TARGETING OF CYTOTOXIC PEPTIDES TO HAEMATOLOGICAL MALIGNANCIES

by

Alexandra Jane Marks

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Department of Haematology,
Royal Free and University College School of Medicine,
University of London

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Amphipathic peptides with an α-helical structure disrupt membranes rich in negatively charged phospholipids and have antibiotic properties. They are toxic to eukaryotic cells if internalised by a suitable targeting mechanism. We have targeted one such peptide $D(KLAKLAK)_2$ to haemopoetic cells by conjugating it to monoclonal antibodies which recognise lineage-specific cell-surface molecules. An anti-CD33: peptide conjugate was cytotoxic to one of three CD33-positive myeloid leukaemia lines, whereas an anti-CD19: peptide conjugate efficiently killed three out of three B lymphoid lines with IC_{50} in the low nanomolar range. Cell lines which did not express the relevant antigen were resistant to the conjugates at the concentrations used. Peptide conjugated to anti-CD19 and anti-CD33 antibodies had cytotoxic activity towards cells isolated from the peripheral blood of patients with chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML) respectively. CLL cells isolated from heavily pre-treated patients or from poor risk category individuals, as determined by elevated expression of the ZAP-70 protein tyrosine kinase, were as sensitive to the conjugate as were untreated or good risk category patients. Cell death was shown to be by an apoptotic mechanism and independent of the level of expression of anti-apoptotic proteins Bcl-2, Mcl-1 and XIAP. In addition the conjugate acted synergistically with chlorambucil. The data here suggest that anti-CD19 $D(KLAKLAK)_2$ displays a highly selective and potent cytotoxic activity against CLL cells and may be of therapeutic value in the treatment of this incurable leukaemia. We also panned a phage display library in an attempt to isolate a peptide that would bind to CD33 and thereby create a peptide with both targeting and cytotoxic activity towards malignant myeloid cells.

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. 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.

Data on IgVH gene mutation and ZAP-70 status of CLL samples was generously provided by Veronique Duke and Najeem Folarin respectively.

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Abbreviations

ADCC	Antibody dependent cell mediated cytotoxicity
ADEPT	Antibody directed enzyme therapy
AIF	Apoptosis inducing factor
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
Ant	Adenine nucleotide transporter
Apaf-1	Apoptotic protease activating factor 1
APAAP	Alkaline phosphatase-anti-alkaline phosphatase
APC	Antigen presenting cell
APL	Acute promyelocytic leukaemia
Ara-C	Cytosine arabinoside
ASO	Anti-sense oligonucleotide
ATM	Ataxia telangiectasia mutated
ATP	Adenosine 5-triphosphate
ATRA	All-trans retinoic acid
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated protein X
BH	Bcl-2 homology
BSA	Bovine serum albumin
CDC	Complement dependent cytotoxicity
CHO	Chinese hamster ovary
CML	Chronic myelogenous leukaemia
CTL	Cytotoxic T-lymphocyte
CR	Complete remission
DgA	Deglycosylated ricin A chain
DIABLO	Direct IAP binding protein with low pI
DLI	Donor lymphocyte infusion
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DT	Diptheria toxin
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence

EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra-acetic acid
EF-1	Elongation factor 1
EGTA	Ethylene glycol tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage colony stimulating factor
GVHD	Graft versus host disease
HAMA	Human anti-mouse antibody
HBSS	Hanks Balanced Salts Solution
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
Id	Idiotype
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
KLH	Keyhole limpet haemocyanin
LAK	Lymphokine activated killer
Mab	Monoclonal antibody
MDS	Myelodysplastic syndrome
MGG	May Grümwald Giemsa
MHC	Major histocompatibility complex
MM	Multiple myeloma
MMP	Mitochondrial membrane permeabilisation
MOPS	N-morpholino propanesulfonic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor-kappa B
NHL	Non Hodgkin's lymphoma
NK	Natural killer
PARP	Poly (ADP) ribose polymerase

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Pseudomonas exotoxin
Pfu	Plaque forming unit
PIG-3	p53 induced gene 3
PMSF	Phenylmethylsulfonyl fluoride
PT	Permeability transition
PVP	Polyvinylpyrolidone
RARα	Retinoic acid receptor α
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
SCT	Stem cell transplant
SDS	Sodium dodecyl sulfate
Smac	Second mitochondria-derived activator of caspase
SPDP	N-Succinimidyl-3- (2-Pyridyldithio) Propionate
ScFv	Single chain fragment
TAA	Tumour associated antigen
TGF-β	Transforming growth factor B
TIL	Tumour infiltrating lymphocytes
TM	Transmembrane
T-PLL	T-cell prolymphocytic leukaemia
TNF	Tumour necrosis factor
UV	Ultra violet
VDAC	Voltage dependent anion channels
VH	Heavy chain variable region gene
VL	Light chain variable region gene
VOD	Veno-occlusive disease
XIAP	X-linked inhibitor of apoptosis
ZAP-70	Zeta-associated protein 70

Chapter 1. Introduction

1.1.Introduction to targeted therapy

The goal of targeted therapy in malignant disease is to develop protocols which kill cancer cells while leaving normal healthy tissue untouched. The advantage of this specificity is to decrease side effects, and by doing so, to allow higher and more effective doses of drug to be administered. It also allows treatment of groups of patients who would otherwise be considered poor risk in terms of age and coexistent disease. This is especially relevant in view of the increasing age of the patient population.

All chemotherapeutic agents in use today are in principle, targeted to a certain extent, in that they affect the malignant cells more profoundly than healthy cells. Most conventional agents target DNA, exploiting the fact that the tumour cells differ from normal cells in their mitotic rate. Alkylating agents such as cyclophosphamide and chlorambucil alkylate DNA, while antimetabolites such as methotrexate, a folic acid antagonist and purine /pyrimidine antagonists such as cytarabine interfere with nucleic acid precursor synthesis. The plant alkaloids such as vincristine cause microtubule depolymerisation, thus disrupting chromosome segregation during mitosis. These agents have side effects related to the drug effects on the rapidly dividing cells of the normal bone marrow, gastrointestinal tract and hair follicles. Side effects that occur as a result of these toxicities means that chemotherapeutic agents are often given at sub optimal doses, resulting in eventual failure of therapy, which may be accompanied by the evolution of drug resistance and onset of metastatic disease.

1.1.1 Targeting specific genetic lesions in leukaemia

With increasing knowledge and understanding of the molecular basis of many haematological malignancies, novel therapies have emerged that target more specific pathways. In some diseases, the elucidation of specific genetic changes and their altered gene products has been exploited. For example, in acute promyelocytic leukaemia (APL), all trans retinoic acid (ATRA), an active derivative of vitamin A, binds directly to the pml-rarα fusion protein, overcoming the differentiation block which is characteristic of this disease [1]. In chronic myelogenous leukaemia (CML), a reciprocal translocation occurs in the haemopoietic stem cell leading to the creation of a BCR-ABL gene on the resultant Philadelphia chromosome (t (9; 22)). This encodes a protein with dysregulated tyrosine kinase activity. Imatinib mesylate (or STI-571) competitively inhibits the interaction of adenosine triphosphate (ATP) with this bcr-abl tyrosine kinase thus decreasing its ability to phosphorylate and activate downstream target proteins [2].

There are many other leukaemias where relatively disease specific mechanisms are being targeted and new classes of drugs are in development. Some of these are summarised in Table 1.1

Drug class	Target pathway	Ref
Inhibitors of farnesyl transferase (FTase)	Farnesylation is an important post-translational modification step for RAS proteins involved in signal transduction and growth regulation. Ras mutations are a common finding in haematological and other malignancies	3
Inhibitors of DNA methyltransferase Azacytidine, Decitabine	DNA hypermethylation is associated with suppression of regulatory genes involved in cell proliferation and differentiation is a common phenomenon in myelodysplastic syndrome (MDS), acute myeloid leukaemia (AML) and CML.	4
FLT 3 inhibitors	FLT3 is a transmembrane tyrosine kinase growth factor receptor selectively expressed on haematopoietic cells Activating FLT3 mutations occur in up to 41% of AML	5
Proteasome inhibitors PS-341	The ubiquitin-proteasome pathway is the principal intracellular pathway responsible for the regulated degradation of proteins, including many that participate in cell cycle regulation and tumour growth. Inactivation of the transcription factor NF-κB is thought to be important in the anti neoplastic activity of this class of drug especially in multiple myeloma (MM).	6
Bcl-2 antisense oligonucleotide	Bcl-2 overexpression occurs in many B cell non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukaemia (CLL) and is also associated with treatment resistance in AML, CLL and NHL.	7
Thalidomide derivatives	Main action is to target angiogenesis in MM, MDS	8

Table 1.1 New drugs which target specific pathways in haematological malignancies

1.1.2 Immunotherapy

Immunotherapy represents one of the most important arms of the targeted therapy approach. Tumour cells often express specific receptors or other molecules, on their surface membranes, which distinguish them from their surrounding normal tissues. These are called tumour-associated antigens (TAAs). TAAs include tumour specific antigens, which are the result of mutations or gene rearrangements, viral antigens in cancers associated with viruses, and tissue specific differentiation antigens, which are also expressed by normal cells from which the malignant clone arose. TAAs may be truly specific as in idiotype surface immunoglobulin in B cell malignancies or may be overexpressed in the malignant population compared to healthy cells.

Many tumour-associated antigens may be recognised by the immune system and play a critical role in the host – tumour interaction. Immunotherapy exploits this by either stimulating the patient's immune system to augment this response using cytokines or vaccination strategies (active immunotherapy), or by manipulating or mimicking certain aspects of the normal immune response to target the malignant cells (passive immunotherapy).

As a general rule, foreign viral antigens and mutation-related tumour specific antigens are more immunogenic than self-antigens. Some key TAAs expressed by haematological malignancies are summarised in Table1.2.

Antigen type		Haematological malignancy
Tumour specific antigens		
Fusion gene products	bcr-abl	CML
	AML/ETO	AML
	PML-RARα	APL
Idiotype epitopes	Id Immunoglobulin	B-cell malignancies
	T-cell receptor idiotypes	T-cell malignancies
Viral associated antigens		
Epstein Barr virus		Burkitt's lymphoma
		Hodgkin's lymphoma
HTLV-1		Adult T-cell leukaemia
Over expressed normal antigens		
Proteinase 3		AML, CML
WT-1		AML, CML, ALL
MUC-1		MM
Mutated oncogenic proteins		
p53		Numerous malignancies
RAS		
Tissue specific differentiation antigens		
CD33		AML
CD19		CLL, NHL, B-cell acute lymphocytic leukaemia (ALL)

Table1. 2 Tumour associated antigens expressed by haematological malignancies

1.1.2.1 Immune response to TAAs: immunosurveillance and escape mechanisms

Burnet and Thomas first coined the term 'immunosurveillance' in 1967[9]. This described the concept that the immune system can recognise and destroy tumour cells. Initially, the finding that several immunocompromised mouse strains showed no significant increase in incidence of common tumours, with the exception of the viral associated tumours, dampened enthusiasm for this hypothesis[10]. However more recent epidemiological evidence from immunosupressed post-transplant populations shows increased incidence of a broad range of tumours with no apparent viral aetiology [11]. The finding in many malignancies that the presence of tumour infiltrating lymphocytes (TIL) correlates with improved survival further supports the theory of immunosurveillance [12]. Microarray studies have further deomonstrated that the moelucular features of TILs in lymphoma biopsy specimens have a profound effect on survival[13]

Many tumour specific antigens can be shown to evoke an anti - tumour T cell response in vitro and in vivo [14]. Major histocompatability (MHC) molecules on antigen presenting cells (APC), present TAAs to lymphocytes that participate in the innate immune response (NKT, NK$\gamma\beta$ T cells) which are then stimulated to produce interferon-γ. This has several effects, which include recruitment of other immune cells, a direct antiproliferative effect and the activation of the cyocidal action of macrophages and NK cells. Tumour cell debris ingested by dendritic cells is trafficked to draining lymph nodes where tumour specific CD4+ T helper cells and CD8+ T cells are produced. CD8+ T cells ultimately differentiate into cytotoxic T lymphocytes (CTL) and lyse tumour cells [15].

Despite all the mechanisms described above, it is exceptional for such a response to

spontaneously eliminate an established malignancy. There are several reasons for this. The first is that most tumours do not make distinctive antigenic peptides or do not express the co-stimulatory molecules necessary to initiate an adaptive immune response. Second, many tumour cells can use their genetic instability to their advantage by developing escape mechanisms by which they can avoid or suppress the adaptive immune response. This may involve down regulating the expression of MHC class 1 molecules[16] or the tumour antigens themselves, or secreting immunosuppressive cytokines such as transforming growth factor β[17] (TGF-β) and IL-10[18]. Thirdly, tumour cells may induce T-cell apoptotic clonal deletion by upregulating Fas ligand, which initiates cell killing mechanisms by engaging Fas molecules on the T cells[19].

1.1.2.2 Active immunotherapy

The aim of active immunotherapy is to stimulate the patient's immune system to augment the anti-tumour response; this can be achieved by using therapeutic cytokine or vaccination strategies.

a. Cytokines

Cytokines are small hormone-like proteins (of ~20 kDa) that act by binding to specific receptors, leading to activation of signalling pathways which result in changes in gene expression in the nucleus. Cytokines are secreted by a variety of cells, and can act on the cells that produce them (autocrine action), on other cells in the immediate vicinity (paracrine action), or on cells at a distance (endocrine action). Cytokines have role in controlling the growth, development, and functional differentiation of the cells of the immune system. A variety of cytokines are used alone or in combination with other therapies in the treatment of haematological malignancies.

Interferon -α (IFN-α)

IFN-α is secreted mainly by monocytes and lymphoid cells. This cytokine is used alone or in combination with chemotherapy and/or STI 571 in patients with Ph-positive CML who are not candidates for allogeneic stem cell transplantation (SCT). As a single agent it induces durable cytogenetic responses and prolongs the duration of the chronic phase and of survival compared with conventional chemotherapy[20]. In the maintenance treatment of MM, IFN-α has been shown to prolong the plateau phase of the disease following high dose chemotherapy or autologous transplantation[21]. It is also used in the treatment of hairy cell leukaemia[22]. The exact mechanism of action of IFN-α in these diseases is not known. It may have a direct anti-proliferate effect or an indirect one on the immune system through non-specific enhancement of anti-leukaemic cell -mediated responses[23]. In some diseases it is thought to stimulate the synthesis of tumour associated cell surface antigens.

Granulocyte macrophage colony stimulating factor (GM-CSF)

GM-CSF is secreted by activated T-cells and macrophages. Its main functions are to stimulate growth and differentiation of leukocytes. It is chemotactic for neutrophils and enhances microbicidal activity, oxidative metabolism, and phagocytotic activity of neutrophils and macrophages.

The main uses for GM-CSF in the treatment of haematological malignancies are to enhance the reconstitution of the haematopoietic system post chemotherapy or bone marrow transplant and as an adjuvant in vaccination therapies to induce the differentiation and activation of dendritic cells (see whole cell vaccines p29).

Interleukin-2 (IL-2)

IL-2 is secreted by CD4- positive T-cells, NK cells and lymphokine-activated killer cells (LAK). IL-2 is a central regulator of the immune response; it induces the proliferation of T-cells and NK cells and in combination with IL-4 also promotes proliferation of activated B-cells. It stimulates the synthesis of IFN-γ in peripheral leukocytes and induces the secretion of IL-1and TNF.

In the treatment of haematological malignancies IL-2 has been shown, in some cases to induce some tumour response in B cell NHL, but with no increase in overall survival[24]. Its use has been limited by non-specific toxic side effects. IL-2 is, however, an important component of adoptive immunotherapy strategies, where it is used to stimulate the production and ex vivo proliferation of LAK cells and TILs (see p30).

Chimeric fusion toxins obtained by the fusion of cDNAs of IL-2 and the coding regions of toxic protein molecules such as Diphtheria toxin have been developed for the treatment of T- cell malignancies that express the IL-2 receptor[25].

Cytokine gene transfer is another strategy being developed with the aim of increasing the immunogenicity of tumours by incorporating a functional IL-2 gene[26].

b. Vaccination

In 1796 Edward Jenner used vaccination with cowpox to induce immunity to smallpox, eventually leading to eradication of the disease 180 years later [27].

William Coley introduced the idea of vaccination for cancer therapy in the 1890s[28]. The objective of cancer vaccination is to elicit an active immune response against antigens expressed by tumour cells leading to specific rejection of disseminated malignant cells and ideally providing long-lived immunological memory capable of protecting the vaccinated patient against relapse.

Active immune responses against tumour cells can be induced in normal animals and can render them immune to subsequent challenge with the tumour. However successful vaccination against actively growing tumours is much more difficult to achieve. This may suggest that vaccination would have the most clinical benefit either used in the setting of disease remission or minimal residual disease or even as a prophylactic treatment in individuals deemed to be at high risk of developing a specific malignancy.

Tumour specific idiotype vaccines for B-cell malignancies

B-lymphocytes express an immunoglobulin (or idiotype Id) that is the product of a unique combination of gene rearrangements. All of the malignant cells that arise from a single B cell will express the same idiotype. Using Id as a vaccination target would require generation of a different vaccine for each patient. By generating a hybridoma from tumour cells obtained at lymph node biopsy fused to a myeloma cell line, a group at Stanford University were the first to purify large quantities of idiotype protein. This was then chemically coupled to keyhole limpet haemocyanin (KLH), a foreign protein, to make it more immunogenic. This approach was used in a trial of 32 patients with low-grade follicular lymphoma in first remission post chemotherapy. Approximately half the patients mounted an immunological response to the vaccine, mainly by generating anti-Id antibodies. This response strongly correlated with freedom from disease progression and overall survival[29]. Dendritic cells, isolated from peripheral blood or generated in vitro from monocytes, and then pulsed with Id proteins, have been used as an alternative route for Id vaccination[30]. Other variations include co-administration of GM-CSF and/or IL-2 as an adjuvant[31]. Anti-idiotype vaccine

strategies of this type are currently being tested in two large Phase III randomised trials in first complete remission (CR) patients[32].

DNA Id vaccination involves cloning the genes encoding idiotype variable regions in a mammalian vector and delivering it, often in the form of a fusion protein with a fragment of tetanus toxin, directly into muscle[33]. The mechanisms by which antigen is presented to the immune system after DNA vaccination is complex. It involves a process called 'cross-priming', in which antigen translated in the muscle cells is released and taken up by bone marrow derived antigen presenting cells, as well as the direct transfection of dendritic cells. DNA vaccines are therefore capable of inducing both humoral and cellular responses, as opposed to mainly humoral responses in conventional Id vaccination strategies[34,35].

Bcr-abl peptide vaccination for CML

Although the bcr-abl fusion protein is an intracellular peptide, Clark et al demonstrated that smaller peptides which span the bcr-abl junction, and are derived from the full length peptide, are expressed on the surface of CML cells in association with HLA class I molecules [36]. Furthermore they demonstrated an in-vitro cytotoxic T-cell response to these peptide fragments that was capable of killing autologous CML cells. Another groups used bcr-abl peptide fragments to vaccinate 12 patients with Philadelphia positive CML, 3 patients demonstrated specific proliferative immune responses but none showed any evidence of cytotoxic T lymphocyte activity[37].

Whole cell vaccines

This is an approach that has been used when no specific TAA has been identified. It involves using genetically altered autologous tumour cells transduced to secrete cytokines such as GM-CSF or IL-12 or express co-stimulatory molecules such as CD40 ligand or B7, to make the tumour cells more immunogenic[38,39]. Tumour vaccines that secrete GM-CSF, which induces differentiation and activation of dendritic cells, have been shown to induce an increased immune response in mouse models of AML[40].

Another novel experimental approach to tumour vaccination is the use of heat-shock proteins isolated from tumour cells. The underlying principle of this therapy is that one of the physiological activities of heat-shock proteins is to act as intracellular chaperones of antigenic peptides. There is evidence for receptors on the surface of professional antigen-presenting cells that take up certain heat-shock proteins together with any bound peptides. Uptake of heat-shock proteins via these receptors delivers the accompanying peptide into the antigen-processing pathways leading to peptide presentation by MHC class I molecules[41].

1.1.2.3 Passive immunotherapy

Passive immunotherapy involves the administration of pre-formed immune system components, or monoclonal antibodies, to target diseased cells without directly activating the patient's immune system.

a. Adoptive immunotherapy

In this strategy tumour specific lymphocytes are removed from the patient and expanded in vitro, in the presence of cytokines, before being returned.

Lymphokine activated killer (LAK) cells

Initial experiments in adoptive immunotherapy involved removing lymphocytes from the blood of a patient and growing them in the presence of IL-2. The cells were then returned to the patient. These lymphocytes were called lymphokine-activated killer (LAK) cells and are now thought to be derived mainly from NK cells. In an early trial, out of 25 patients with advanced chemotherapy resistant cancers, 10 patients showed a 50% or greater reduction in tumour size [42].

Tumour infiltrating lymphocytes (TIL)

The results of adoptive immunotherapy with blood derived LAK cells led to a search for more specific cytotoxic cells. CD4 + and CD8+ lymphocytes which infiltrate tumours can be isolated from the tumour tissue and expanded in the presence of IL-2, at lower concentrations than those required for LAK cells[43]. These cells have tumour-targeting properties and have shown a much stronger anti-tumour effect than LAK cells on a per cell basis in animal models. The mechanism of anti-tumour activity of TILs is not known, though there is some evidence that these cells secrete cytotoxins and cytokines capable of killing tumour cells and recruiting other immune cells. Due to their tumour homing properties TILs are suitable targets for T-cell mediated gene therapy and some groups have engineered TILs with genes encoding cytokines or cytotoxins[44,45].

At the present time the clinical use of TILs is limited because of the difficulty in finding sufficient numbers of TILs at diagnosis and the long ex vivo expansion process.

Donor lymphocyte infusion (DLI)

DLI has proved to be very effective, particularly in patients with CML, restoring a state of haematological remission after leukaemic relapse following an allograft in approximately 70% of patients[46,47]. Furthermore most of these remissions are sustained indicating the eradication of the clonogenic leukaemia cells. DLI has also been used to reverse relapse in patients with acute leukaemia, NHL and MM. However the response rates of patients with other haematological malignancies is significantly lower than for patients with CML[48]. DLI also has a role in the treatment of EBV induced post transplant lymphoproliferative disorders[49].

DLI cells may target MHC differences, minor histocompatibility antigens, or tumour-specific antigens. Myelosupression and graft versus host disease (GVHD) are the two main side effects of DLI. One approach to limit the morbidity associated with GVHD is to infuse thymidine kinase gene-transduced DLI, followed by treatment of the patient with gancyclovir if this side effect occurs[50].

