most abundant cytosolic proteins in eukaryotes. It is a peptide-binding protein that provides essential chaperone support by folding intermediates of various signal transduction molecules, including certain steroid hormone receptors (SHR), and more than 12 kinases that include serine kinase Raf and the tyrosine kinases. Ansamycins, such as HMA and geldanamycin, have been shown to bind to a conserved pocket that has homology to the DNA gyrase ATPase in hsp90 and alter the function of this chaperone protein. Occupancy of this pocket by ansamycins results in the degradation of a subset of signalling molecules, which include SHR, Raf, as well as certain transmembrane tyrosine kinases, such as the ErbB receptor family. It has also been reported that in mammalian cells, hsp90 can interact with mutant p53 and treatment with geldanamycin increases p53 levels. In a variety of tumour cell lines, treatment with HMA results in growth inhibition and subsequent cell death. One mechanism involves PI3-K/Akt pathway. Growth factor-stimulated translation of D-type cyclins has been shown to occur through the PI3-K/Akt pathway. The D-type cyclins associate with cyclin dependent kinases Cdk4 or Cdk6 to form an active complex that phosphorylates and inactivates RB. Ansamycins inhibit this pathway, in part by degrading upstream transmembrane tyrosine kinases, and downregulating D-type cyclin expression. However, in B-CLL cells HMA does not block activation of Akt. Geldanamycin can also destabilise Raf-1, which
leads to disruption of the Raf-1-MEK-mitogen activated protein kinase (MAPK) signalling pathway. HMA has also been shown to inhibit the formation of the hsp90/Src family tyrosine kinase complex. Src family protein tyrosine kinases play a key role in cell homeostasis. The viral pp60v-src kinase (v-Src), an oncogenic protein encoded by the Rous sarcoma virus, is in its active state when attached to the plasma membrane. It activity causes uncontrolled, oncogenic proliferation of cells. Only the soluble form of v-Src forms a stable complex with hsp90, which suggests that hsp90 is involved in maturation of the kinase. The cellular counterpart c-Src has also been shown to require hsp90 for maturation. Lck and c-fgr, which are members of the Src family have been reported to be expressed in B-CLL cells. It is possible that these Src-tyrosine kinases in B-CLL cells can be activated by plasma, and the actions of ansamycin antibiotics on the stability of these proteins may warrant an investigation. In chronic myelogenous leukaemia, it has been reported that brief exposure of cells to geldanamycin decreases the association of v-Src with Hsp90, which was followed by proteasome induced degradation of v-Src.

Since hsp90 can interact with a large array of cell regulatory proteins, the precise identification of interactions and activation inhibited by HMA presents a formidable challenge. The use of geldanamycin may also be of some interest in treating B-CLL cells in combination with cytotoxic agents.

In order to expand on observations in this thesis, studies could be under-taken to explore the effect of LY294002, HMA and geldanamycin in combination with cytotoxic agents on Mdm2, NF-κB, Src and hsp90 expression and regulation in B-CLL cells when cultured in plasma. It is also important to find the constituent in plasma that protects B-CLL cells from apoptosis. Neutralising antibodies to various cytokines and growth factors failed to block protection of plasma and failure to activate JAK1 and JAK2 confirms that various cytokines and growth factors do not contribute to the anti-apoptotic action of plasma. In addition, enzyme-linked immunosorbent assays (ELISAs) also confirmed that plasma from patients did not contain detectable levels of IL-4, IL-6 or IFN-α. Fractionation studies have been initiated in an attempt to identify these unknown
cytoprotective agents, which mediate the potent cytoprotective actions of plasma on B-CLL cells. Identification of these components may suggest further strategies to enhance the actions of cytotoxic drugs on B-CLL cells.
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**Publications related to this thesis**

**Full papers:**


**Abstracts:**


Jones, D.T., Wickremasinghe, R.G., Mehta, A.B., Prentice, H.G., Hoffbrand, A.V. and Ganeshaguru, K. The role of induced FAS expression in the killing of B-Chronic...