Genetically modified T-cells

Advances in gene therapy have allowed the manipulation of T-cells in an effort to improve the immunological response to adaptive therapy. T-cells can be transduced with genes that encode antigen receptors thus enabling the recognition of antigens that are poorly immunogenic. T-cell proliferation and function can be increased by manipulating cells to express modified co-stimulatory receptors, or be genetically protected from immunosuppressive factors such as TGF-β. T cells can also be transduced with regulatable suicide genes, thereby improving the safety of adoptive cell therapy [51].

b. Antibody therapy

Monoclonal antibodies can be used either alone or conjugated to cytotoxic drugs or radioisotopes.

i.Unconjugated monoclonal antibodies

Rituximab

The unconjugated monoclonal antibody that has been the most extensively studied and used clinically is the chimeric anti-CD20 monoclonal antibody Rituximab. This antibody consists of murine variable regions grafted onto a human IgG1 constant region. In vitro results have shown that Rituximab acts by complement dependent cytotoxicity (CDC), antibody-dependent cell mediated cytotoxicity (ADCC) and induction of apoptosis, but it is not clear by which mechanism Rituximab works clinically[52].

The first pivotal trial of Rituximab in patients with relapsed indolent NHL, showed a 48% overall response rate[53]. More recent studies have shown a significant benefit of Rituximab used as maintenance therapy following standard chemotherapy regimes in newly diagnosed follicular lymphoma[54]. Two trials of Rituximab as single agent therapy in aggressive NHL have shown overall response rates of around 30%[55,56].

Rituximab has used most successfully in combination with other chemotherapeutic regimes, most notably in combination with CHOP. A large Phase III randomised trial by Coiffier et al evaluated 397 patients aged 60-80 years, with newly diagnosed aggressive NHL who were randomised to CHOP alone versus CHOP + Rituximab (CHOP-R). In this trial, the CR rate, event-free and overall survival rates were superior for the combination arm [57]. The superiority of CHOP-R over CHOP alone for 1st line

treatment of patients with newly diagnosed advanced follicular lymphoma has also been recently demonstrated[58].

Rituximab has also been shown to have therapeutic activity in CLL[59] and Waldenstroms macroglobulinaemia[60] and has been reported to be effective in certain non-malignant autoimmune disorders such as idiopathic thrombocytopenic purpura, auto-immune haemolytic anaemia, cold agglutinin disease, rheumatoid arthritis[61] and acquired haemophilia[62].

Alemtuzumab

Alemtuzumab (CAMPATH-1H anti-CD52) is a humanised monoclonal antibody that targets CD52, an antigen present on 95% of all normal human B and T lymphocytes, monocytes and macrophages, as well as in most B-cell and T-cell lymphomas. It acts by a combination of CDC and ADCC and has also been shown in vitro to directly induce apoptosis.

The main clinical uses of Campath 1-H are in the treatment of CLL and as a method of T-cell depletion to prevent GVHD in the allogenieic transplant setting. In a pivotal study, 93 patients with advanced CLL who had failed other therapies gave an overall response rate of 33%[63]. Other studies have also shown significant results in the treatment of T-PLL[64]and first line treatment of CLL[65]. Ongoing studies are investigating the efficacy of Campath –1H in combination with other therapies such as fludarabine and Rituximab.

Other unconjugated monoclonal antibodies for B-cell malignancies

Apolizumab/ Hu1d10, a humanised monoclonal antibody, is directed against a polymorphic determinant of HLA-DR expressed on normal and malignant B-cells and is capable of inducing ADCC, CDC and direct apoptosis of lymphoma cell lines. A phase I dose escalation study in 20 B-cell lymphoma patients demonstrated that 4 of 8 follicular lymphoma patients achieved durable responses. A Phase II randomised study is currently being performed in patients with relapsed or refractory follicular, small lymphocytic or marginal zone/MALT B-cell lymphoma. [66]

Epratuzumab is a humanised monoclonal antibody directed against the CD22 antigen, present on 75% of B-lymphocytes. Overall response rates of 43% and 34% have been shown in phase I/II trials in follicular lymphoma and diffuse large cell lymphoma respectively[66].

IDEC-152 is a primatised monoclonal antibody that targets CD23 on B-cell malignancies, it has been shown to induce ADCC, CDC and apoptosis in CLL cells in vitro and also has synergistic actions with both fludarabine and rituximab[67].

HuM-195

HuM195 (anti-CD33) is a humanised monoclonal antibody directed against the cell surface antigen, CD33, which is detectable in over 90% of patients with acute myeloid leukaemia. It has been shown to induce cell death by activation of complement as well as by ADCC, but there is no evidence that binding of the antibody to the CD33 antigen initiates intracellular signalling leading to direct activation of apoptosis. As a single agent in patients with relapsed or refractory AML, HuM195 showed only modest results, with just two complete and one partial remission out of 49 evaluable patients[68].

HuM195 however showed better efficacy in minimal residual leukaemia in a study of patients with APL in CR with minimal disease documented by RT-PCR for PMLRARα positivity. In this trial, 11 of 22 patients converted to a RT-PCR negative state following maintenance therapy with Hum195 [69].

ii. Conjugated monoclonal antibodies

Gemtuzamab ozogamicin

This consists of a humanised anti-CD-33 antibody conjugated to a potent anti-tumour antibiotic, calicheamicin. As with unconjugated Hum195 it is used in the treatment of AML.

The combined results of 3 multicenter phase II clinical trials of Gemtuzamab ozogamicin in 142 patients with relapsed AML showed a remission rate of 30%. An analysis of the leukaemic blasts from the above study showed a positive correlation of P-glycoprotein drug efflux pump surface expression and treatment failure[70]. Side effects of this immunoconjugate noted during this trial included a transient post transfusion syndrome with fever, chills and hypotension and notably an increase in transaminases in 17% of patients with one report of fatal hepatic veno-occlusive disease (VOD), a clinical syndrome consisting of weight gain, ascites, painful hepatomegaly and jaundice. There have also been additional reports of VOD in approximately 16% of patients treated with Gemtuzamab ozogamicin after myeloablative therapy and haemopoetic cell transplantation. It is thought that this toxicity arises from uptake of the conjugate by CD33-positive cells in hepatic sinusoids[71].

The role for Gemtuzamab ozogamicn in the treatment of AML is currently being investigated as part of the UK MRC 15 AML trial[72].

Immunotoxins

Immunotoxins are composed of internalising monoclonal antibodies or other ligands linked to extremely potent plant or bacterial toxins or modified toxin subunits, which kill cells by inactivating protein synthesis.

Initial studies in the 1980s were performed with monoclonal antibodies linked to toxins which had been chemically or genetically altered to reduce normal tissue binding. Trials of patients with NHL, CLL, and peripheral T-cell lymphoma resulted in few durable remissions[73]. In the 1990s, genetic engineering was used to fuse the catalytic domains of toxins to single-chain Fv (scFv) antibody fragments and cytokines to produce fusion toxins[74]. Some immunotoxins currently in clinical trials are summarised in Table1. 3.

With a few exceptions, immunotoxin therapies have not yet shown impressive results. They are associated with higher levels of systemic toxicities than other therapies including infusion related events and vascular leak syndrome as well as immunogenicity of both the antibody and the toxin.

Trade name	Indication	Ligand /toxin	Ref.
Dinileukin Difitox (Ontak) FDA approved	Cutaneous T-cell lymphoma CLL	IL2-DT fusion protein	[75,76]
Anti-B4 blocked ricin	NHL	Murine anti CD19 - blocked ricin	[77]
HD-37-dgA	Refractory B cell lymphoma	Anti-CD22 – dgA	[78]
RFB4-dgA	Refractory B cell lymphoma	Anti-CD19- dgA	[78]
RFT5-dgA	Hodgkin's lymphoma	Anti-CD25- dgA	[79]
Anti-Tac (Fv)-PE38 (LMB2)	CD25+ haematological malignancies	Anti-CD25 Fv-truncated PE fusion protein	[80]
RFB4-dsFv-PE38 (BL22)	Hairy cell leukaemia	Disulphide stabilised anti-CD22 Fv-truncated PE fusion protein	[81]
DT388GMCSF	AML	GM-CSF-DT fusion protein	[82]

Table1.3 Immunotoxins currently in clinical trials
DT: diphtheria toxin
dgA: deglycosylated ricin A chain
PE: pseudomonas exotoxin

iii. Radioimmunotherapy

In this mode of therapy, monoclonal antibodies with selectivity for target cells are linked to radionucleotides with high linear energy transfer (LET) such as beta (^{131}Iodine,^{90}Yttrium) or alpha (^{213}Bismuth,^{211}Astatine) emitters that deliver radiation to the targeted cell and the neighbouring cells (cross fire effect). Beta particles have a penetration range of millimetres and are used for therapy of bulky disease, whereas alpha particles have a penetration range of only a few cell diameters and are suitable for treatment of micrometastases and circulating tumour cells. The cytotoxicity of radioimmunotherapy results from a combination of direct radiation activity causing DNA strand breaks and the effects of the antibody itself such as CDC, ADCC and direct induction of apoptosis. Radioimmunotherapy regimes often require a pretherapy step in which cold mouse antibody is administered, either as a predose or concomitantly, which improves the biodistribution of the labelled antibodies to the tumour cells[83].

Radioimmunotherapy has been most successful in the treatment of NHL. The first trial for B-cell lymphomas used a ^{131}Iodine labelled anti-HLA DR10 β subunit (Lym1). In this trial objective remissions were seen in 50% of patients treated with advanced relapsed B-cell malignancies[84].

The most commonly used antibody used in the radioimmunotherapy of B-cell malignancies is anti-CD20: murine anti-CD20 conjugated to ^{90}Yttrium (Zevalin) has been approved for use in NHL. A randomised controlled trial demonstrated responses to this treatment are significantly better than to unlabeled anti-CD20 Rituximab, with overall responses of 80% vs. 56% for the unlabeled antibody. However these higher response rates have not yet translated into improved overall survival[85].

Tositumomab, an ^{131}Iodine labelled murine anti-CD20 antibody (Bexxar) has also had encouraging results, with response overall response rates of 47-68% in patients with relapsed or refractory follicular lymphoma[86,87] and a recently reported overall response rate of 95% when used as initial therapy in newly diagnosed patient[88].

Other therapies which are currently in phase I/II clinical trials include ^{90}Yttrium-Anti-CD22 for B-cell malignancies, ^{90}Yttrium-humanised anti-Tac/CD25 for T-cell leukaemia and ^{213}Bismuth-humanised anti-CD33 for AML.

The main side effects of these treatments are the development of human anti-mouse antibodies (HAMA) and myelosupression. Fears of increased incidence of therapy related MDS or AML as compared to conventional therapy have so far been unfounded[89].

iv. Antibody directed enzyme therapy (ADEPT)

This novel targeting strategy involves a two step approach: in the first step non-internalising monoclonal antibodies are used to localise enzymes to target cells bearing specific tumour antigens. After any unbound antibody-enzyme conjugate has cleared from the circulation, prodrugs are administered, which are converted to active drug by the targeted enzyme. This strategy allows high concentrations of drug to localise to the tumour site, where it can be taken up passively both by antigen positive cells and antigen negative bystander cells. Many enzymes and prodrugs are available for use with ADEPT technology, including β-lactamase which cleaves β-lactam rings to release active drugs such as doxorubucin and cytosine deaminase, which converts 5-flurocytosine to the active drug 5-flurouracil.

To date there have been no clinical trials of ADEPT technology for haematological malignancies but phase I trials in colorectal cancer have shown only modest anti-

tumour responses and significant immune responses against the targeted enzyme have been observed[90].

1.2. Vectors for targeted therapy

1.2.1 Monoclonal antibodies (mAbs)

IgG molecules are the most common antibodies employed in cancer therapy. Antibodies are Y shaped (Fig 1.1), having two arms attached to a single stem. The arms of the Y are the variable regions, the tips of the arms containing specific complementarity - determining regions, with the stem being the constant region. The constant region triggers effector cell function i.e. ADCC and CDC by linking the complex to other cells of the immune system.

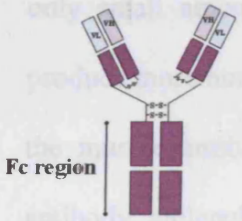
In 1953 Pressman and Korngold demonstrated that antibodies could specifically target tumour cells [91]. The advent of hybridoma technology 20 years later, when Kohler and Milstein discovered that the antibody producing cells from a mouse spleen could be immortalised by fusion with myeloma cells, allowed the production of unlimited supplies of monoclonal antibody directed against a specific antigen [92]. In 1979 the first patient was treated with a monoclonal antibody directed against a lymphoma-associated antigen [93].

1.2.1.1 Structural modifications of monoclonal antibodies

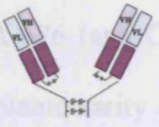
a. Antibody fragments

In an effort to increase the ability of relatively large monoclonal antibodies to penetrate solid and often poorly vascularised tumours, antibody fragments have been used in the place of the whole antibody molecule (Fig.1.1). Fragments consisting of the specific binding regions with much of the constant region deleted: Fab or F $(ab')_2$ can be prepared by digestion of whole antibody using papain or pepsin proteases respectively.

Recombinant antibody technology has allowed the production of even smaller single chain Fvs antibody fragments (sCFvs), consisting of only the variable light chain (VL) and variable heavy chain (VH) domains covalently connected by a polypeptide linker [94]. These antibody fragments have the potential to penetrate deeper into tumour masses. There are however, disadvantages to using smaller antibody fragments, they have significantly reduced serum half-life when compared to the whole antibody, binding avidity that comes from the presence of two binding sites on the molecule is lost, and lack of the Fc portion of the antibody means that much of the host effector response (CDC, ADCC) is diminished.



IgG 150kD



F (ab')$_2$ 95kD

Fab 47kD

sFv 25kD

Fig 1.1 Relative sizes of an antibody molecule and antigen binding fragments

b. Humanisation of monoclonal antibodies

The first generation of monoclonal antibodies were murine antibodies derived from mouse B cell hybridomas[92]. However it soon became clear, that they had limited clinical potential as the murine mAbs were recognised by the immune system as foreign material, resulting in the production of human anti-mouse antibodies (HAMA). The HAMA response alters the pharmakokinetics of mAb therapy, leading to rapid clearance of the agent and limiting the ability to retreat patients[95]. Furthermore, murine mAbs are inefficient at triggering host effector cell responses.

An initial strategy to humanise monoclonal antibodies was to produce human hybridomas using human B cell lines. However these cell lines were unstable yielding only small amounts of antibody. Subsequently, genetic manipulation was used to produce more human-like monoclonal products by splicing the antigen binding parts of the murine antibody into a human antibody backbone[96]. The extent to which the antibody molecule is humanised can vary; a chimeric mAb such as Rituximab is approximately half human and half murine, whereas a humanised antibody such as CMA-676 (anti-CD33 part of gentuzamab ozogomicin) is 95% human with only the complementarity determining regions being derived from the mouse. (Fig1.2). Fully human antibodies can be obtained using phage display libraries or transgenic mice, both of which make use of repertoires of human V-regions[97].

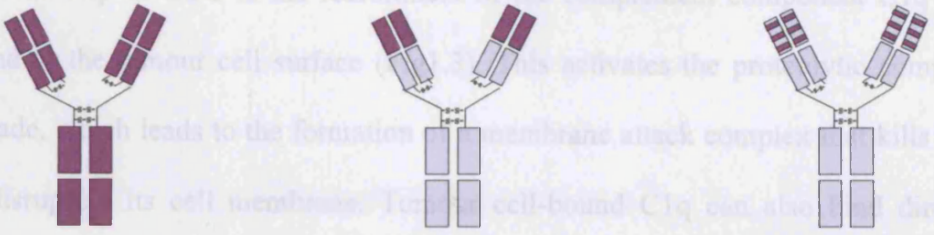


Fig. 1. 2 Murine, chimeric and humanised monoclonal antibodies

1.2.1.2 Mechanism of action of monoclonal antibodies

Monoclonal antibodies can kill tumour cells in one of 4 general ways:

a. By inducing fatal immunological injury via host effector mechanisms: CDC and ADCC.

b. By binding to cell-surface receptors resulting in signal transduction events leading to apoptosis.

c. By blocking binding of growth factors and/or survival factors to the cell-surface receptor.

c. By targeting cytotoxic therapy to the cell (conjugated antibodies).

a. Antibody dependent cell mediated cytotoxicity and complement dependent cytotoxicity.

ADCC is triggered by the interaction between the Fc region of an antibody that has bound, through its antigen-binding region to a tumour cell, and the Fcγ receptors on immune effector cells (Fig1.3). Effector cells include phagocytic cells such as macrophages and neutrophils which engulf and destroy antibody coated cells, and other cells such as NK cells which are triggered to release stored cytoplasmic cytotoxic granules containing perforin and granzyme when their Fc receptors are engaged, leading to cell lysis.

The first step of CDC is the recruitment of the complement component C1q by IgG bound to the tumour cell surface (Fig1.3). This activates the proteolytic complement cascade, which leads to the formation of a membrane attack complex that kills the cell by disrupting its cell membrane. Tumour cell-bound C1q can also bind directly to complement receptors on effector cells, which can trigger cell-mediated tumour lysis or phagocytosis.

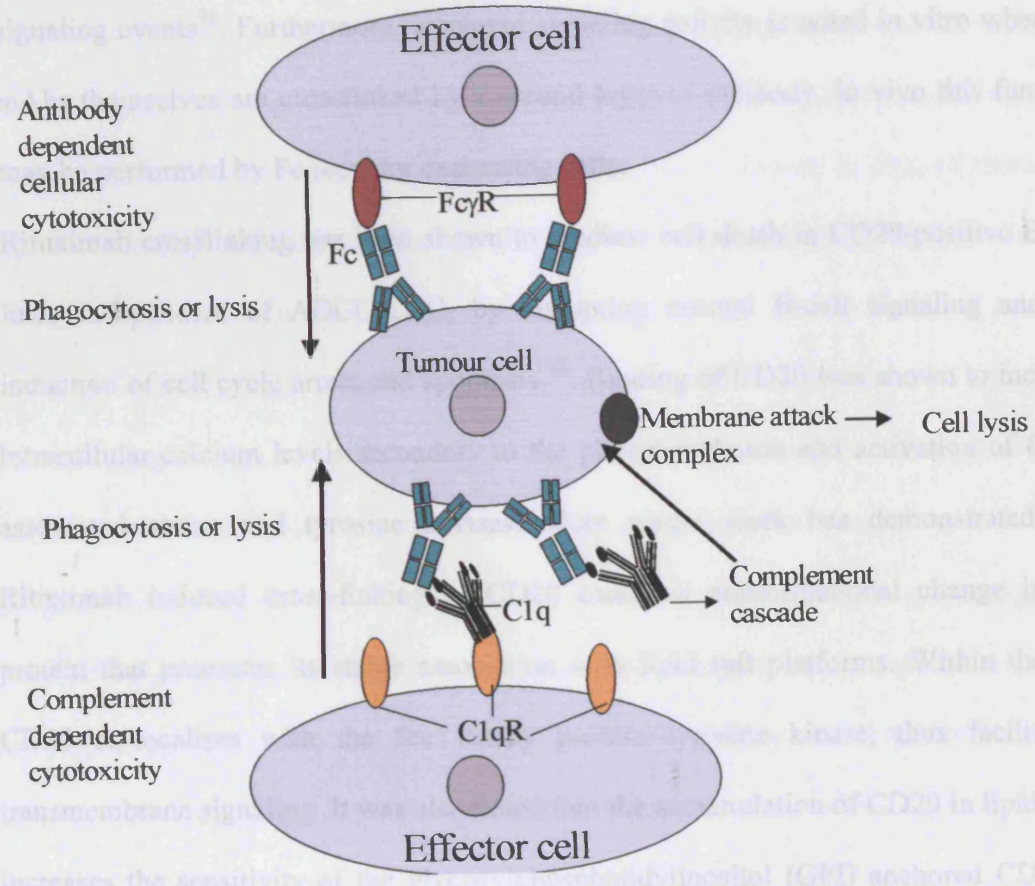


Fig 1.3 Antibody Effector Function[97]

b. Signaling events

Several antibodies have shown evidence of direct signaling effects consequent to binding to their ligands; these include chimeric anti-CD20 (Rituximab), humanised anti-CD52 (Campath 1-H) and humanised anti-HLA-DR (Apolizumab/ Hu1d10).

Crosslinking of target antigens by mAb seems to be important in the induction of signaling events[98]. Furthermore, increased signaling activity is noted in vitro when the mAbs themselves are crosslinked by a second layer of antibody. In vivo this function may be performed by Fc receptor expressing cells.

Rituximab crosslinking has been shown to mediate cell death in CD20-positive B-cell lines independent of ADCC/CDC, by disrupting normal B-cell signaling and the induction of cell cycle arrest and apoptosis [99]. Binding of CD20 was shown to increase intracellular calcium levels secondary to the phosphorylation and activation of CD20 associated serine and tyrosine kinases. More recent work has demonstrated that Rituximab induced cross-linking of CD20 causes a conformational change in the protein that promotes its stable association with lipid raft platforms. Within the raft CD20 co-localises with the Src family protein tyrosine kinase, thus facilitating transmembrane signaling .It was also noted that the accumulation of CD20 in lipid rafts increases the sensitivity of the glycosylphosphatidylinositol (GPI) anchored CD55 C defence protein to phospholipase C and D, which may alter the complement defence of cells that bind to Rituximab in vivo. [100,101]

Crosslinking of Campath-1H has similarly been shown to trigger tyrosine phosphorylation events in human T and CLL cells in vitro, however CD52 ligation did not lead to any detectable changes in intracellular calcium levels[102,103]. CD19 has also been shown to be physically and functionally associated with the src family protein

tyrosine kinases and that antibody binding to CD19 initiates multiple intracellular signal transduction cascades including increases in intracellular calcium ions [104].

c. Growth factor blockade

Monoclonal antibodies can be used directly to affect the survival of tumour cells by depriving them of essential extracellular proliferation and/or survival signals such as those mediated by growth factors through their cell-surface receptors. Examples of these are growth factor receptors such as epidermal growth factor receptor (EGF) which is overexpressed in a variety of tumours[105], HER2 overexpressed in 30% of metastatic breast cancers[106] and vascular endothelial growth factor receptor (VEGF) which is essential for angiogenesis and may be important in the pathogenesis of multiple myeloma as well as numerous solid tumours[107].

Increased surface growth factor receptor is often associated with increased production of the growth factor by the tumour cells, indicating autocrine growth control in the progression of these tumours. Binding of the growth factors to their receptors triggers a cascade of cellular biochemical events including receptor autophosphorylation and activation. Receptor autophosphorylation activates in turn the RAS GTP-binding protein, thus initiating a cascade of protein phosphorylation events culminating in activation of ERK1/2 and consequent triggering of the cell cycle[108]. Blocking growth factor-receptor interaction with monoclonal antibodies can therefore effectively decrease tumour cellular proliferation or neovascularisation of solid tumours.

d.Conjugated monoclonal antibodies

Most conjugated antibodies need to be internalised to deliver their toxic conjugate. Exceptions to this are ADEPT enzymes, which need to be present on the cell surface to convert non-active prodrugs into active cytotoxic molecules, and radiolabelled antibodies, which are for the most part non-internalising.

Internalisation of receptor bound antibody occurs through clathrin-mediated endocytosis. Internalised molecules are delivered to early endosomes where receptor – ligand uncoupling occurs in the mildly acidic environment of the lysosomes. Some receptors are then recycled back to the plasma membrane through cycling endosomes, whereas ligands and downregulated receptors are transported to late endosomes and lysosomes for degradation[109].

Many factors need to be taken into account when selecting the most suitable antigen for targeted drug delivery to malignant cells; these include level of antigen expression on malignant and healthy tissues, the degree of shredding of the antigen into the circulation and the effect of the antigen binding to the ligand such as receptor mediated internalisation into the endosome and other signaling events.

1.2.1.3 Choice of target antigen for targeting of cytotoxic molecules to haematological malignancies

a. Targeting lymphoid malignancies

Idiotype protein

The idiotype protein would seem to be the ideal antigen to target for delivering therapy to B-cell malignancies, since it is completely specific to the malignant clone and is rapidly internalised on binding to the antigen. Early studies targeted the idiotype with

individual monoclonal antibodies designed for a particular patient's lymphoma. Many of these patients had direct anti-tumour responses that lasted several years and were associated with very little toxicity[110]. However as well as the practical issues of having to make patient specific antibodies, idiotype also has the disadvantage of being shed in large amounts into the circulation where it competes with the target cells for binding and forms complexes that are rapidly cleared. Another problem with idiotype immunoglobulin is that it undergoes rapid somatic mutations generating tumour clones that fail to bind the antibody[111].

Pan B-cell antigens

Targeting pan-B-cell antigens with cytotoxic molecules leads to the temporary elimination of normal as well as malignant B-cells. However as long as the antigen is not also expressed on the pluripotent stem cell, repopulation of the bone marrow haemopoetic cells can occur post-treatment. Although there is also a theoretical risk of increased susceptibility to infections during the period of repopulation, it has not been a major problem associated with the use of conjugated antibodies vs. pan B-cell markers, presumably because plasma cells do not express many of the antigens concerned and serum immunoglobulin levels are therefore not adversely affected.

CD19 and CD22 are the most commonly used pan B-cell antigens used for drug delivery to lymphoid malignancies. They are both members of the immunoglobulin supergene family. CD19 is the earliest of the B-lineage restricted antigens to be expressed and is detectable on the surface of pre-B cells at approximately the same time as Ig. heavy chain rearrangement occurs whereas CD22 is only detectable on the cell surface at more mature stages of B-cell differentiation. The expression of both antigens is lost during terminal differentiation to plasma cells. CD19 is expressed on almost all

B-cell malignancies whereas CD22 is expressed on 75-80% of B-cell NHLs and is more variably expressed cell to cell than CD19[112]. Neither receptor is shed from the cell surface and both are internalised on binding to the ligands. CD22 is thought to be internalised particularly rapidly[113]. Anti-CD19 antibodies conjugated to toxins such as blocked ricin [77] and tyrosine kinase inhibitors[114,115] have been used in therapeutic trials for the treatment of B-cell malignancies. BL22, a recombinant immunotoxin consisting of the Fv domain of anti-CD22 fused to a fragment of the pseudomonas exotoxin has shown impressive activity in purine analog resistant hairy cell leukaemia[81] and a phase 2 trial in CLL is currently underway.

The IL-2 receptor and lymphocyte activation markers

The IL-2 receptor comprises three subunits, the alpha (also known as Tac, p55 or CD25), beta and gamma chains. The IL-2 receptor is overexpressed on the malignant cells in adult T-cell lymphoma, peripheral T-cell leukaemia/lymphomas, cutaneous T-cell lymphomas, B-cell NHL, Hodgkin's disease, hairy cell leukaemia, CLL and AML as well as on activated T-cells.

Immunotoxins have been targeted to the IL-2 receptor using anti-CD25 antibodies[79,80] or IL-2 fusion toxins[116,117].

CD30 is another lymphocyte activation marker which is also expressed on Reed Sternberg cells in Hodgkin's disease and anaplastic large cell lymphoma. As with CD25, it internalises on binding to the ligand and in phase I/II clinical trials anti-CD30 immunotoxins have shown some efficacy in heavily pretreated refractory patients with Hodgkin's disease[118,119].

b. Targeting myeloid malignancies

CD33

CD33 is a member of the immunoglobulin supergene family that shares sequence similarity with CD22. Expression is restricted to cells of the myelomonocytic lineage, it is not expressed on pluripotent stem cells but is first detected on a sub-population of mixed colony forming cells, expression then continues along the myelomonocytic pathway until it is downregulated on granulocytes but retained by monocytes and macrophages[120]. CD33 is the principal antigen used for targeting myeloid malignancies. CD33 is expressed on blast cells in AML and to a lesser extent in MDS and CML[121]. Anti-CD33 antibodies have been conjugated to toxins[102,122,123]and more recently the anti-tumour antibiotic calicheamicin for the targeted therapy of AML[70].

1.2.2 Non antibody-derived targeting vectors

Non-antibody ligands can be used to target toxic vectors (Table1.4). Non-antibody ligands are often readily available, inexpensive and easy to manufacture as compared to monoclonal antibodies or antibody fragments. The disadvantage is that their binding is often less selective than with monoclonal antibodies.

Non-antibody vectors can be the natural ligands that bind specific known receptors on target cells such the folate or transferrin receptor, which are overexpressed in a variety of malignancies. Alternatively, phage display technology can be used to identify short peptides sequences that bind to specific molecules, cells, tissues or organs. Some examples of non-antibody vectors used to target toxic ligands can be found in Table1.4.

* Indicates sequence identified by phage display.

Vector	Target	Target tissue	Ref.
Folate	Folate receptor	Cancer cells that overexpress the folate receptor	[124]
Transferrin	Transferrin receptor	Cancer cells that overexpress the transferrin receptor	[125]
GM-CSF	GM-CSF receptor	Leukaemic blasts	[82]
*RGD	Cellular adhesion molecules such as αvβ3 integrin	Vascular endothelial cells in solid tumours	[126]
*NGR	Aminopeptidase N (CD13)	Vascular endothelial cells in solid tumours	[126]
*SMSIARL	Prostate vasculature	Prostate vasculature	[127]
*THALWHT	Human airway epithelial cells	Human airway epithelial cells	[128]

Table 1.4 Non-antibody vectors used to target toxic ligands .

1.2.3 Phage display technology

Phage display comprises a selection technique in which a peptide or protein (including antibody or enzymes) is expressed as a fusion with the coat protein of a bacteriophage, resulting in the display of the fused protein on the exterior surface of the phage virion. Phage display libraries of peptides and proteins can be screened, both in vivo and in vitro, to rapidly identify those compounds that bind with high affinity and high specificity to targets of interest. Each library contains millions of different peptide sequences. The in vitro method of screening these libraries to select phage that bind to a specific target with high affinity is termed biopanning (Fig1.4). In it's simplest form biopanning is carried out by incubating a library of phage displayed peptides with a plate (or bead) coated with the target, washing away the unbound phage, and eluting the

specifically- bound phage. The eluted phage is then amplified and taken through additional cycles of biopanning and amplification to successively enrich the pool of phage in favour of the tightest binding sequences. After 3-4 rounds, individual clones are characterised by DNA sequencing

Phage display technology has been used in this way to identify scFv against many purified haematological cell surface antigens including blood group antigens and malignant cell surface molecules such as CD19 expressed in B-cell malignancies, CD38 in multiple myeloma and CD30, which is overexpressed on Reed Sternberg cells in Hodgkin's disease[129,130]. These scFvs can be linked to radionucleotides or fluorophores for imaging purposes (having better tumour penetration than larger immunoglobulin) or to toxins. The therapeutic usefulness of these immunotoxins however can be limited by their rapid renal clearance and short half-life.

Phage display may also be performed to select peptides that bind to immunoglobulin on the surface of myeloma[131] or CLL cells[132] or to identify peptides that bind whole cells such as fibroblasts[133] or more recently whole CLL cells[134].

A method of in vivo phage selection has also been reported: by injecting phage display libraries into the tail vein of mice, sacrificing the mice after several minutes, then isolating and amplifying phage which bound to specific organs or tissues, specific homing peptides have been identified[135].

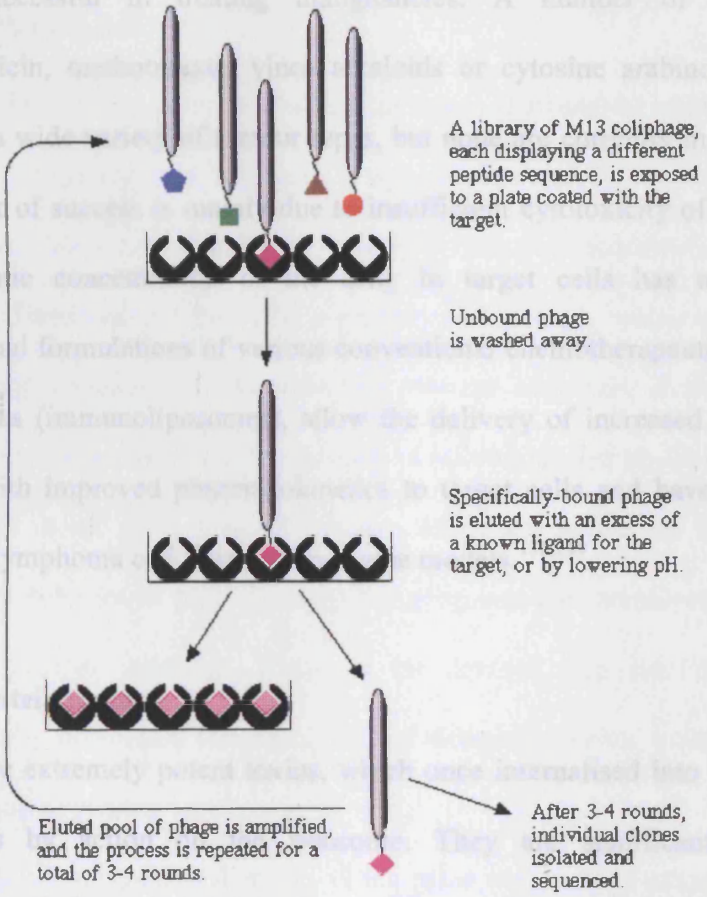


Fig 1.4 Schematic representation of biopanning a phage display library [136]

1.3. Toxic ligands used in targeted therapy

1.3.1 Conventional chemotherapeutic agents

Conventional chemotherapeutic agents linked to monoclonal antibodies have not been very successful in treating malignancies. A number of agents incorporating doxorubicin, methotrexate, vinca alkaloids or cytosine arabinoside have been used against a wide variety of tumour types, but none are currently in clinical development. The lack of success is mainly due to insufficient cytotoxicity of the agents, because a therapeutic concentration of the drug in target cells has not been achieved[137]. Liposomal formulations of various conventional chemotherapeutic drugs conjugated to antibodies (immunoliposomes), allow the delivery of increased concentrations of the drugs with improved pharmakokinetics to target cells and have been investigated in vitro in lymphoma cell lines and in mouse models.[138,139]

1.3.2 Protein toxins

These are extremely potent toxins, which once internalised into a cell, disrupt protein synthesis by action on the ribosome. They are significantly more toxic than conventional chemotherapeutic agents. Whereas conventional chemotherapeutic agents act stoichiometrically, protein toxins act catalytically. Thus a single catalytic toxin molecule in the cytoplasm of a cell can cause cell death.

Because the toxins themselves show widespread binding to many normal tissues, which might override the specific binding of the ligand, their binding domains are often removed or mutated to prevent them from binding to normal cells[140]. They are then fused or chemically conjugated to a ligand specific for tumour cells Alternatively,

inserting the DNA encoding the fusion toxin into an expression plasmid can produce recombinant toxins.

Ribosome targeting toxins include plant and bacterial toxins:

1.3.2.1 Plant toxins

Plant toxins exist in nature as holotoxins (class II ribosome inactivating proteins) such as ricin, which contains a binding domain connected to an enzymatic domain via a disulphide bond, or hemitoxins (class I ribosome inactivating proteins) such as pokeweed antiviral protein (PAP), saporin and gelonin, which contain enzymatic, but no binding domains. It is thought that only the enzymatic domain translocates to the cytosol and hence the binding domains of holotoxins are removed by reduction of the disulphide bond prior to translocation. After internalisation into endosomes, plant toxins are believed to traffic through the golgi and then translocate into the cytosol via the endoplasmic reticulum. Once in the cytosol they inhibit intracellular protein synthesis by preventing the association of elongation factor 1 and 2 (EF-1, 2) with the 60s ribosomal subunit[141].

Originally the enzymatic domains of the plant toxins were conjugated to their ligands via disulphide bonds, however even without its binding domain ricin, was taken up non-specifically by macrophages and hepatic parenchymal cells. The hepatic uptake was due to glycosylated side residues on the ricin enzymatic domain (ricin A chain) binding to mannose receptors on the liver cells. Chemical deglycosylation, blocking the ricin A chain or using a recombinant ricin A chain improved the therapeutic index of these immunotoxins[142]

1.3.2.2 Bacterial toxins

The two bacterial toxins used in immunotoxin therapy are Pseudomonas exotoxin (PE) made by Pseudomonas aeruginosa and Diphtheria toxin (DT), made by Corynebacterium diptherium. These toxins are single chain proteins that contain both binding and catalytic domains. They prevent protein synthesis by catalysing the ADP ribosylation of EF-2, resulting in its inactivation.

Both DT and PE undergo proteolysis and disulphide bond reduction to separate their binding and catalytic domains. PE undergoes further processing, resulting in a 37kDa carboxy terminal toxin fragment ending in the residues REDL. This binds to a REDL receptor and is transported via the golgi to the endoplasmic reticulum and then the cytosol. In contrast, the catalytic A chain of DT is translocated into the cytosol directly from the endosome[141].

BL22, an immunotoxin consisting of truncated PE linked to CD22, has been shown to cause apoptotic cell death in CLL cells.[143]

1.3.2.3 Limitations of protein toxins

The extreme potency, non-specific binding and immunogenicity of the protein toxins have been a major limitation in their clinical use. Although immune responses against antibodies can be reduced by antibody engineering, the toxins themselves are immunogenic and cannot be humanised. New strategies such as linking polyethylene glycol to ricin molecules have significantly reduced the immunogenicity of the toxin, with no effect on its ability to inhibit protein synthesis[144]. Another way to circumvent the immunogenicity of non human proteins is to use human proteins as toxins, for example the human ribonuclease angiogenin has been genetically fused to fragments of

an anti-transferrin receptor antibody and found to be selectively cytotoxic for transferrin receptor bearing cells[145].

Vascular leak syndrome has been the major dose limiting toxicity in immunotoxin therapy, especially in patients who have received prior radiotherapy[78]. It involves damage to vascular endothelial cells, resulting in fluid and protein extravasation, leading to weight gain and peripheral oedema and in its more severe form, renal dysfunction and pulmonary oedema[146]. Baluna et al have identified a structural motif LDV, which is common to several molecules that exhibit vascular toxicity including ribosome inactivating proteins, IL-2 and disintegrins. They also demonstrated that this motif is not essential for the protein synthesis inhibiting function of these toxins[147].

1.3.3 Non protein toxins

Maytansinoids [148] and calicheamicins[149] are the most extensively studied small molecule toxins. These molecules, which are 100-1000 times more potent than conventional chemotherapeutic agents can be delivered to target cells as inactive prodrugs by conjugating them to monoclonal antibodies. Activation of the prodrug on release of the drug from the antibody and occurs in the tumour cells following receptor binding and antibody internalisation.

Maytansine kills cells by interfering with microtubule formation. Derivatives of maytansine have cured mice that bear human tumour xenografts [150] and two phase I trials for antibody-maytansinoid conjugates are currently in progress.

Calicheamicin γ^1 is a member of the very potent enediyne family of natural antitumour antibiotics, originally isolated from Micromonospora echinospora dsp calichenis.

The anti-tumour activity of Calicheamicin is approximately 4000 x greater against mouse tumours than is adriamycin [151]. It binds to the minor groove of DNA causing

double strand breaks and ultimately apoptotic cell death. As the toxic ligand of Gemtuzumab ozogamicin, it has yielded promising results for the treatment of AML.

1.4. Apoptosis and mitochondria

Apoptosis is an evolutionarily conserved mechanism of programmed cell death, in which cells undergo carefully orchestrated self-destruction and phagocytosis without eliciting an inflammatory response[152]. In contrast, necrotic cell death, ensuing from damage to the cell membranes, results in the release of proteins and nucleic acids into the intracellular space.

Apoptotic cells display characteristic morphological features, which include DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly into membrane-enclosed fragments[152].

All of the above changes occur as a result of the action of a group of intracellular cysteine proteases called caspases. These become activated in response to a wide variety of cell death stimuli. Caspases are organised in cascades, with upstream (initiator) caspases responsible for activating downstream (effector) caspases.

1.4.1 Apoptotic pathways

There are two major pathways by which effector caspases become activated (Fig 1.5):

1. The extrinsic pathway: this involves ligation of cell surface death receptors of the TNF family, which activates a series of events leading to the recruitment of death effector protein Fadd/Mort and activation of caspase 8[153].

2. The intrinsic pathway: this occurs in response to disturbances of intracellular homeostasis or damage to specific organelles secondary to stress conditions such as ultraviolet (UV) irradiation, cytotoxic drugs or growth factor deprivation. In this pathway, caspase 9 is activated, via a poorly understood mechanism involving changes in mitochondrial membrane permeabilisation (MMP), cytochrome *c* release into the

cytosol and the dATP/ATP dependent assembly of the apoptosome, a complex made up of cytochrome *c*, apoptotic protease activating factor 1 (Apaf-1) and pro-caspase 9[154]. Both pathways culminate in the activation of downstream effector caspases. The cleavage targets of these effectors are a restricted set of cellular substrates. Up to 60 different caspase substrates have been identified[155], some of which are listed in table 1.5. The net result of the action of the effector caspases on their substrates is the morphologically distinct apoptotic cell.

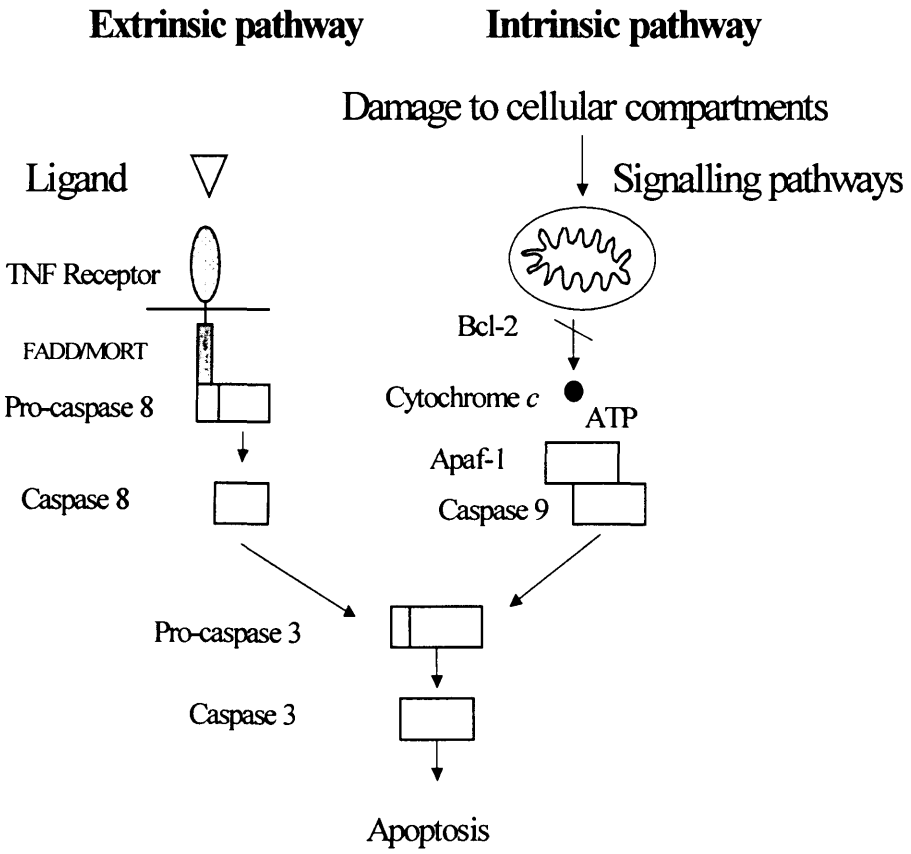


Fig.1. 5 Pathways of apoptotic cell death.

Substrate	Caspase	Consequences
Cytoskeletal scaffold proteins		
Lamin: scaffold protein of nuclear envelope	6	Chromatin condensation and pyknosis
Gelsonin: actin depolymerising enzyme	3	Actin depolymerisation and membrane blebbing
α-fodrin and focal adhesion kinase: make up focal adhesion complex which links plasma membrane to the extracellular matrix	3,6,7	Cell body shrinkage and detachment from basement membrane and neighbouring cells.
Inhibitor of p21-activated kinase (iPAK-2)	3	Activation of PAK2,a GTPase involved in actin dynamics,leads to formation of apoptotic bodies
Repair and housekeeping enzymes		
Poly (ADP) ribose polymerase (PARP)	3,7	Blockade of DNA repair
DNA metabolism		
Inhibitor of caspase-activated Dnase Protein kinase C (ICAD)	3,7	Activation of ICAD nuclease, leading to DNA fragmentation

Table1. 5 Classes of caspase substrates [155]

1.4.2 Recognition of apoptotic cells by phagocytes

One of the important distinctions between apoptotic and necrotic cell death is the lack of an inflammatory response during apoptosis. This is in part due to the fact that apoptotic cells are engulfed with their outer membrane intact thereby preventing the release of intracellular components, but also because the macrophages themselves do not elicit an inflammatory cytokine response as they do when phagocytosing necrotic or infective tissue. There are a number of macrophage receptors such as CD14, vitronectin and other integrins that play a role in recognising markers displayed by apoptotic cells. The best characterised of the signals in the apoptotic cell is a change in the pattern of phospholipid distribution in the cell membrane with exposure of phosphatidylserine residues on the outer surface of the membrane. This is recognised by a newly identified macrophage receptor, the PtdSer receptor, which not only initiates phagocytosis, but also seems to be coupled to anti-inflammatory clearance mechanisms, stimulating macrophage TGF-β secretion and inhibiting TNF-α release[156].

1.4.3 Role of mitochondria in apoptotic (and non-apoptotic) cell death

1.4.3.1 Triggers of mitochondrial disruption

Mitochondria, the organelles responsible for cellular oxidative phosphorylation and ATP production, also play a central role in apoptotic (and non-apoptotic) cell death. A wide variety of triggers can cause mitochondrial disruption. Many cellular insults such as radiation, drug-induced DNA damage, osmotic stress or cytokine withdrawal can trigger the mitochondrial apoptotic pathway[154].

DNA damage triggers the mitochondrial apoptotic pathway via a mechanism

involving activation of ataxia telangiectasia mutated (ATM) with consequent stabilisation of the tumour suppressor protein p53[157]. 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 and Rad3 related). ATM and DNA-PK can bind directly to free DNA ends, which then triggers kinase phosphorylation cascades to transmit damage signals to checkpoint and repair proteins. p53 is a tumour suppressor protein that is normally maintained at low levels within the cell through its interaction with Mdm2, a ubiquitin ligase that escorts p53 from the nucleus and targets it for protein degradation by the 26S proteosome 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. A key consequence of p53 induction is the increased transcription of the pro-apoptotic genes Puma and Noxa. p53 also enhances reactive oxygen species (ROS) production by transcriptionally activating genes such as PIG3 (p53 induced gene 3). 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[157].

1.4.3.2 Consequences of mitochondrial disruption

Disruption of the mitochondria leading to changes in MMP and cytochrome *c* release commit a cell to die, either by a rapid apoptotic mechanism involving Apaf-1 mediated caspase activation or by a slower necrotic process due to collapse of electron transport due to depletion of cytochrome *c* from the mitochondria[154]

1.4.3.3. Cytochrome *c* and other mitochondrial pro-apoptotic factors

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. Once released into the cytosol, cytochrome *c* associates with Apaf 1 and then pro-caspase 9 (and possibly other proteins) in a dATP/ATP dependent process, to form the apoptosome. In the apoptosome juxtaposition of up to twelve pro-caspase 9 molecules leads to their activation, either by conformational change or by autocatalysis to form an active dimeric protease. Active caspase 9 subsequently activates caspase 3 resulting in apoptosis[158].

Other mitochondrial pro-apoptotic factors include apoptosis inducing factor (AIF), Smac/DIABLO (second mitochondria-derived activator of caspase, also called direct IAP binding protein with low pI) and pro-caspases 2, 3 and 9.

AIF is a death effector, which is synthesised as a precursor in the cytosol and imported into the intermembrane space of the mitochondria where 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 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* [159].

Smac/DIABLO is also released from the mitochondria into the cytosol during mitochondrial apoptosis. It eliminates the inhibitory effects of IAPs (inhibitors of apoptosis) on caspases[160].

1.4.3.4 Mitochondrial structure

Mitochondria are bound by two membranes: the inner and outer membranes, which are separated by the intermembranous space. The inner membrane is highly convoluted; forming a series of infoldings called cristae .The space bound by the cristea is called the matrix. Oxidative phosphorylation takes place in the inner mitochondrial membrane whereas the reactions of the citric acid cycle and fatty acid oxidation take place in the matrix.

The outer membrane contains protein structures called porins or voltage dependent anion channels (VDAC), which in normal conditions are permeable to solutes up to about 5000 Da. The inner membrane is almost is almost impermeable in physiological conditions (although transient increases in permeability may occur to selected ions and metabolites of less than 1500 Da as a consequence of various ion channels and uniporters), thereby allowing the respiratory chain to create an electrochemical gradient ($\Delta\Psi_M$). The $\Delta\Psi_M$ results from electron transport chain mediated pumping of protons across the inner mitochondrial membrane thus driving ATP synthase which phosphorylates ADP to ATP. ATP generated on the matrix side of the inner

mitochondrial membrane is then exported in exchange for ADP by the adenine nucleotide transported (Ant).

The permeability transition (PT) pore is a poorly characterised conductance channel, which is proposed to include both inner and outer membrane proteins (Ant and VDAC), operating in concert, at inner and outer mitochondrial membrane contact sites[161].

1.4.3.5 Models for cytochrome *c* release into the cytosol

Several models have been proposed to explain the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol [162,163]. One model involves mitochondrial swelling and rupture of the outer membrane leading to cytochrome *c* release. Mitochondrial swelling in this model may occur as a consequence of the interaction of PTP openers such as Bax interacting with Ant leading to to opening of the PTP in the inner membrane[164] or to mitochondrial hyperpolarisation secondary to closure of the VDAC[165]. Against this model is the observation that cytochrome *c* release and apoptotic cell death can occur in the absence of mitochondrial swelling.

Other models propose that cytochrome *c* release occurs through channels in the outer mitochondrial membrane. Some pro-apoptotic members of the Bcl-2 family which bear structural resemblance to channel – forming bacterial toxins can form ion channels in synthetic lipid bilayers[166,167] and have been demonstrated to induce release of cytochrome *c* from isolated mitochondria in the absence of mitochondrial swelling[168]. Another theory proposes that Bax interacts with VDAC leading to conformational change in the channal thus allowing the passage of cytochrome *c* into the cytosol[169].

1.4.4 Bcl-2 family of proteins

The mitochondria–mediated pathway of apoptosis is regulated by the Bcl-2 family of anti-apoptotic and proapoptotic proteins which all share at least one Bcl-2 homology (BH) domain.

The Bcl-2 family of apoptosis regulating proteins fall into three sub-families (Fig 1.6)

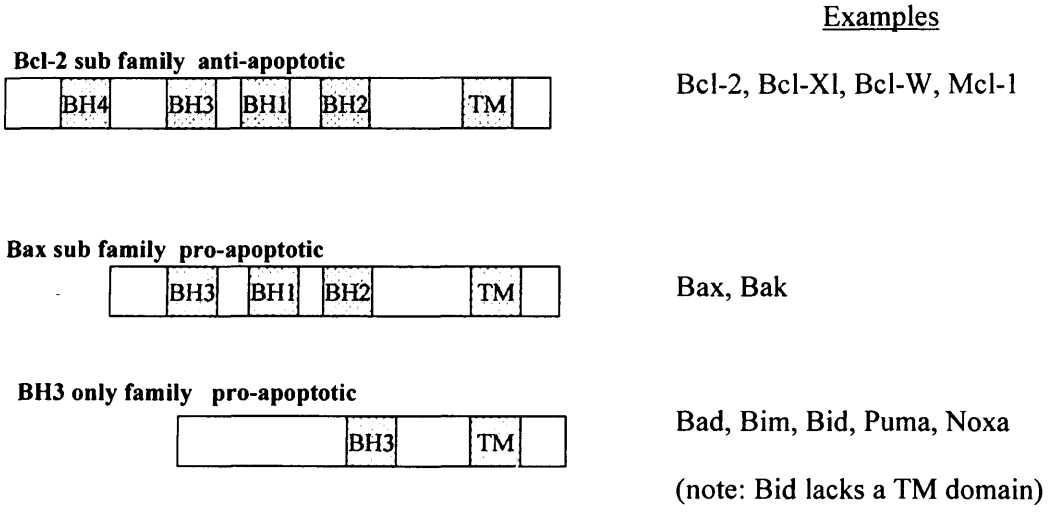


Fig.1.6. The Bcl-2 protein family
The Bcl-2 homology (BH) domains 1-4 and the transmembrane (TM) domain are shown aligned for comparison.

1.4.4.1 Pro-survival Bcl-2 subfamily

Bcl-2 is a 26-kDa membrane-associated protein with a hydrophobic carboxyl terminal transmembrane domain that anchors it to the cytoplasmic face of the endoplasmic reticulum, outer mitochondrial membrane, and the nuclear envelope.

Bcl-2 can protect cells from death induced by growth factor deprivation, γ-irradiation, or cytotoxic drugs but not from cell death induced by cytotoxic T lymphocytes.

In the mitochondria, Bcl-2 exerts its antiapoptotic effects by interacting with BH3 -only pro-apoptotic family members. A hydrophobic groove made up of the BH1, BH2 and BH3 regions of Bcl-2 binds the BH3 α-helix region of the death agonists thereby forming inactive heterodimers[170].

The BH4 region of Bcl-2 is thought to be responsible for a stabilising effect on the VDAC channel preventing permeabilisation of the outer membrane[165]. It is also thought to be involved in cell cycle regulation and interaction with regulatory proteins outside the Bcl-2 family.

BH1 and BH2 domains, which are present in both pro-apoptotic (Bax, Bak) and anti-apoptotic (Bcl-2, Bcl -xl) members of the family, have a 3 dimensional structure resembling the pore forming domains of some types of bacterial toxins such as Diphtheria toxin[171], and can form ion channels when added to synthetic membranes[166,167]. Bcl-2 can also homodimerize and form small ion channels in the outer mitochondrial membrane, which may stabilise the membrane and prevent loss of $\Delta\Psi_M$ and cytochrome *c* release[172].

Bcl-X, like Bcl-2 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.

1.4.4.2 Pro-apoptotic BH3-only subfamily

BH3-only proteins act as damage sensors, detecting intracellular damage and exerting their pro-apoptotic effect by interacting with the pro-apoptotic Bax subfamily proteins. BH3-only proteins are normally held in check by diverse mechanisms and are released or synthesised de novo in response to specific apoptotic signals[170], resulting in a rapid increase in their availability. For example Bim and Bmf are sequestered by binding to the microtubules or actin cytoskeleton respectively via interaction with a dynein light chain and are released in response to microtubule stress. Bad, after phosphorylation by kinases such as Akt and protein kinase A, is bound by 14-3-3 scaffold proteins but is released following withdrawal of growth factors and the subsequent downregulation of Akt activity. Other members of the BH3-only family such as Noxa and Puma are controlled primarily at the transcriptional level. Expression of these proteins is upregulated by p53[170]. Bid is inactive until proteolytically cleaved by caspase 8 forming tBid. This excerts its actions by causing conformational change and translocation of Bax to the outer mitochondrial membrane where it oligomerises leading to pore formation and cytochrome c release[173]. tBid is also thought to act at the cardiolipin-containing contact sites where the inner and outer mitochondrial membranes interact[174].

1.4.4.3 Pro-apoptotic Bax subfamily

Bax (Bcl-2 associated protein X), a 21kDa protein, exists as a cytosolic monomer in healthy cells. Like Bcl-2, Bax protein contains a hydrophobic carboxy terminus. However in Bax this tail occludes the BH1-3 hydrophobic groove. In the presence of a stress signal the carboxy tail is thought to flip out allowing integration into the outer mitochondrial membrane and consequent oligomerisation.[170] BH3-only proteins are thought to act catalytically in displacing the c-terminal tail. Therefore the increase in

free BH3-only peptides, brought about by diverse apoptotic stimuli (section 1.4.4.2) is a key event in initiating the mitochondrial pathway of cell killing.

Bax can interact with the mitochondria by three separate mechanisms:

Firstly, at the outer mitochondrial membrane Bax is known to accelerate the opening of VDAC with subsequent release of cytochrome *c*[161,175].Secondly, Bax interacts with Ant at the inner mitochondrial membrane, thus enhancing permeability[164]. Thirdly, when expressed at high levels Bax can oligomerize, leading directly to pore formation at the mitochondrial membrane[166].

Bak (Bcl-2 homologous antagonist killer) is an oligomeric integral mitochondrial membrane protein, which is also thought to change conformation in during apoptosis. Bak is expressed in a wide variety of tissues.

The antiapoptotic function of the pro-survival family members (Bcl-2, Bcl-xl) is thought to be mediated by their ability to bind and neutralise the BH3-only proteins, thus blocking their ability to catalyse the translocation of Bax and/or Bak, to the mitochondria. Thus, the susceptibility of cells to apoptotic stimuli is critically dependent on the relative expression of Bcl-2 family members versus the Bax subfamily members. Earlier reports that members of these sub-families may bind each other directly are probably based on an artefact induced by detergent lysis of cells.

1.4.5 Mechanisms of resistance to apoptosis of haematological malignancies

In most malignancies the balance between proliferation and programmed cell death is disturbed, and defects in the apoptotic pathways allow cells with genetic abnormalities to survive. Most chemotherapeutic strategies, including radiation, ultimately kill cancer cells by causing irreparable cellular damage that triggers apoptosis. Consequently, the efficacy of cancer treatments depends not only on the cellular damage they cause but

also on the cell's ability to respond to the damage by engaging the apoptotic machinery. Accordingly, mutations affecting the apoptotic pathway may result in resistance to drugs and radiation. The two most studied genes in relation to resistance to apoptosis are p53 and Bcl-2.

p53 Deletion and/or mutation of p53 alleles results in the generation of tumours with impaired expression of functional p53 protein and are the most frequently observed genetic lesions in human cancers[176].With the exception of Burkitt's lymphoma and L3 ALL in which mutations are observed in 30% and 55% respectively[177], p53 changes are relatively rare in haematological malignancies and tend to be associated with progression or transformation of disease i.e. blast crisis in CML or Richter's immunoblastic transformation in CLL[177]. As with other malignancies p53 mutations are associated with aggressive disease, drug resistance and poor survival[178].

Bcl-2: Abnormalities in Bcl-2 expression represent the single most common molecular event associated with lymphomas. The t (14; 18) translocation that places the Bcl-2 gene on chromosome 18 under the aberrant transcriptional control of the immunoglobulin heavy chain gene located on chromosome 14, is present in 90% of follicular NHL, 50% of undifferentiated B-cell lymphomas and 10% of large cell diffuse B-cell lymphomas[179]. Generally, close associations have been found between the presence of translocation and the expression of Bcl-2 protein. However in many haematological malignancies, such as mantle cell lymphoma, MM, CLL and AML, high level expression of Bcl-2 occurs in the absence of translocations and are associated with poor response to therapy[180].

1.4.6 Drugs designed to specifically target the mitochondrial apoptotic pathway

Targeting the mitochondrial apoptotic pathway may have advantages over conventional chemotherapeutic strategies that act mainly through causing DNA damage and rely on the p53 pathway to induce apoptosis. By acting at the mitochondrial level these drugs can bypass the block caused by p53 mutations and may overcome the chemoresistant effects of raised Bcl-2 expression. Bcl-2 is an attractive therapeutic target due to its overexpression in a wide variety of haematological malignancies and its association with oncogenesis as well as the development of chemoresistance. Furthermore normal cells can tolerate reduced Bcl-2 levels, as mice that lack one Bcl-2 allele are completely healthy [170]. Bcl-2 knockout mice survive embryonic development and develop normally, only later exhibiting marked lymphoid apoptosis. [181].

1.4.6.1 Bcl-2 anti-sense oligonucleotides (ASO)

One strategy to inhibit Bcl-2 involves liposomal oligonucleotides. These consist of chemically modified single-strand DNA molecules that have a nucleotide sequence that is complementary to the Bcl-2 mRNA. ASOs inhibit gene expression by hybridisation with the mRNA, followed by cleavage of the mRNA by recruitment of RNAse-H.

Bcl-2 ASOs have been shown to induce apoptosis in HL60 cells and primary AML blasts and to sensitise cells to the treatment with Ara-C [182]. Increased sensitivity to chemotherapy has also been demonstrated in NHL and MM[183] .

In a phase I study in relapsed patients with Bcl-2-positive lymphomas, disease stabilisation was seen in 43% (9 of 21) and improvements were seen in 14% (3 of 21, including one complete responder)[184]. Bcl-2 ASOs are currently in phase III trials.

1.4.6.2 BH3 mimetics

Another strategy involves drugs that mimic the structure of BH3 domains. Synthetic peptides which represent the α-helical BH3 domains of various Bcl-2 family proteins that have been modified to incorporate cell permeable moieties have been shown to have specific effects on the mitochondrial apoptotic pathway. These effects include inducing MMP, interaction with ANT and stimulating channel formation by recombinant Bax.[185-188].

HA14-1 is a small organic molecule identified by screening a virtual library of compounds using the binding of Bak's BH3 domain to the BH1-3 pocket of bcl-2 as a model. HA14-1 has been demonstrated to bind to bcl-2 in vitro and to induce apoptosis in HL60 cells[189]. Another small cell permeable compound BH31-2' has been reported to bind to the hydrophobic pocket of Bcl-X_L in a similar way to BaxBH3 peptide[190], and has been demonstrated to have effects at both the inner and outer mitochondrial membranes[185]. A further BH3 mimetic identified by nuclear magnetic resonance screening has been shown to lead to apoptosis in lymphoma and small cell carcinoma cell lines and had been reported to induce solid tumour regression in mouse models[191].

Gossypol is a natural product found in cotton seed and used as a herbal medicine in China. It interacts with the BH3 binding pocket on the surface of anti-apoptotic Bcl-2 family of proteins. Semi-synthetic analogs of this compound are currently in development.[192]

Some antibiotic peptides with a α-helical structure have also been shown to have toxic effect on mitochondria; these will be discussed further in the next section (Section 5).

1.5 Antibiotic peptides

1.5.1 Naturally occurring antibiotic peptides

Antibiotic peptides form a key component of the innate immune system of most multicellular organisms. To date several hundreds of structures with antimicrobial activity have been described in almost all groups of animal and plants [193]. The peptide antimicrobial responses have been most extensively studied in insects, where they have been shown to be mediated by structurally homologous signalling molecules such as nuclear factor (NF)-κB transcription factors, which induce expression of multiple genes encoding antimicrobial peptides in both blood cells (haemocytes) and the insect body fat (liver equivalent)[194]. Inducibility of antimicrobial proteins and peptides is also a feature of the innate defence both systemically and at epithelial sites in plants, amphibians and mammals. In mammals these peptides are carried in the cytosolic granules of leukocytes and platelets or are secreted from the liver as part of the acute phase reaction[195].

These peptides are generally membrane active cationic proteins whose affinity for negatively charged bacterial membranes depends on both electrostatic interactions and their tertiary structure. Many share common structural patterns, one of the best-described groups being the linear alpha helix forming peptides. They are also described as amphipathic as they contain both hydrophilic and hydrophobic residues. These peptides are very potent, with IC_{50} in the submicromolar range and act through disrupting the bacterial membrane. They are made up of up to 40 amino acid residues and are basic in character. They have the common feature of adopting an unfolded conformation in aqueous solution. However, on contact with the hydrophobic environment of the membrane, they stabilise in an alpha helical conformation. In this form, membrane permeation is thought to occur in one of two ways:(a) a

transmembrane pore formation via a "barrel-stave" mechanism – in which the peptide inserts into the membrane in a perpendicular orientation, or (b) a carpet like mechanism in which the peptide lies over and parallel to the membrane surface.[196] (Fig 1.7).

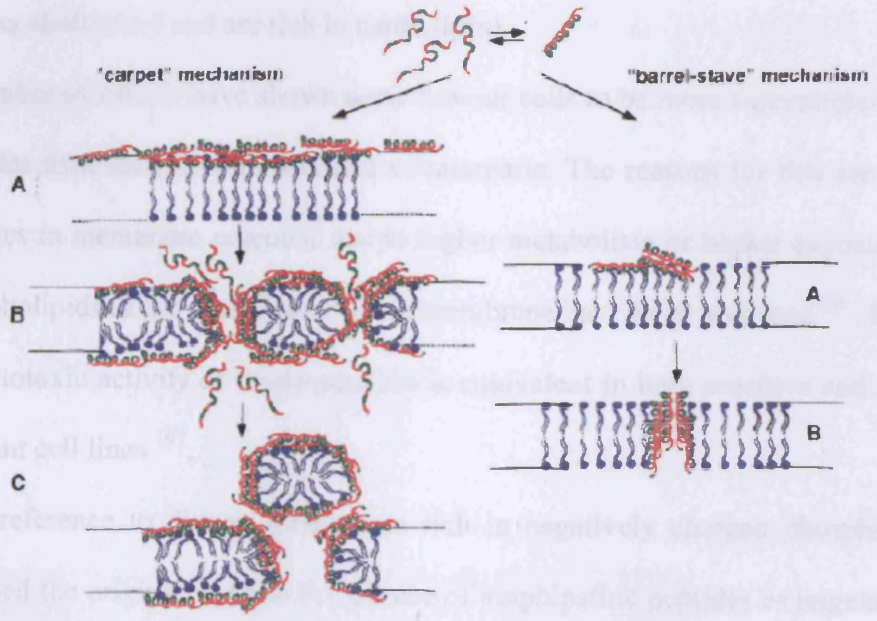


Fig.1.7 Mechanisms of membrane permeation by amphipathic peptides[196]

The selectivity of these peptides towards bacterial rather than eukaryotic membranes is based on differences in nett charge of these membranes. The cationic peptides preferentially bind bacterial membranes which are rich in negatively charged phospholipids rather than eukaryotic cell membranes which in which the majority of the phospholipds are zwitterionic in character, having little overall charge. These differences in charge reflect differences in phospholipid composition between eukaryotic and bacterial membranes (ie bacterial membranes generally lack sterols such as cholesterol and are rich in cardiolipin).

A number of studies have shown some tumour cells to be more susceptible to antibiotic peptides than their non-transformed counterparts. The reasons for this are unclear but changes in membrane potential due to higher metabolism or higher exposure of acidic phospholipids in the outer leaflet of the membrane have been proposed[193] . Furthermore the cytotoxic activity of these peptides is equivalent in both sensitive and multidrug – resistant cell lines [197].

The preference to disrupt membranes rich in negatively charged phospholipids also provided the original impetus for the use of amphipathic peptides as tatgeted cytotoxic agents. Mitochondrial membranes have a similar phospholipid compostion to bacterial membranes (being rich in cardiolipin), possibly reflecting the common ancestry of bacteria and mitochondria.[198]. Therefore, although antibiotic peptides are generally not toxic to eukaryotic cells , if they are internalised into the cell they have the potential ability to disrupt the mitochondrial membrane, possibly leading to the activation of the caspase cascade and apoptotic cell death. However it should be stressed that this mechanism has not been directly demonstrated in any study to date and that the presise mechansim underlying the toxicity of these agents when internalised by eukaryotic cells remainunclear.

1.5.2 Synthetic antibiotic peptides

Synthetic forms of antibiotic peptides have been developed by several groups , mainly for use against antibiotic resistant bacteria[199,200,201]. These molecules are often constructed using "non-natural" amino acid residues such as the β or D-conformations. These peptides have the advantage of avoiding proteolytic degradation whilst maintaining their membrane disrupting properties , which are not dependent on chirality.

A D-isomeric form of a synthetic antibiotic peptide $(KLAKLAK)_2$ which was specifically designed to have low mammalian cell toxicity[202] has been used as a cytotoxic agent in a number of human disease models.

Fig 1.8 illustrates the structure of $D(KLAKLAK)_2$. It is composed of 14 amino acids in an alpha helical formation with alternating cationic hydrophilic residues (in red) and non-polar hydrophobic residues (in green).

$_D$(Lys - Leu - Ala - Lys - Leu - Ala - Lys)$_2$
K - L - A - K - L - A - K

Fig 1.8 Structure of $D(KLAKLAK)_2$[203]

1.5.3 Cytotoxicity of D (KLAKLAK)$_2$

A compound peptide consisting of D(KLAKLAK)$_2$ fused to a protein transduction domain (RRQRRTSKLMKR),which allows receptor independent internalisation into cells, has been shown to induce tumour cell apoptosis and to reduce tumour volume when directly injected into day 7 established MCA205 murine fibrosarcoma tumours in a mouse model.[204].

Other groups have targeted D(KLAKLAK)$_2$ to specific tissue types, using short targeting peptides identified by phage display technology. Arap et al showed that systemic intravenous injection of a chimeric molecule consisting of D(KLAKLAK)$_2$ linked to a peptide, selected by phage display to specifically recognise the vasculature of the prostate, caused tissue destruction in the prostate of mice , but not in any other organs[127]. Another peptide (RGD-4C), identified by phage display to bind to integrins expressed on newly forming blood vessels but not on establised vasculature has been used to target and internalise D(KLAKLAK)$_2$ to synovial neovasculature in mouse model of inflammatory arthritis[205]. The identical chimeric peptide has also been shown to be effective in treating nude mice carrying human MDA-MD-435 breast carcinoma xenografts by inhibiting tumour angiogenesis.[203]. In both these studies, the targeted angiogenic cells showed the morphological features of apoptotic cell death, and caspase 3 processing was also demonstrated.

1.6. Aims

The aim of this project was to generate novel cytotoxic reagents that specifically target antigens expressed on leukaemia or lymphoma cells, leading to cell death.

The initial aim of the project was to link antibodies against CD19 and CD33 to the small antibiotic peptide D (KLAKLAK) $_2$. Targeting of D (KLAKLAK) $_2$ by linking it to antibodies will, in principle, allow selective killing of cells that express the relevant antigen whilst leaving cells that do not express these antigens intact.

There are potential advantages of using peptides rather than antibodies for targeting: They can be easily and economically synthesised. Purification is also easier as they are less likely to be contaminated by DNA and viruses. They are less immunogenic than antibodies and due to their small size may have better tumour penetration than larger antibody conjugates. However peptide sequences, which bind to CD19 and CD33, have not as yet been described. Therefore the second aim of the project was to identify such peptide sequences using phage display technology and create peptide – peptide conjugates which will have a selective cytotoxic effect on leukaemia/lymphoma cells.

Chapter 2 Materials and methods

2.1 Antibody conjugation protocol and characterisation of the conjugates

2.1.1 Monoclonal antibodies used in the conjugation reaction

Rat monoclonal anti-human CD45 (azide free, clone YTH 23.5) and mouse monoclonal anti-human CD33 (azide free, clone NHM 30.1.3.1) were both from Geoff Hale, Therapeutic Antibody Centre, Oxford, UK.

Mouse monoclonal anti-human CD19 (azide free, clone B-C3) was from Immunodiagnostic systems Ltd – Diaclone Research, Boldon, UK.

2.1.2 Conjugation protocol

The conjugation of D (KLAKLAK)$_2$ to monoclonal antibody using N-Succinimidyl-3-(2-Pyridyldithio) Propionate (SPDP, Pierce Chemical Company, Tattenhall, UK) as a cross-linker, was carried out using a method adapted from a published protocol.[206]

Initial experiments designed to investigate the effect of changing reaction conditions on the conjugation efficiency used anti-CD45 as the monoclonal antibody, as it was readily available in the laboratory.

1 ml of carrier solution containing 1mg of antibody was buffer exchanged in a centricon 50 device (Amicon Bioseparations - Millipore, Edinburgh, UK) into phosphate buffered saline (PBS) buffer (0.1M PBS, 0.9%NaCl, 5mM EDTA, pH 8.5) and concentrated to approximately 500µl. This was then transferred into a 1.5ml microfuge tube. 16µl of 5mM SPDP in dimethylsulfoxide DMSO (a 1 in 12 molar ratio of antibody to SPDP) was added and rotated at room temperature for 5 minutes. The reaction mixture was subjected to gel filtration using a PD-10 Sephadex column (Amersham Pharmacia, Little Chalfont, UK), equilibrated in phosphate buffer (0.1M

PBS, 0.9%NaCl, 5mM EDTA, pH 6.6) and re-concentrated in a centricon 50. A 0.5 mM solution of the amphipathic peptide L (CGG) D $(KLAKLAK)_2$ (Alta Bioscience, Birmingham, UK) in PBS pH6.6 was prepared, and reduced with 20mg of Cleland's Reductacryl reagent (Calbiochem, Nottingham,UK) by rotating for 15 minutes at room temperature. Following centrifugation at 10,000rpm for 5 minutes to separate out the resin, 240µl of the peptide solution, a 1 in 18 molar ratio of antibody to peptide, was added to the antibody – SPDP conjugate. The stoichiometry of the coupling reaction between the peptide and the antibody –SPDP conjugate was monitored in a glass cuvette in the spectrophotometer at 343nm and the final peptide: antibody ratio was calculated from the plateau absorbance value using the molar extinction coefficient of pyridine-2-thione ($8.08 \times 10^3 M^{-1}.cm^{-1}$) as described[207].The resulting conjugate was then subjected to gel filtration using a PD-10 column equilibrated with RPMI (Gibco, Life Technologies, Paisley, U.K) and concentrated in a centricon 50. Antibody conjugates were stored in 50µl aliquots.

2.1.3 Analysis of conjugates

2.1.3.1 Bio-Rad assay

The protein concentration of the final conjugate was quantified using Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, UK), a dye-binding assay, based on the differential colour change of a dye in response to various concentrations of proteins. The colour change was measured using a spectrophotometer at 595nm. Murine IgG (Sigma, Poole, UK) was used as a standard.

2.1.3.2 Gel electrophoresis under reducing conditions

NuPAGE Tris Glycine 4-12% precast gradient gel plates (NOVEX, Paisley, UK) were assembled according to manufacturer's instructions. 20µl of each conjugate were mixed with 7.5µl of 1 x sodium dodecyl sulfate (SDS) sample buffer (NOVEX), 2.5µl of 1M dithiothreitol (DTT) reducing agent. Controls consisted of anti-CD45 antibody and unconjugated peptide at known concentrations. Samples were heat denatured for 10 minutes at 70°C in a water bath, centrifuged briefly and loaded onto gels. Gels were run at 200 V using 1x N-morpholino propanesulfonic acid (MOPS) running buffer and NuPAGE running buffer antioxidant (NOVEX). The gels were simultaneously fixed and stained by immersing in a solution of methanol, glacial acetic acid and Coomassie Brilliant Blue (Sigma) for 1 hour on a shaking platform. Gels were then destained overnight by soaking in a methanol/acetic acid solution.

2.1.3.3 Western blot analysis of antibody conjugates

Antibody conjugates were prepared as before (section 2.1.2) using anti-CD33 and a biotinylated version of the peptide, biotin-L (CGG) D $(KLAKLAK)_2$. Gels were prepared as in section 2.1.3.2. Two Whatman filter papers and one nylon membrane (Hybond C, Amersham, UK) per gel were soaked in blotting buffer (25 mM Bicine, 35mM Bis Tris, 1mM EDTA, 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, 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) to ensure equal and satisfactory transfer of proteins. The membrane was then placed on top of a plastic

sheath, rolled up and placed in a 50ml Falcon tube with 10ml blocking buffer (0.02M Tris, 0.14M NaCl, 5% new-born calf serum and 5% polyvinylpyrolidone (PVP, Sigma) [pH7.6]) and rotated at room temperature for 1 hour. 0.5μl of streptavidin-horseradish peroxidase (HRP) (Pharmingen, Cowley, UK) was added to the blocking solution and the Falcon tube was rotated overnight in a rotisserie at room temperature. The blot was washed in 0.02M Tris, 0.14M NaCl and 0.02% Tween 20 for three 15 min washes followed by two 5 min washes. The enhanced chemiluminescence method (ECL,Amersham) was used to detect immunoreactive protein bands as detailed in the protocol provided.

Another blot prepared in an identical way to the first was probed by the same method using anti-mouse immunoglobulin-HRP (Dako, Glostrup, Denmark).

2.2 Cell lines and patient samples

2.2.1 Cell lines

The following mycoplasma-free leukaemia/lymphoma cell lines were used in this study: Raji, Daudi and 721.221, all human B cell lymphoblastic cell lines, Jurkat, a human acute T cell leukaemia cell line, THP1, a human acute monocytic leukaemia cell line, HL60, a human promyelocytic cell line, K562, an erythromyeloblastic transformation of human chronic myelogenous leukaemia (CML) and U937, a histiocytic lymphoma cell line.

All cells were cultured in 50ml tissue culture flasks (Nalge Nunc International Rochester USA) at 37^0C in a humidified 5% CO_2 incubator. Culture medium consisted of RPMI 1640 (Gibco, Life Technologies, Paisley, U.K) supplemented with 10% foetal calf serum (FCS), 100 units/mL penicillin, 2mM L-glutamine, and 100 μg/mL

streptomycin (all from Invitrogen Paisley, UK). Cell concentrations were maintained at between 0.2 and 0.6 x10^6 cells/ml by the addition of fresh medium.

CD33, CD19 and CD2 expression by each cell line was assessed by flow cytometry. 0.5 million cells were washed twice in Hanks Balanced Salts Solution (HBSS; Gibco, Life Technologies) and incubated with 10µl of fluorescein isothiocyanate (FITC)-conjugated primary antibody in 50µl of PBS. Antibodies used were CD19, CD33 and CD2 (Becton Dickinson, San Jose, USA) and mouse isotype control. After 20 minutes incubation at 4°C the cells were washed once in PBS, resuspended in 0.5ml of PBS and analysed by flow cytometry (FACScan, Becton Dickinson, Cowley, UK) equipped with Cell Quest software (Becton Dickinson).

Table 2.1 details the antigen expression pattern by human cell lines used

CELL LINE	CD33	CD19	CD2
RAJI	Negative	Positive	Negative
DAUDI	Negative	Positive	Negative
721.221	Negative	Positive	Negative
HL-60	Positive	Negative	Negative
KG-1	Positive	Negative	Negative
U937	Positive	Negative	Negative
K562	Negative	Negative	Negative
JURKAT	Negative	Negative	Positive

Table 2.1 Antigen expression by human cell lines

2.2.2 Patient's cells

Mononuclear cells were isolated from patient's peripheral blood samples or bone marrow with informed consent. Clotting was prevented by the addition of preservative free heparin. Plasma was withdrawn after sedimentation at 700xg. Patient samples were then diluted three to four fold in HBSS and 30ml carefully layered onto 20ml lymphoprep (Axis-Shield, Oslo, Norway). Tubes were centrifuged at 600xg for 30 minutes. The top layer of the medium was removed and the interface, consisting of mononuclear cells, was transferred to another universal tube and washed twice in HBSS. Cell concentrations were estimated using a Coulter cell counter. Cells were then resuspended in supplemented RPMI culture medium as before at a concentration of 2 x 10^6 cells/ml.

2.3 Cell viability assays and imaging

2.3.1 MTT assay

200µl of cells at a concentration of 0.25 x 10^6/ml (for the cell lines) and 2 x 10^6/ml (for the AML/CLL cells) were cultured with increasing concentrations of the antibody peptide conjugate, made up to 20µl in RPMI, in flat bottom 96 well plates. At 24, 48 and 72 hours 100µl of cells from each well were transferred into 96 well round bottom plates with 10µl of 5mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma)[208] and cultured for a further 4 hours at 37°C. Plates were centrifuged at 800 xg for 5 minutes, the supernatants were withdrawn and 100 µl per well of Dimethylsulfoxide (DMSO) were added. The absorbance of the purple formazan generated by reduction of MTT by viable cells was quantified by recording absorbance at 540 nm on an Anthos plate reader.

2.3.2 Clonogenic assay

1 ml aliquots of cell lines ($10^6.ml^{-1}$) were incubated at 37°C in the presence or absence of antibody-peptide conjugate for 48h. 10µl of these suspensions were mixed with 290µl of Iscove's modification of Dulbecco's medium (Invitrogen) and added to semi-solid methylcellulose culture medium with recombinant cytokines (Stem Cell Factor, GM-CSF, IL-3, IL-6, G-CSF, Eryhtropoeitin) (Methocult H4435; Stem Cell Technologies, Meylan, France). Triplicate aliquots (1 ml) were plated in 35 mm Petri plates and incubated further at 37°C. Colonies were counted at 7 days using an inverted microscope.

2.3.3 Cytospin preparation

Raji and Jurkat cells at a concentration of 0.5 x 10^6/ml were incubated with 10nM anti-CD19-D $(KLAKLAK)_2$ for 48 hours. Cytospin preparations were then made of these cells along with untreated controls. Slides were stained with May Grümwald Giemsa (MGG) and viewed under the light microscope. Apoptotic cell death was quantified by estimating the proportion of cells with condensed chromatin or fragmented nuclei[209] . Slides were examined in random order and at least two hundred cells in four different fields were counted per cytospin preparation.

2.3.4 Immunofluorescent microscopy

The biotinylated amphipathic peptide biotin-L (CGG) D $(KLAKLAK)_2$ was conjugated using the method described in section 2.1.2, to human monoclonal anti body at a molar ratio of 1:6. 1 ml of various cell lines at a concentration of 0.5 x 10^6/ml was incubated with 10nM of the conjugate solution. Cytospin preparations were made at 0,

5, 15, 30 and 60 minutes. Slides were fixed in ice-cold methanol overnight and rehydrated in PBS containing 1% bovine albumin. Spots were incubated with either 10 µl of FITC-conjugated streptavidin (Pharmingen) or 100µl of a 1/10 dilution of FITC-labelled anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) for half an hour in the dark. Following three rinses with PBS, 0.1% tween 20, 2% FCS, the slides were mounted in PBS:glycerol (1:1), viewed and viewed in an Olympus IX70 confocal microscope.

2.3.5 Electron microscopy

1 ml of cells isolated from the peripheral blood of a patient with CLL at a concentration of 2 x 10^6/ml were incubated with 10nM of anti-CD19 D(KLAKLAK)$_2$. After 24 hours the cell suspension was transferred to a 1.5 ml microfuge tube and concentrated by centrifugation to a volume of 50µl. 0.5ml of gluteraldehyde was then slowly added to the tube, whilst vortexing gently, to fix the cells. Electron microscopy was kindly carried out by Jackie Lewin at the Royal Free Hospital.

2.3.6 Flow cytometry of annexin V/Propidium iodide stained cells

Phosphatidylserine on the outer membrane of treated cells was detected by flow cytometry using a human Annexin V FITC kit (Bender Medsystems, Austria) following the manufacturer's instructions. 1 ml of cells at a concentration of 0.5 x 10^6/ml (for the cell lines) and 2 x 10^6/ml (for the CLL cells) were incubated with either no additions (negative control), with 10nM anti-CD19 D (KLAKLAK)$_2$ or 20µg/ml chlorambucil (positive control). At various time points (1, 3, 8 and 24 hours) cells were washed in PBS and resuspended in pre-diluted binding buffer at a concentration of 5 x 10^5/ml. 5µl of annexin V-FITC was then added to 195µl of the cell suspension and incubated for

ten minutes at room temperature in the dark. Cells were then washed once in PBS, resuspended in 190µl of pre-diluted binding buffer, and 10µl of 20µg/ml propidium iodide stock solution were added. Samples were immediately analysed on a Becton Dickinson flow cytometer using Cell Quest software. A published equation was used to compute agent-specific apoptosis[210].

$$\text{Specific apoptosis} = \frac{\%\text{ apoptosis in treated sample} - \%\text{of apoptosis in untreated control}}{100 - \%\text{ of apoptosis in untreated control}}$$

2.3.7 Flow cytometry to demonstrate antibody internalisation into Raji cells

1 ml of Raji cells at a concentration of 0.5 x 10^6/ml were incubated with either no addition (negative control) or 10nM anti-CD19 D $(KLAKLAK)_2$ for half an hour. Cells were then washed with HBSS and resuspended in culture medium. Aliquots were taken for flow cytometric analysis immediately (0 hour) and following incubation for 1,3 and 6 hours. Cells were stained with FITC-labelled anti-mouse immunoglobulin either directly or following permeabilisation and fixation with IntraStain (DAKO).

Samples were then analysed on a Becton Dickinson flow cytometer using Cell Quest software.

2.3.8 Quantification of protein expression in Raji and CLL cells by western blotting

Western blotting was used to quantitate the expression of various anti-apoptotic proteins in untreated CLL cells and also to estimate PARP cleavage in Raji and CLL cells after treatment with anti-CD19 D$(KLAKLAK)_2$ or chlorambucil for 24 hours.

Proteins were extracted from between 2-5 million cells. Cells were washed once in cold HBSS, transferred to microfuge tubes and resuspended in 50 to 100µl of lysis buffer

(20mM Hepes-KOH [pH 7.5], 50mM NaCl, 2% nonidet P40, 0.5% sodium deoxycholate, 0.2% SDS, 1mM sodium vanadate, 10mM sodium fluoride, 1mM ethylene glycol tetra-acetic acid (EGTA)), 0.02M DTT and 1mM 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 microfuge tube and stored at –70°C until use.

Protein concentrations were estimated as described in section 2.1.3.1 using bovine serum albumin (BSA,Sigma) as a standard. Western blots were then prepared as described in section 2.1.3.3 using 15μg of protein per well. Blots were blocked as before with 5% PVP blocking buffer and incubated overnight with primary antibody (listed in Table 2.2), followed by washing and incubation with the appropriate HRP linked secondary antibody (DAKO Denmark, 1/5000 dilution) for 1-3 hours. The ECL method was used as before to detect immunoreactive protein bands.

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. Band intensities were determined using Quantity 1 software (BioRad).

Antibody	**Source**
XIAP	BD bioscience, Oxford, UK
Bcl-2	Dako, Glostrup, Norway
Mcl-1	Santa Cruz, CA, US
P116 PARP	Pharmingen, Cowley, UK
P85 PARP	Promega, Southampton, UK
Actin	Sigma, Poole, UK

Table 2.2 Antibodies used in this study

2.3.9 Zeta-asociated protein 70 (ZAP-70) and IgVH mutation analysis estimation

ZAP-70 expression CLL samples was carried out by Najeem Folarin, using a flow cytometric method. Mouse monoclonal anti-ZAP-70 clone 2F3-2 (Upstate, Lake Placid, NY) and an isotype control mouse IgG2A (Dako, Glostrup, Denmark) were labelled with Alexa Fluor 488 immediately before use, using a Zenon antibody-labelling kit (Invitrogen). The procedure for determination of ZAP-70 expression status was carried out as described[211]. ZAP-70 mean cell fluorescence was simultaneously determined in the B and T lymphoid populations of CLL isolates, distinguished by labelling with phycoerythrin-conjugated anti-CD3 and PerCP-conjugated anti-CD19 (Becton-Dickinson, Oxford, UK) respectively. The ratio of ZAP-70 mean cell fluorescence of the B and T lymphoid populations was determined. A ratio above 0.75 was considered to indicate elevated ZAP-70 expression. This cut-off value was determined by analysis of B and T lymphocytes from 20 normal subjects and represents the mean plus two standard deviations of the ratio determined for normal B cells.

IgVH gene sequencing was carried out by Veronique Duke using polymerase chain reaction products obtained by amplification of genomic DNA using Framework Region 1 and J_H primers as described[212].

2.4 Phage display studies

2.4.1 Cell line

A CD33-Fc secreting CHO cell line was kindly supplied by Paul Crocker at Dundee University. Cells were cultured in $80cm^2$ tissue culture flasks at 37^0C in a humidified 5% CO_2 incubator. Culture medium consisted of Glasgow's Modified Eagles Medium supplemented for CHO culture with glutamine synthetase (GS) expression system (First Link, Brierley Hill UK) +5% dialysed immunoglobulin depleted foetal calf serum

(Gibco, Life Technologies) +200μM methionine sulfoximine (Sigma). Supernatant was removed weekly and stored in 50ml aliquots at -20°C.

2.4.2 ELISA for CD33

100μl of protein AG (5μg/ml) was placed in the wells of a protein immobiliser microwell plate (Exiqon, Vedbaek, Denmark) for 2 hours on an agitator at room temperature. Plates were then washed 3 times with TBS + 0.1% Tween 20, to remove any unbound protein.100μl of CD33-Fc containing supernatant were then added to each well and the plate placed on the agitator for a further 2 hours. After another 3 washes with TBS-Tween, non-specific protein binding was blocked by filling the wells with TBS-Tween + 10% foetal calf serum for 1 hour. Murine anti-human CD33 clone Him-3 (Pharmingen) (100μl of a 1:2000 dilution in TBS-tween/FCS) was then added to each well and incubated for 1 hour. After 3 further washes, 100μl of a 1:2000 dilution of HRP-conjugated goat anti mouse immunoglobulin (Dako) was added to each well and incubated for an hour. The wells were washed a further 3 times with TBS-Tween /FCS and 100μl of substrate reagent (R&D Systems catalogue number DY999) were added to each well. The reaction was stopped after 10 minutes on the agitator using 100μl of 2N sulphuric acid solution. The plates were then read spectrophotometrically at 450 nm on an Anthos plate reader.

2.4.3 Phage display biopanning

The CD33-Fc containing supernatant was dialysed into 0.1M $NaHCO_3$ (pH 8.6). 150μl of this solution was added to a single well of a 96 well flat bottom plate and incubated overnight at 4°C in a humidified container. The excess supernatant was removed with a pipette and the well was filled with blocking buffer (0.1M $NaHCO_3$ pH 8.6 + 5mg/ml

BSA + 0.02% NaN_3) and left for one hour at 4°C. This was then discarded and the well was washed 6x with TBS Tween 20 0.1% + 0.1% BSA. A phage display library (Ph.D 7mer peptide library, New England Biolabs, Ipswich, US) was used to pan the protein to select phage that bound to CD33. 100µl of phage medium consisting of 10µl of phage, 20µl of human IgG (Sigma) and 70µl of TBS Tween 0.1% were added to the well. After an hour on the agitator the excess medium containing unbound phage was pipetted off and the well was washed ten times with TBS Tween as before. The bound phage were then eluted by adding 100µl of 0.2M glycine HCL pH2.2 +1mg/ml BSA to the well for 8 minutes. The eluate was then transferred into a microfuge tube and neutralised with 15µl of 1M Tris HCL pH9.1.

A small amount of the eluted phage was then titred as follows: dilutions of phage (10^1,10^2,10^3 and 10^4) were prepared in LB media .E.coli ER2738 culture, in mid log phase was dispensed into four microfuge tubes and 10µl of diluted phage added .One at a time , the infected cell suspensions were transferred to culture tubes containing melted agarose top. The tubes were vortexed quickly and the media immediately poured onto pre-warmed LB/IPTG/Xgal plates. The plates were allowed to cool for 5 minutes before inverting and incubating at 37°C overnight. The number of blue plaques were then counted.

The remainder of the phage from biopanning was amplified by adding to 20 ml of E.coli culture in early log phase. The culture was incubated with vigorous shaking for 4.5 hours. The culture was then transferred to a centrifuge tube and centrifuged at 10,000 xg at 4°C for 10 minutes. The supernatant was transferred to a fresh tube and centrifuged again. The upper 80% of supernatant was then transferred to a fresh tube, 1/6 volume of PEG/NaCl was added and the solution kept a 4°C overnight to precipitate the phage.

The following day another culture of ER2738 was set up in order to titre the amplified phage. Again, four LB/IPTG/Xgal plates were placed in an incubator at 37°C and agarose top was melted and kept at 45°C until required.

The amplified phage precipitated overnight was centrifuged at 10,000 xg for 15 minutes. The supernatant was removed and the phage centrifuged briefly. Residual supernatant was removed with a pipette. The phage pellet was resuspended in 1ml TBS and transferred to a microfuge tube. It was centrifuged for 5 minutes at 4°C to pellet any residual cells and the supernatant transferred to a fresh microfuge tube. The phage was reprecipitated by adding 1/6 volume of PEG/NaCl and leaving on ice for 1 hour. The precipitated phage was centrifuged for 10 minutes at 4°C, the supernatant discarded and the phage briefly re-centrifuged. The remaining supernatant was removed with a pipette. The phage pellet was resuspended in 100µl TBS +0.02% NaN_3.The phage solution was centrifuged for one minute to remove any insoluble matter and the supernatant transferred to a fresh tube for storage at 4°C. The amplified eluate was then titered as before, but this time using dilutions of 10^8-10^{11} fold.

Three further rounds of biopanning were performed in this way, each time using approximately $2x10^{11}$ pfu (plaque forming units) of amplified eluate from the previous round as input phage. The method for subsequent rounds was exactly the same as for the first except that the concentration of TBS Tween was raised to 0.5%.

2.4.4 Sequencing of selected phage clones

After the fourth round of biopanning, clones were picked from the plate that had been used to titre the unamplified phage .Ten blue plaques were picked from the plate that contained less than 100 colonies and that had been incubated for not more than 16 hours. The plaques were transferred to separate tubes containing a culture of E.coli ER2738 in 10ml LB medium, and incubated at 37°C with vigorous shaking for 4.5 hours. Cultures were then transferred to centrifuge tubes and centrifuged for 30 seconds.500µl of the phage-containing supernatants were then transferred to large microfuge tubes to which 200µl of PEG/NaCl were added, and allowed to stand at room temperature for 10 minutes. After discarding the supernatant each pellet was suspended in 100µl of Iodide buffer (10mM Tris-HCl pH8.0, 1mM EDTA, 4M NaI), to which 250µl of ethanol were added and left for 10 minutes at room temperature to precipitate the phage DNA. The samples were then centrifuged for 10 minutes, and after discarding the supernatant, the remaining pellets were washed in 70% ethanol and dried briefly under vacuum. The pellets were then suspended in 30µl TE buffer (10nM Tris-HCl pH8.0, 1mM EDTA).

PCR mixture for each clone to be sequenced was prepared with 2µl –96 gIII sequencing primer (10pmol/µl), 1µl Big Dye Perkin Elmer, 7µl dilution buffer and 8µl water.2µl of DNA in TE buffer were added to each PCR reaction tube. PCR was carried out for 25 cycles: 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. The PCR products were then transferred to 0.7ml tubes and precipitated by adding 2µl of Na Acetate 3M and 50µl 100% Ethanol to each tube. These were then left on ice for 30 minutes, centrifuged for 30 minutes at full speed before removing the supernatant. The

DNA was washed with 150 µl of 70% ethanol, centrifuged for 10 minutes. After removing the ethanol, pellets were allowed to air dry.

An acrylamide gel was prepared for sequencing. Loading buffer was prepared using a 1in 6 dilution of concentrated loading buffer in formamide. For each reaction tube, 4.5µl of loading buffer were added and after vigorous vortexing the DNA was denatured at 95°C for 2 minutes. Prior to loading the denatured DNA was kept on ice . 2µl samples were loaded onto the acrylamide gel and the DNA sequencer (Applied Biosystems, Foster City, US) was run overnight to sequence the selected phage.

2.5 Statistical methods

Statistical analyisis were carried out using Prism software.

The apoptotic and loss of viability data in chapter 4 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 parametric 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

Synthesis and characterization of antibody –D (KLAKLAK)$_2$ conjugates

3.1 Introduction

In order to create conjugates that were selectively cytotoxic to haematological malignant cells, we conjugated D(KLAKLAK)$_2$ to commercially available monoclonal antibodies with specificities against antigens expressed on lymphoid and myeloid cells.

3.1.1 Choice of antibody

Anti-CD19 and anti-CD33 were chosen for this purpose, as they are expressed on early lymphoid and myeloid progenitors cells respectively (Fig. 3.1). Neither of these antigens are expressed on the pluripotent stem cell, thus theoretically allowing repopulation of bone marrow haemopoetic cells post-treatment. They also both have the additional favourable characteristics of internalising on binding to the ligand, providing a method of delivery of the cytotoxic peptide to the cytosol.

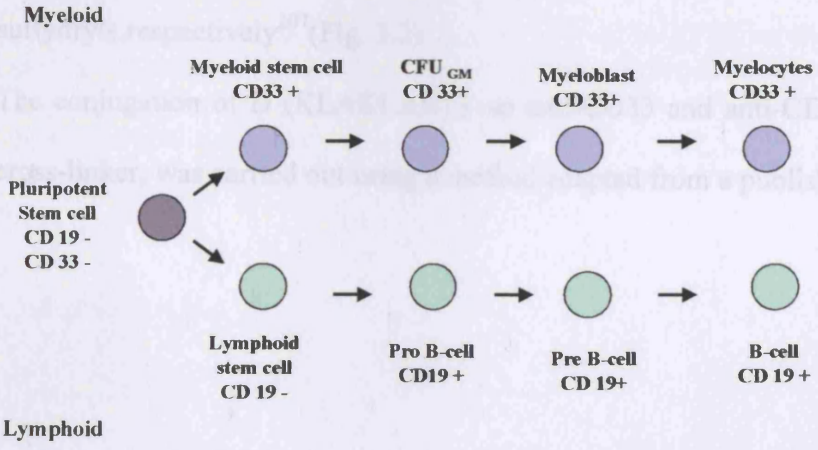


Fig 3.1 Expression of CD19 and CD33 on maturing haemopoietic cells

3.1.2 Choice of linker molecule

Antibodies can be chemically linked to peptides via cross-linking reagents. Most cross linking reagents have two reactive groups connected by a flexible spacer arm; reagents differ in their spacer arm length, cleavability and reactive groups. Homo-bifunctional linkers have the same reactive group at both ends and which can lead to high levels of polymerisation; alternatively bifunctional reagents have different reactive groups at the ends. Most protein cross-linking reactions are side chain reactions and are nucleophillic and pH dependent. Most proteins including antibodies have lysine residues available at the surface of the proteins, providing primary amines; many proteins also have cysteine residues that are not involved in disulphide bonds providing free sulfydryls. These are the two most commonly used groups in protein cross-linking strategies. Cross-linking strategies may also use carbohydrates, carboxyls or other reactive functional groups.[206]

N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) is a cleavable heterobifunctional cross-linking agent which contains one N-hydroxysuccinimide (NHS) residue and one pyridyl disulphide residue which react with primary amines and sulfydryls respectively[207](Fig. 3.2).

The conjugation of D $(KLAKLAK)_2$ to anti-CD33 and anti-CD19 using SPDP as a cross-linker, was carried out using a method adapted from a published protocol .[206]

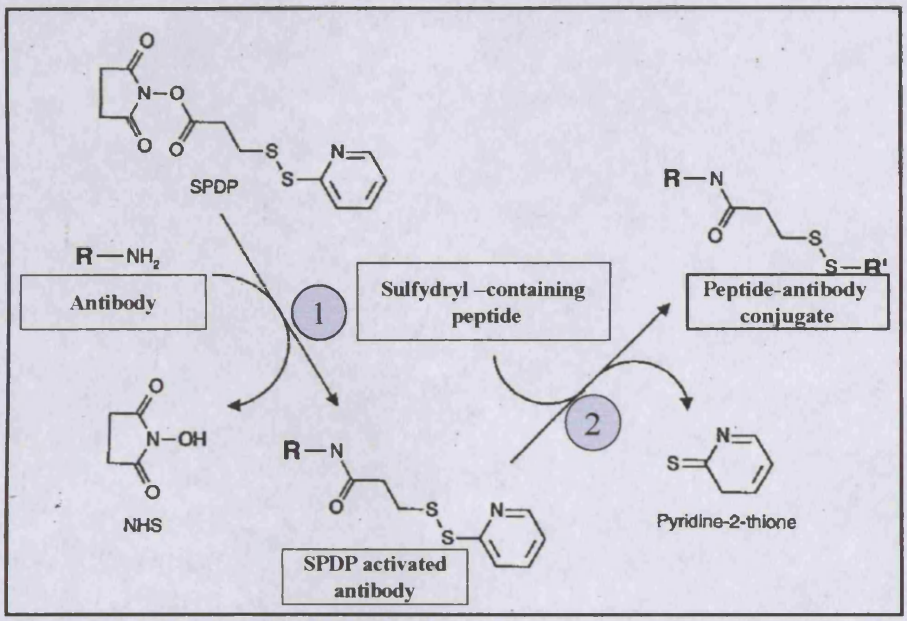


Fig 3.2 SPDP (*N*-Succinimidyl 3-(2-pyridyldithio) propionate conjugation
Step 1, the activated NHS end of the SPDP reacts with amino groups on lysine residues of the antibody.
Step 2, the 2-pyridyldithiol group at the other end of the SPDP moiety reacts with sulfydryl residues on the peptide releasing Pyridine-2-thione in the process.Pyridine-2-thione can be detected spectrophotometrically at 343nm,allowing the monitoring of the progress of the conjugation.
The molar extinction coefficient of pyridine-2-thione at 343nm is 8.08 x $10^3 M^{-1}cm^{-1}$

Adapted from ref[206].

3.2 Results

3.2.1 Spectrophotometric monitoring of the conjugation reaction.

The concentration of pyridine-2-thione released during the conjugation reaction (step 2 fig. 3.2) was determined by measuring the change in absorbance in a u.v. spectrophotometer at 343nM. The molar extinction coefficient of pyridine-2-thione was used to calculate the number of SPDP molecules attached per antibody molecule and hence the final stoichiometry of the conjugation reaction (assuming the SPDP: D (KLAKLAK) $_2$ ratio to be 1:1). Fig 3.3 illustrates the change in absorbance occurring during the reaction of 1mg antibody (6.67 x10^{-9} mols) with SPDP in 3 different molar ratios.

Example of calculation (see fig 3.3)

Increase in absorbance at 343nm at peptide: antibody molar ratio 18:1 = 0.1

Molar extinction coefficient of pyridine -2-thione at 343nm = 8.08 x 10^3/M/cm

Standard u.v cuvette path length = 1 cm

0.1/ (8.08x 10^3) =1.24 x10^{-5} molar increase in concentration =1.24x10^{-5}/ litre

The volume of the cuvette is 500µl therefore moles of pyridine-2-thione released in 500µl = 6.2 x 10^{-9}.

Ratio of antibody to SPDP = 6.67:6.2 approximately 1:1

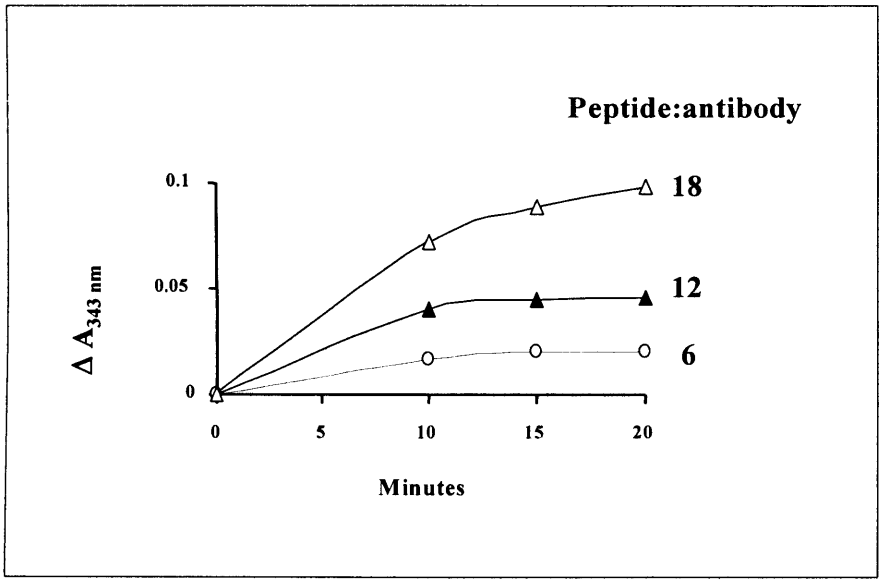


Fig. 3.3 Effect of increasing the peptide/SPDP : antibody ratio
Increasing the peptide/SPDP : antibody ratio results in a corresponding increase in the change in A_{343nm} and hence in the number of peptides incorporated per antibody molecule.

An SPDP to antibody ratio of only 1:1 using an 18:1molar ratio of peptide to antibody was an unexpectedly low efficiency. To determine the reason for this we first estimated the protein loss during the conjugation reaction by biorad assay of pre and post conjugation samples compared to a standard curve generated using known concentrations of anti-CD45. This was found to be approximately 30% and therefore did not account for the poor conjugation efficiency.

3.2.2 Effect of pH on the efficiency of the conjugation reaction

In our initial conjugation protocol adapted from a standard published method a conjugation buffer with pH 7.2 was used. However other methods in the literature[213] noted an increased efficiency of SPDP conjugation efficiency at pH between 8.0 and 8.5. We investigated this by repeating the conjugation reaction in three buffers of varying pH (7.0, 8.0, 8.5) using the same molar ratio of SPDP to antibody of 6:1 in each experiment (Fig.3.4). In these reactions DTT was used instead of $D(KLAKLAK)_2$ to reduce the disulphide bond and release the pyridine-2-thione (step 2 Fig 3.2).

The results of these experiments demonstrated a conjugation efficiency of approximately 50% (i.e. final SPDP antibody ratio 3:1 using initial molar ratio of 6:1) using buffers with pH 8.0 and 8.5 compared to 7% with the buffer at pH 7.0. These calculations took into account the 30% protein loss during conjugation as estimated by biorad assay above.

The conjugation protocol was modified accordingly and a pH of 8 was used in all subsequent conjugations.

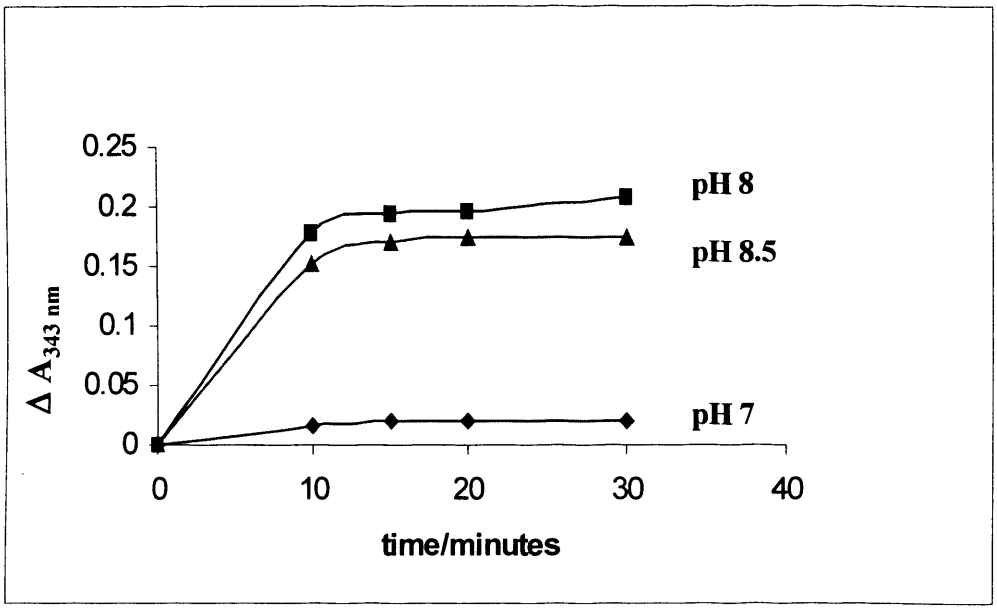


Fig.3.4 Effect of pH on the SPDP conjugation reaction
Small changes in the pH of the reaction buffer resulted in relatively large increases in efficiency of the conjugation reaction.

3.2.3 Characterization of conjugates

3.2.3.1 Gel electrophoresis of antibody conjugates

In addition to spectrophotometric monitoring, the stoichiometry of the final conjugates was assessed by gel electrophoresis (Fig.3.5). 5 µl of peptide conjugates of anti-CD45 (CD45K), and 20 µl of anti-CD33 (CD33K) and anti-CD19 (CD19K), and known amounts of unconjugated peptide and unconjugated anti-CD45 were fractionated by SDS- PAGE. The gel was stained with Coomassie blue. Intensities of bands were quantified using Quantity 1 software (BioRad).

These values were then used to estimate the concentration of the antibody conjugates and the number of $D(KLAKLAK)_2$ peptides per antibody molecule as follows:

i. Standard unit from known amounts of anti-CD45 control

Total µg antibody applied to lane	Absorbance units/µg antibody = (absorbance of heavy chain + absorbance of light chain) / µg antibody applied to lane
20µg	27.7 + 12.24 / 20 = 2
10µg	13.08 + 6.86 / 10 = 1.99
5µg	7.27 + 3.77 / 5 = 2.21

For further calculations it was assumed that a total absorbance of 2 corresponded to 1µg of antibody.

ii. Calculation of concentrations of antibody conjugates

e.g. anti-CD33K : 7.18 + 3.6/2 = 5.39μg in 20μl .

Therefore the antibody concentration is 0.27μg/μl, (a value of 0.3μg/μl was obtained by biorad assay of the same conjugate).

iii. Estimation of the proportion of the ratio of D(KLAKLAK)$_2$ to antibody

Absorbance of [D(KLAKLAK)$_2$ /(heavy chain + light chain + D(KLAKLAK)$_2$] *100

For example, this was computed at 9.9% for the anti-CD33-D(KLAKLAK)$_2$ conjugate.

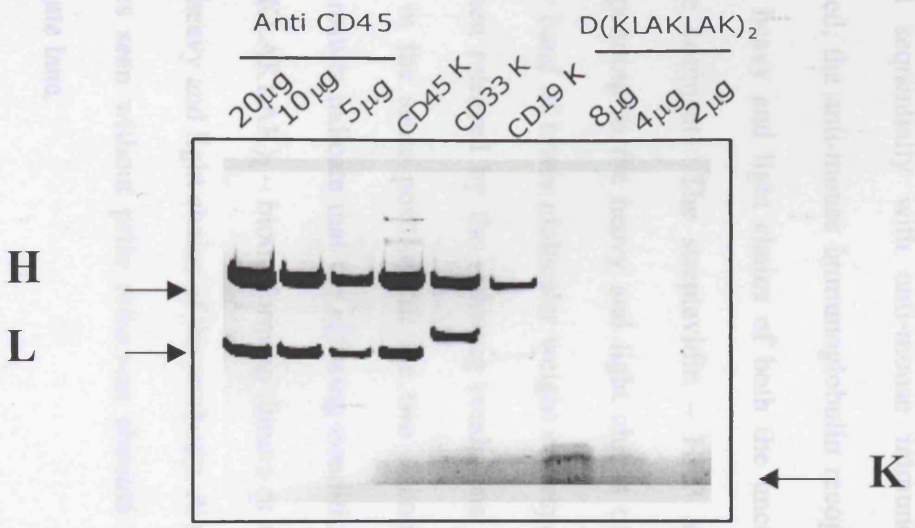
Molecular weight of D(KLAKLAK)$_2$ = 2000

Molecular weight of unconjugated antibody = 150 000

Using the formula = [2000x /(150 000 +2000x)] * 100 = 9.9 , where x represents the number of molecules of D(KLAKLAK)$_2$ per molecule of antibody.

This represents approximately 8 D (KLAKLAK)$_2$ peptides per antibody molecule.

Spectrophotometric monitoring of the same conjugate estimated a conjugation stoichiometry of 10 peptide molecules per antibody molecule.



	anti-CD45 20ug	anti-CD45 10ug	anti-CD45 5ug	anti-CD45K 5ul	anti-CD33K 20ul	anti-CD19K 20ul	K 8ug	K 4ug	K 2ug
Heavy chain	27.7	13.08	7.27	14.1	7.18	3.07			
Light chain	12.29	6.86	3.77	7.75	3.6	1.47			
D(KLAKLAK)2				0.73	1.19	0.92	2.14	0.86	0.34

Fig.3.5 Gel electrophoresis of antibody conjugates

Peptide conjugates of anti-CD45 (CD45K), anti-CD33 (CD33K) and anti-CD19 (CD19K) , unconjugated peptide and unconjugated anti-CD45 were fractionated by SDS- PAGE. The gel was stained with Coumassie blue. Intensities of bands were quantified and are presented in the table.

H,heavy chain L,light chain K, $D(KLAKLAK)_2$

3.2.3.2 Western blot analysis of antibody conjugates

Conjugates were also characterised by western blotting (Fig 3.6). In these experiments anti-CD33 was conjugated to a peptide consisting of $D(KLAKLAK)_2$ with the addition of a biotin tag . This was fractionated by SDS-PAGE along with a similar amount of unconjugated anti-CD33 and two concentrations of unconjugated biotinylated peptide. All the samples were reduced by heating to 70°C with 170mM DTT. The blots were probed sequentially with anti-mouse immunoglobulin and streptavidin-HRP. As expected, the anti-mouse immunoglobulin recognised bands in regions corresponding to the heavy and light chains of both the unconjugated antibody and the antibody-peptide conjugate. The streptavidin – HRP also recognised bands in the regions corresponding to the heavy and light chains of the antibody conjugate as well as a further band of lower molecular weight corresponding to the biotinylated peptide that had been released by the reducing conditions. The same probe recognised multiple bands in the same position for the two unconjugated biotinylated peptide samples. These results indicate that the reducing conditions used were not sufficient to prevent the $D(KLAKLAK)_2$ – biotin forming dimers or to release all of the peptide conjugated to the heavy and light chains of the antibody. A similar blot (not shown) using the same samples seen without prior reduction showed no free peptide band in the antibody conjugate lane.

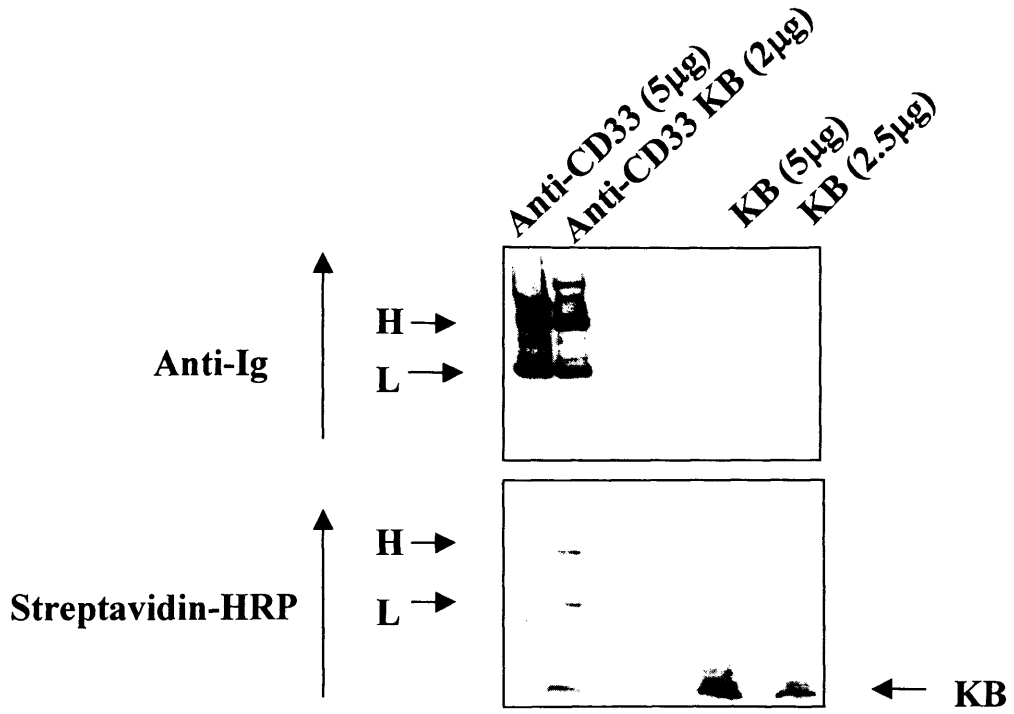


Fig.3.6 Western blot analysis of biotinylated antibody conjugate
Unconjugated anti-CD33, anti-CD33 conjugated to biotinylated $D(KLAKLAK)_2$ (anti-CD33 KB) and unconjugated biotinylated $D(KLAKLAK)_2$ were reduced with DTT and fractionated by SDS-PAGE, transferred to a Hybond membrane and analyzed using anti-mouse immunoglobulin (upper) or sterptavidin- HRP (lower).
H, heavy chain L, light chain KB, biotinylated $D(KLAKLAK)_2$

3.3 Discussion

Using a modified published protocol [206] we have developed a method which allows the conjugation of a predictable number of peptides per antibody molecule and demonstrated reliable stoichiometry by spectrophotometric monitoring of the conjugation reaction and SDS-PAGE electrophoresis. A western blot probed with streptavidin-HRP showed that while there was no free peptide in the unreduced conjugation sample, it was partially released by heating with 170mM DTT.

NHS ester reactions (step 1 Fig 3.2) are normally carried out at pH 7.0-9.0. Reactivity of the lysine group increases as the pH increases to 9.0, but competing NHS hydrolysis also is favored with pH increase[137]. Many of the published protocols for SPDP conjugation (including the manufacturers' recommendations) recommend an initial buffer pH of between 7.0 and 7.5 with a reaction time of at least half an hour and up to twelve hours[137,206,207,214]. However in our hands, a higher pH of 8-8.5 with a shorter reaction time of five minutes as described by Peeters et al[213] gave significantly better conjugation efficiency.

Summary

$D(KLAKLAK)_2$ was conjugated to monoclonal antibodies with specificities against antigens expressed on lyphoid and myeloid cells (CD19 and CD33 respectively). Conjugation of the peptide to the antibody was carried out using the heterobifunctional cross-linking agent SPDP from a method adapted from a published protocol which was optimised by altering the reaction conditions. The resulting conjugate was characterised spectrophotometrically, by gel electrophoresis and Western blotting under reducing conditions.

Chapter 4

Cytotoxicity of D (KLAKLAK)$_2$ antibody conjugates towards leukaemia cell lines

4.1 Introduction

In order to assess the cytotoxicity, potency and specificity of D (KLAKLAK)$_2$ - antibody conjugates we used a number of cell lines of haematological origin.

Cell lines were selected on the basis of the cell lineage and expression of the relevant antigen. (Table 4.1)

Cell line	Cell of origin	Morphology	CD33	CD19	Ref.
Jurkat	T-cell leukaemia	T-lymphoblast	no	yes	215
Raji	Burkitt's lymphoma	B-lymphoblast	no	yes	216
Daudi	Burkitt's lymphoma	B-lymphoblast	no	yes	217
721 221	HLA deficient lymphoblast	B-lymphoblast	no	yes	218
THP1	Acute monocytic leukaemia	Monoblast	yes	no	219
HL60	Acute promyelocytic leukaemia	Myeloblast	yes	no	220
U937	Histiocytic lymphoma (pleural effusion)	Histiomonoblast	yes	no	221
K562	CML in blast crisis (pleural effusion)	Erythromyeloblast	no	no	222

Table 4.1 Characteristics of cell lines used in this study

4.2 Results

4.2.1 Assessment of cytotoxcity of D(KLAKLAK)$_2$ conjugates

4.2.1.1 Anti-CD33-D (KLAKLAK)$_2$ has cytotoxic activity against a CD33 positive cell line as assessed by MTT and clonogenic assay

CD33 expression was first checked by immunophenotyping by flow cytometry and alkaline phosphatase-anti-alkaline phosphatase (APAAP)* staining of cytospin preparations of cells. The cytotoxicity of the anti-CD33-D(KLAKLAK)$_2$ conjugate (antibody: peptide ratio 1:6) was assessed by incubation of increasing concentrations of the conjugate with a CD33 positive cell line (THP1) and three CD33 negative cell lines (Jurkat, Raji and K562). MTT assays were performed at 24, 48 and 72 hours. All MTT assays were performed in duplicate. In the case of the THP1 cell line, the maximal decrease in cell viability of 93% was noted at 24 hours (Fig 4.1) with no further decrease at 48 or 72 hours. The IC_{50} at 24 hours for THP1 cells was 2.53 nM in this experiment, with no decrease in cell viability at these doses being noted in the CD33 negative cell lines (Fig. 4.1).

Two other CD33 positive cell lines were also assayed after incubation with anti-CD33-D(KLAKLAK)$_2$ and showed no decrease in cell viability at 24-72 hours. This will be discussed later in the chapter (section 4.2.3)

*APAAP staining was kindly carried out by Faith Wright of the immunophenotyping laboratory at the Royal Free Hospital.

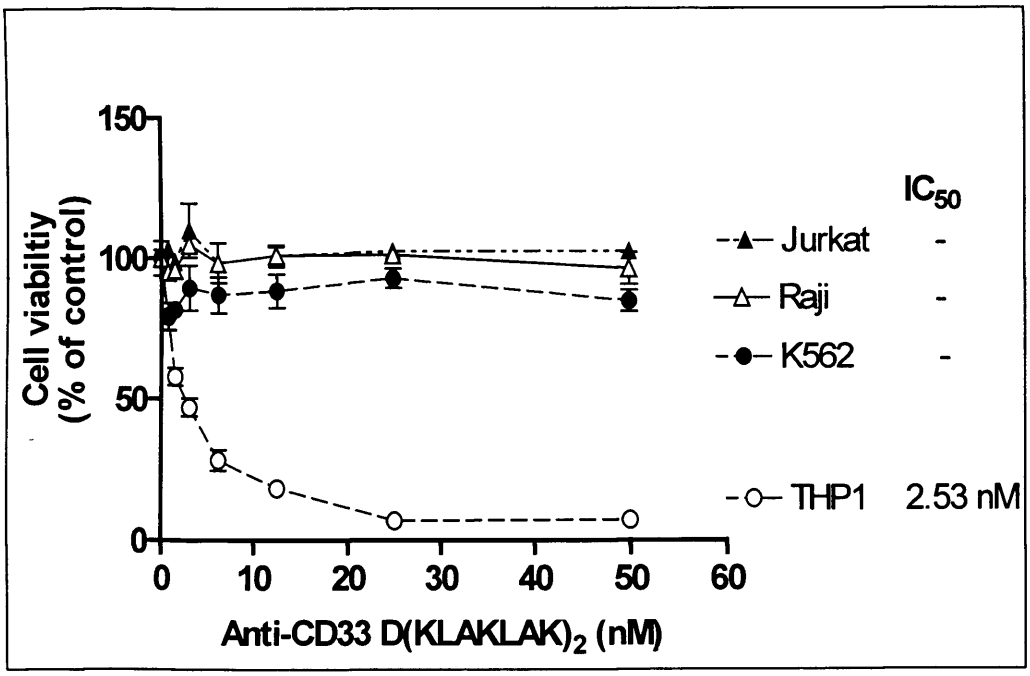


Fig 4.1 Action of anti-CD33 D(KLAKLAK)$_2$ on malignant haemopoietic cell lines
Dose response curve for action of anti-CD33-D(KLAKLAK)$_2$ on CD33 positive (THP1) and CD33 negative (Jurkat,Raji and K562) cell lines. Cell lines were incubated with increasing concentrations of the conjugate. Cell viability was assessed by MTT assay at 24 hours
Data are representative of four independent experiments.
Error bars represent SEM of duplicate determinations.

To ensure that the results for the MTT cell viability assay above correlated with true cell death and loss of proliferative potential, a clonogenic assay and MTT assay were performed in parallel. THP1 cells, either untreated or treated with 10 nM anti-CD33-D(KLAKLAK)$_2$ were incubated for 24 hours, at which time 100μl samples were taken for MTT assay (in duplicate). The remaining cells were suspended in semi solid culture medium in methylcellulose with recombinant cytokines (see section 2.3.2), plated in triplicate Petri dishes and incubated at 37°C. Colonies were counted at one week. The results of this experiment demonstrated equivalent decreases in cell viability of treated cells as compared to untreated controls as measured by both assays: 70.4% decrease in cell viability by clonogenic assay and 66.5% by MTT assay (Fig.4.2).

The effect of altering the antibody to peptide ratio on the cytotoxicity of the conjugates towards cell lines was investigated by incubating THP1 cells with two anti-CD33-D(KLAKLAK)$_2$ conjugates. One of the conjugates had an antibody to peptide ratio of 1:3 and the other of 1:9. MTT assays were performed at 24 hours (Fig 4.3). The results of this experiment indicated that increasing the number of peptides per antibody molecule had no effect on the maximum decrease in cell viability observed: 83% and 81% for the 1:3 and the 1:9 conjugate respectively. However the conjugate with a higher number of peptides per antibody molecule was more potent, with an IC_{50} of 6.3 nM compared to 12.5 nM for the 1:3 conjugate.

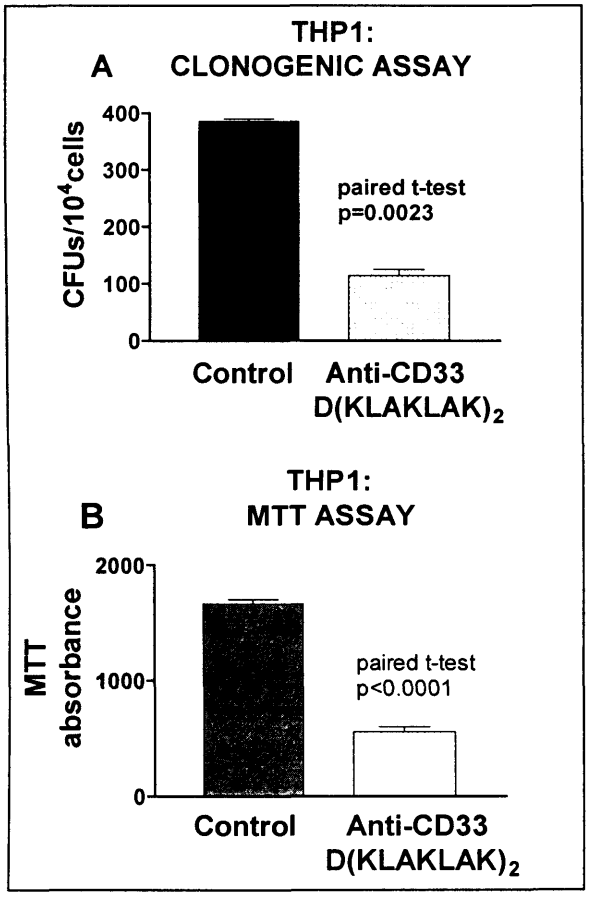


Fig 4.2 Cytotoxicity of anti-CD33-D(KLAKLAK)2 to THP1 cells as assessed by clonogenic (A) and MTT (B) assays

THP1 cells were either untreated (control) or incubated with 10 nM anti-CD33 $D(KLAKLAK)_2$ for 24 hours. Clonogenic assay (A) and MTT assay (B) were then performed in parallel.

A. Clonogenic assay. Colonies were counted at 1 week

B. MTT assay was at 24 hours

Data are representative of two independent experiments.

Error bars represent SEM of triplicate determinations.

Statistical evaluation was by paired Student's t-test.

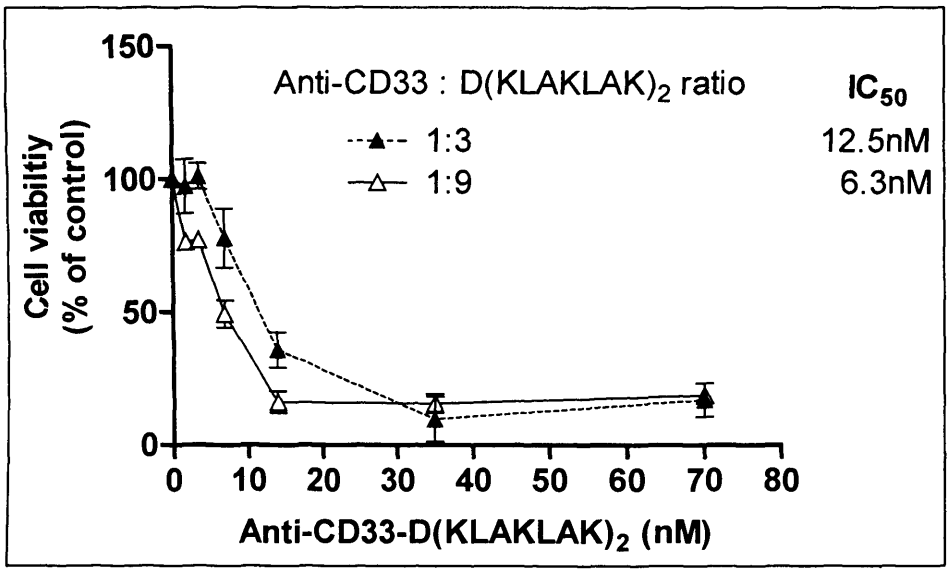


Fig.4.3 Effect of varying the antibody: peptide ratio on cytotoxicity of conjugates
Dose response curves for action of two anti-CD33-D(KLAKLAK)$_2$ conjugates with varying antibody to peptide ratios on THP1 cells. THP1 cells were incubated with increasing concentrations of the two anti-CD33 D(KLAKLAK)$_2$ conjugates, one with an antiboby : peptide ratio of 1:3 and the other with a ratio of 1:9.
Cell viability was assessed by MTT assay at 24 hours.
Data are representative of two independent experiments.
Error bars represent the SEM of duplicate determinations.

4.2.1.2 Anti-CD19-D(KLAKLAK)$_2$ has cytotoxic activity against CD19 positive cell lines as measured by MTT assay and clonogenic assay

Cytotoxicity of anti-CD19-D (KLAKLAK) $_2$ (antibody: peptide ratio of 1:6) was assessed by incubation of increasing concentrations of the conjugate with four cell lines, three of which expressed the CD19 antigen (Raji, Daudi and 721 221) and one of which did not (Jurkat). CD19 expression was first assessed by immunophenotyping by flow cytometry and APAAP staining of cytospin preparations of cells. Samples were taken for MTT assay at 24, 48 and 72 hours. At 24 hours the decrease in cell viability for the three CD19 positive cells was between 37- 83%, mean 61.6% (not shown). Maximal decrease in cell viability 75-97%, mean 87.4%, was observed at 48 hours (Fig 4.4 A). No further decrease in cell viability was noted at 72 hours. IC_{50}s for the three CD19 positive cell lines after 48 hours incubation were in the low nanomolar range (2.04-5.07nM, mean 3.66nM). Jurkat cells showed no decrease in cell viability at the doses of conjugate used in this experiment.

Clonogenic assay confirmed the dose dependent decrease in cell viability of Raji cell treated with three different concentrations of anti-CD19 D(KLAKLAK)$_2$. (Fig 4.4 B). The resistance of Jurkat cells to anti-CD19 conjugate was also confirmed by clonogenic assay (data not shown).

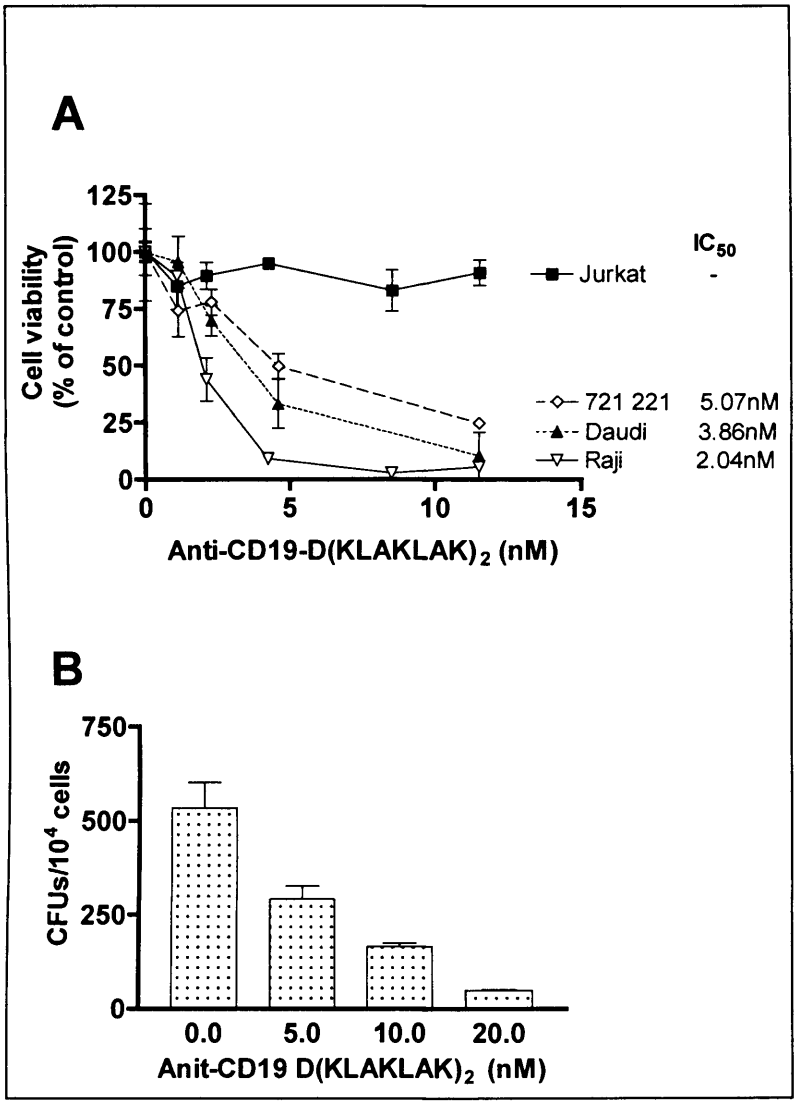


Fig 4.4 Action of anti-CD19 D(KLAKLAK)$_2$ on malignant haemopoietic cell lines
A. Dose response curve for action of anti-CD19 D-(KLAKLAK)2 on three CD19 positive (Raji,721 221 and Daudi) and one CD19 negative (Jurkat) cell lines. Cell lines were incubated with increasing concentrations of the anti-CD19 D(KLAKLAK)$_2$ conjugate. Cell viability was assessed by MTT assay at 48 hours.
B. Clonogenic assay of Raji cells treated with anti-CD19 D(KLAKLAK)$_2$
Raji cells were incubated with three different concentrations of the anti-CD19 D(KLAKLAK)$_2$ conjugate for 48 hours. Colonies were counted at one week.
Data are representative of two independent experiments.
Error bars represent SEM of triplicate determinations.

4.2.1.3 Cytotoxicity of unconjugated antibodies

Raji and THP1 cells were incubated with unconjugated anti-CD19 and anti-CD33 respectively, at the same dose ranges used in the experiments using conjugate. Samples for MTT assay were taken at 48 hours for the anti-CD19 (Fig 4.5A) and at 24 hours for the anti-CD33 antibodies (Fig 4.5B). Neither of the unconjugated antibodies had any significant cytotoxic effect on the cell lines which expressed the relevant antigens at the concentrations used.

4.2.1.4 Cytotoxicity of unconjugated peptide

Five cell lines were incubated with increasing concentrations of unconjugated $D(KLAKLAK)_2$, samples were taken for MTT assay at 24 hours (Fig.4.6). All of the cell lines were susceptible to the cytotoxic action of the unconjugated peptide with decreases in cell viability of at least 97%. IC_{50}s were in the low micromolar range (6.5-19.6μM). Therefore the concentrations of unconjugated peptide required to decrease cell viability by 50% were substantially greater than when the peptide was conjugated to an appropriate internalising antibody.

4.2.1.5 Cytotoxicity of a mixture of unconjugated peptide and antibody

Finally Raji cells were incubated with a mixture of unconjugated peptide and unconjugated anti-CD19 in the same molar proportions as an anti-CD19-$D(KLAKLAK)_2$ conjugate (antibody: peptide ratio 1:6). After 48 hours there was no decrease in cell viability as assessed by MTT in the cells treated with the mixture of agents (Fig.4.7).

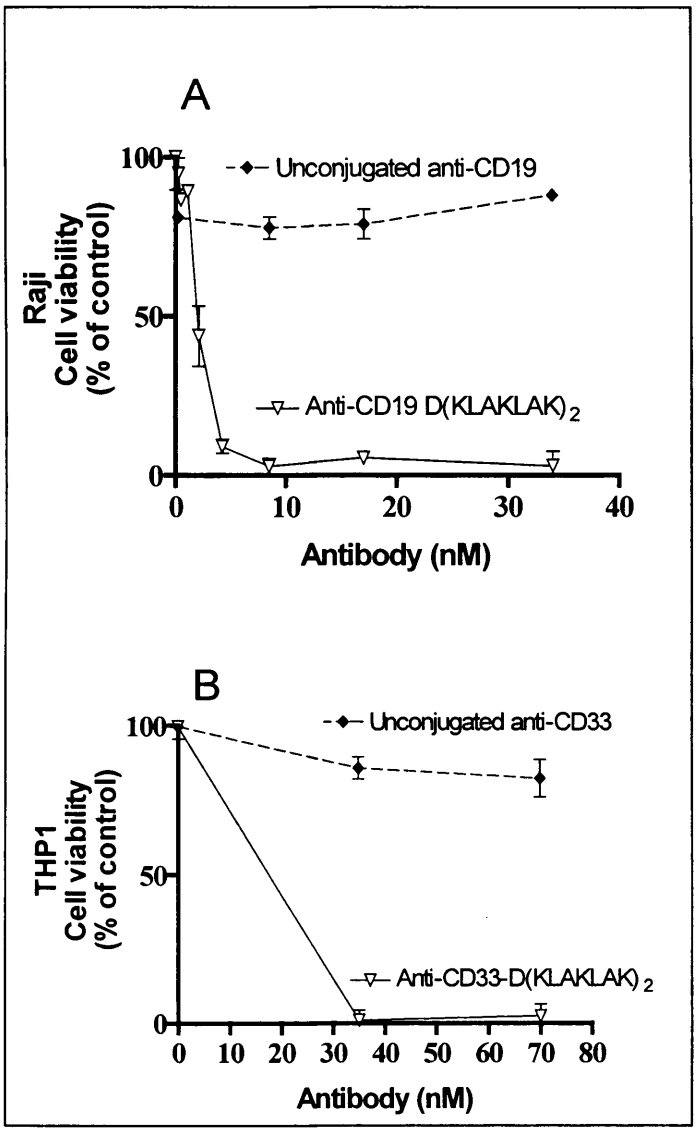


Fig 4.5 Action of unconjugated antibodies on haemopoeitic cell lines
A Dose response curves of the action of unconjugated anti-CD19 and anti-CD19 D(KLAKLAK)$_2$ on Raji cells. Cell viability was assessed byMTT assay at 24 hours.
B. Dose response curve of the action unconjugated anti-CD33 and anti-CD33 D(KLAKLAK)$_2$ on THP1 cells (B). cell viability was assessed by MTT assay at 48 hours.
Data are representative of two independent experiments.
Error bars represent the SEM of duplicate determinations.

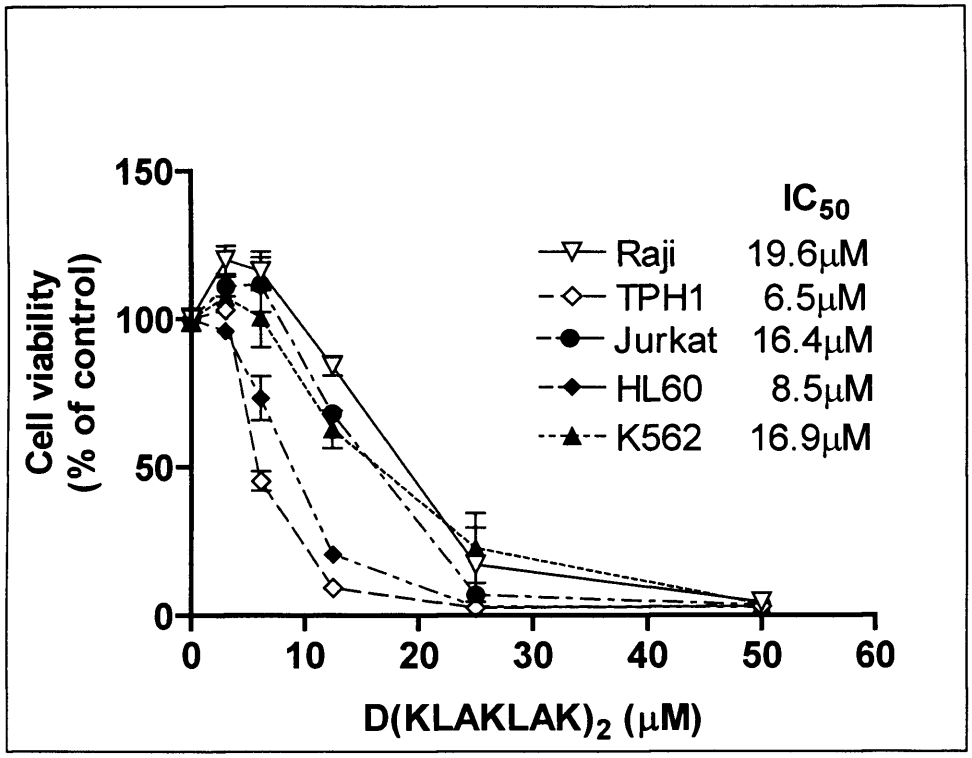


Fig.4.6 Action of unconjugated D(KLAKLAK)2 on malignant haemopoietic cell lines

Dose response curve for action of unconjugated D(KLAKLAK)2 on five cell lines.
Cells were incubated with increasing concentrations of unconjugated peptide.
Cell viability was assessed by MTT assay at 24 hours.
Data are representative of three independent experiments.
Error bars represent the SEM of duplicate determinations.

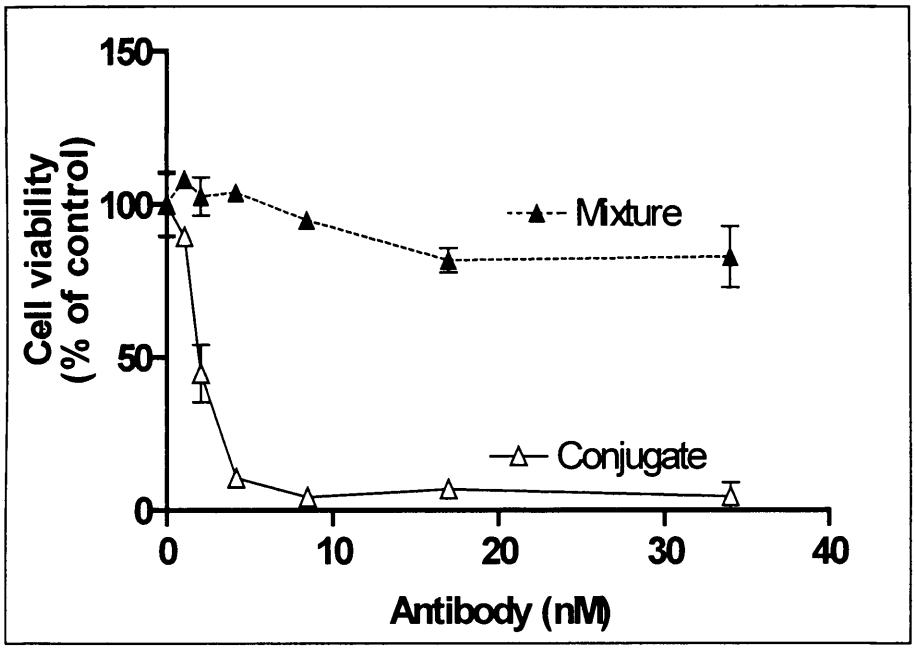


Fig 4.7 Action of a mixture of anti-CD19 and D(KLAKLAK)2 peptide on Raji cells

Dose repsonse curve for the action of a mixture of anti-CD19 and D(KLAKLAK)2 peptide in a molar proportion of 1:6 on Raji cells. Raji cells were incubated with increasing concentrations of a mixture of unconjugated peptide and unconjugated anti-CD19 in the same molar proportions as an anti-CD19 $D(KLAKLAK)_2$ conjugate. Cell viability was assessed by MTT assay at 48 hours. Data representive of two independent experiments.
Error bars represent SEM of duplicate determinations.

4.2.2 Mechanism of action of D(KLAKLAK)$_2$ conjugates

4.2.2.1 D(KLAKLAK)$_2$ conjugates internalise into cells that express the relevant antigen

i. Anti-CD33-D(KLAKLAK)$_2$ conjugates are internalised by CD33 positive cells.

Two CD33 positive cell lines (THP1, HL60) and one CD33 negative cell line (Raji) were incubated with a conjugate of anti-CD33-biotinylated D(KLAKLAK)$_2$ (antibody: peptide ratio 1:9). Cytospin preparations were made and fixed after 0, 15, 30 and 60 minutes incubation. Slides were then stained with streptavidin-FITC and visualised on the confocal microscope. At 15 minutes, internalisation of the biotinylated peptide into vesicular structures was demonstrated in both the cell lines which expressed CD33 but not in the negative control Raji cells. Data not available for HL60 cells. Data for THP1 cells (Fig 4.8).

ii. Anti-CD19-D(KLAKLAK)$_2$ conjugates are internalised by CD19 positive cells.

A similar experiment using a CD19 positive cell line (Raji) incubated with anti-CD19-biotinylated D(KLAKLAK)$_2$, stained with streptavidin - FITC and viewed on the confocal microscope did not yield clear results due to a high level of background staining obscuring visualisation of the biotinylated antibody conjugate (not shown).

To investigate the cause for this high level of background staining a Western blot analysis of the lysate of Raji cells incubated with anti-CD19-biotinylated D(KLAKLAK)$_2$ was performed as in section 2.1.3.3 (Fig 4.9). Staining with streptavidin-HRP revealed a strongly reactive biotin containing protein which was constitutively present in both treated and untreated Raji cells. A much fainter band detected only in the treated cells which co-migrated with a band reactive with anti-mouse immunoglobulin-HRP represented the biotinylated anti-CD19 conjugate.

In view of these findings a different staining method was employed using FITC-labeled anti-mouse immunoglobulin to detect the antibody portion of the conjugate rather than the biotinlyated peptide moiety. In these experiments cells were also incubated with 10μl of Hoechst 33342 as per manufacturers instructions prior to confocal microscopy to allow visualisation of the cell nuclei. When viewed on the confocal microscope, control cells showed no green fluorescence (Fig.4.10 A) but cells incubated with the conjugate showed evidence of antibody internalisation into vesicle-like structures at 30 minutes (Fig. 4.10 B). These cells were then washed free of conjugate and incubated for a further 6 hours, at which point cellular labeling was substantially reduced, compatible with degradation of the immunoglobulin moiety of the conjugate (Fig 4.10C).

Further evidence of anti CD19-conjugate internalisation was obtained by a flow cytometric analysis of Raji cells incubated with anti-CD19 D(KLAKLAK)$_2$ and stained with FITC-labeled anti-mouse immunoglobulin (Fig. 4.11).. Cells were left untreated (control) or were incubated with anti-CD19 conjugate for 30 minutes at 37°C after which cells were washed in Hanks saline and resuspended in culture medium. Aliquots were taken for analysis at 0,1,3 and 6 hours. At each time point cells were analysed without permeabilisation (Fig 4.12 A) or following permeabilisation and fixation with IntraStain (DAKO, Glostrup, Norway)(Fig 4.11 B). Analysis of intact Raji cells incubated with the conjugate for 30 minutes showed cell surface labeling which persisted for one hour after the initiation of a "chase" period with medium alone. However, cell surface labeling was not detectable at 3 or 6 hours. Parallel analysis of permeabilised and fixed cells also provided evidence of cell-associated anti-CD19 conjugate, which declined slowly over a 6 hour "chase" period. The presence of anti-CD19 reactivity in permeabilised cells at 3 and 6 hours, at which time cell surface labeling was undetectable strongly suggested internalisation of the conjugate.

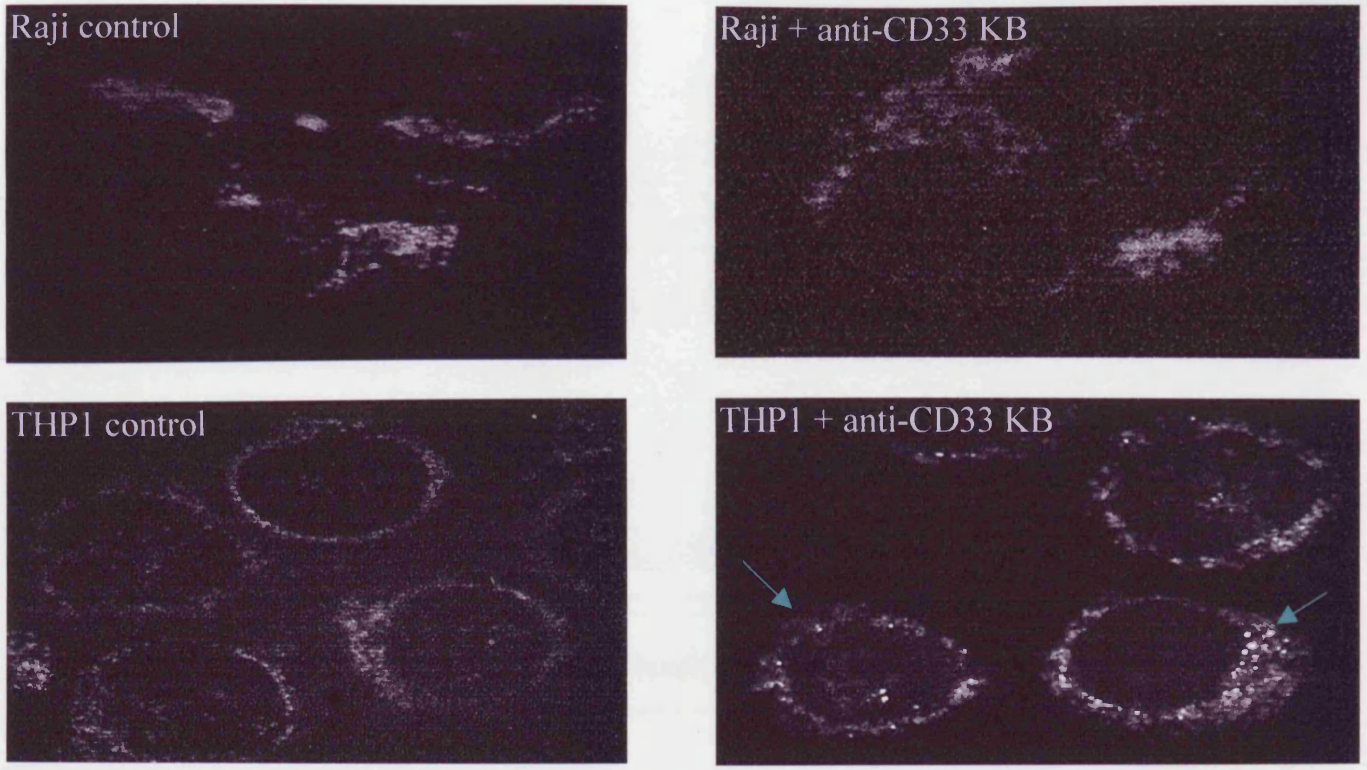


Fig 4.8 Internalisation of anti-CD33D(KLAKLAK)$_2$ by THP1 cells

Confocal microscopy of CD33 positive (THP1) and CD33 negative (Raji) cells treated with anti-CD33 D(KLAKLAK)$_2$biotin (KB)

Conjugate internalisation into intracellular vesicular like structures indicated by green arrow.

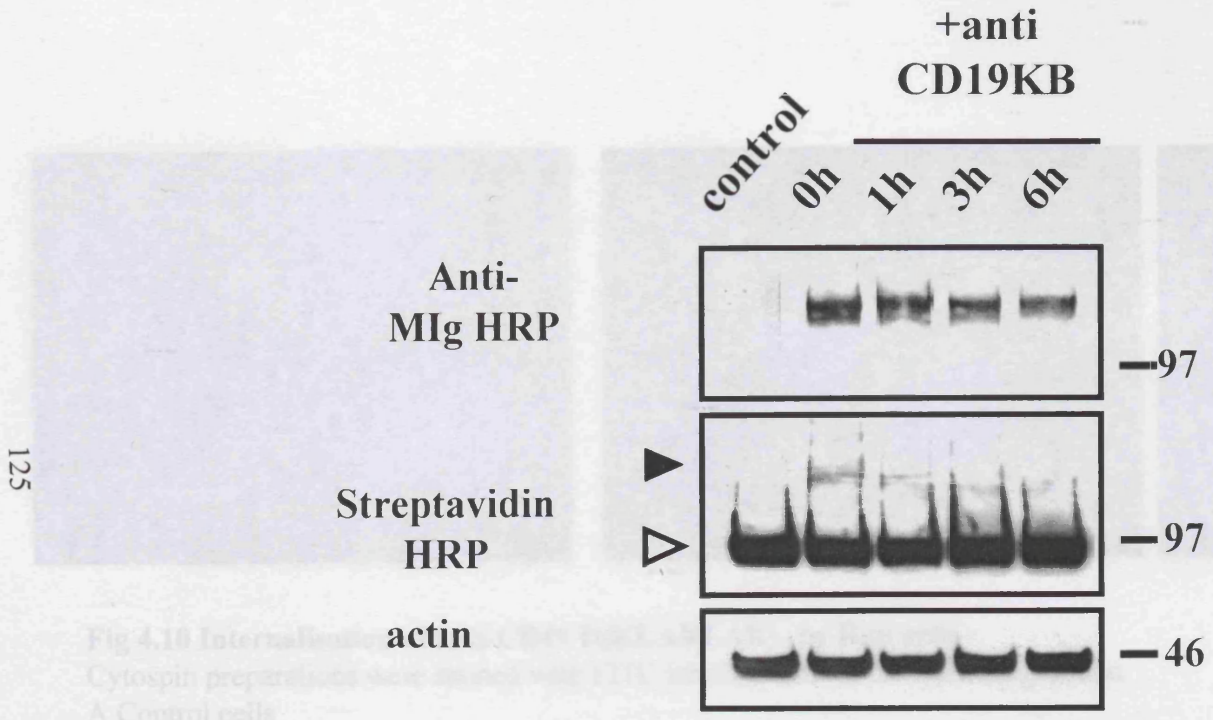


Fig 4.9 Western blots analysis of the lysate of Raji cells treated with anti-CD19 biotinylated D(KLAKLAK)$_2$
Staining with steptavidin- HRP reveals two bands:a stongly reactive biotin containing protein constitutively present in Raji cells (open arrow) and the biotinylated peptide moiety of the anti-CD19 conjugate (closed arrow).
KB biotinylated D(KLAKLAK)$_2$

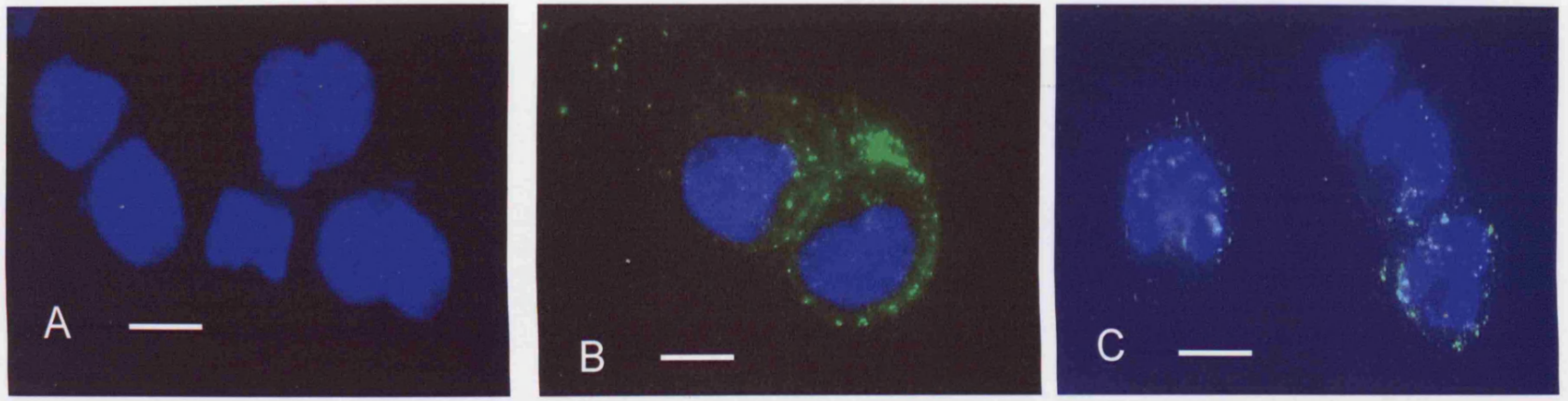


Fig 4.10 Internalisation of anti-CD19 D(KLAKLAK)$_2$ by Raji cells
Cytospin preparations were stained with FITC labelled anti-mouse immunoglobulin
A Control cells
B Cells incubated with anti-CD19 D(KLAKLAK)$_2$ for 30 minutes.
C Cells incubated as in B then washed and cultured in medium alone for an additional 6 hours
Bar = 5μm

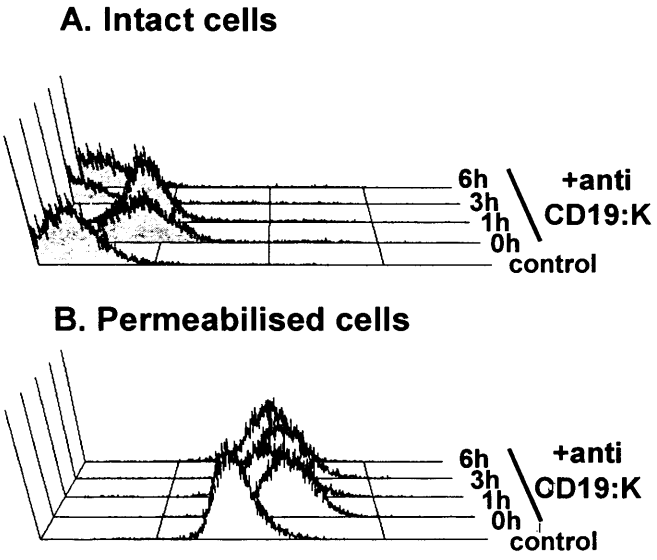


Fig 4.11 Flow cytometric analysis of Raji cells treated with anti-CD19 D(KLAKLAK)$_2$

Raji cells were left untreated (control) or treated with anti-CD19 D(KLAKLAK)$_2$ for 30 minutes at 37°C. Cells were washed with Hank's saline and resuspended in culture medium. Aliquots were taken for flow cytometric analysis immediately (0 hour) or following incubation for 1, 3, or 6 hours. Cells were stained with FITC anti-mouse immunoglobulin either directly (A) or following permabilisation and fixation with Intrastain.(B).

Results are typical of two experiments.

4.2.2.2 D(KLAKLAK)$_2$ conjugates induce apoptosis in cells which express the relevant antigen

Raji and Jurkat cells were incubated with 10nM anti-CD19-D (KLAKLAK) $_2$ for 48 hours. Cytospin preparations were then made of these cells along with untreated controls. Slides were stained with May Grümwald Giemsa (MGG) and viewed under the light microscope (Fig 4.12). For each slide, the number of cells with apoptotic morphology as characterised by cell shrinkage, nuclear condensation or the presence of apoptotic bodies were counted in three separate fields (Fig 4.13). Mitotic cells were also counted in the same way. Statistical evaluation was by Student's t test.

Morphological evidence of apoptotic cell death was seen in all slides at background levels of between 2-12%. The slides of the CD19 positive Raji cells showed a significant increase in the mean percentage of apoptotic cells per field in the treated vs. the untreated controls (27.3%, vs. 3.7%; p=0.04). In the CD19 negative Jurkat cells, the difference was not significant (10% vs. 8%; p=0.64).

The percentage of mitotic cells per field was also counted. The untreated Raji cells showed a background level of 2.1% mitotic cells. In contrast, no mitotic figures were seen in the slide of the treated cells. For the Jurkat cells the difference between the treated cells and the controls was insignificant (2.1% vs. 1.2%; p=0.62).

Further evidence for an apoptotic mode of cell death was provided by western blot analysis of the lysate of Raji cells incubated with anti-CD19 D(KLAKLAK)$_2$ for three hours and probed with antibodies specific for the p116 and p85 fragment of PARP. The p85 fragment is generated from the larger p116 fragment by the action of the apoptotic protease caspase 3[223]. PARP cleavage was seen in cells incubated with conjugate for 3 hours but was not evident at 1 hour or in untreated control cells.(Fig 4.14).

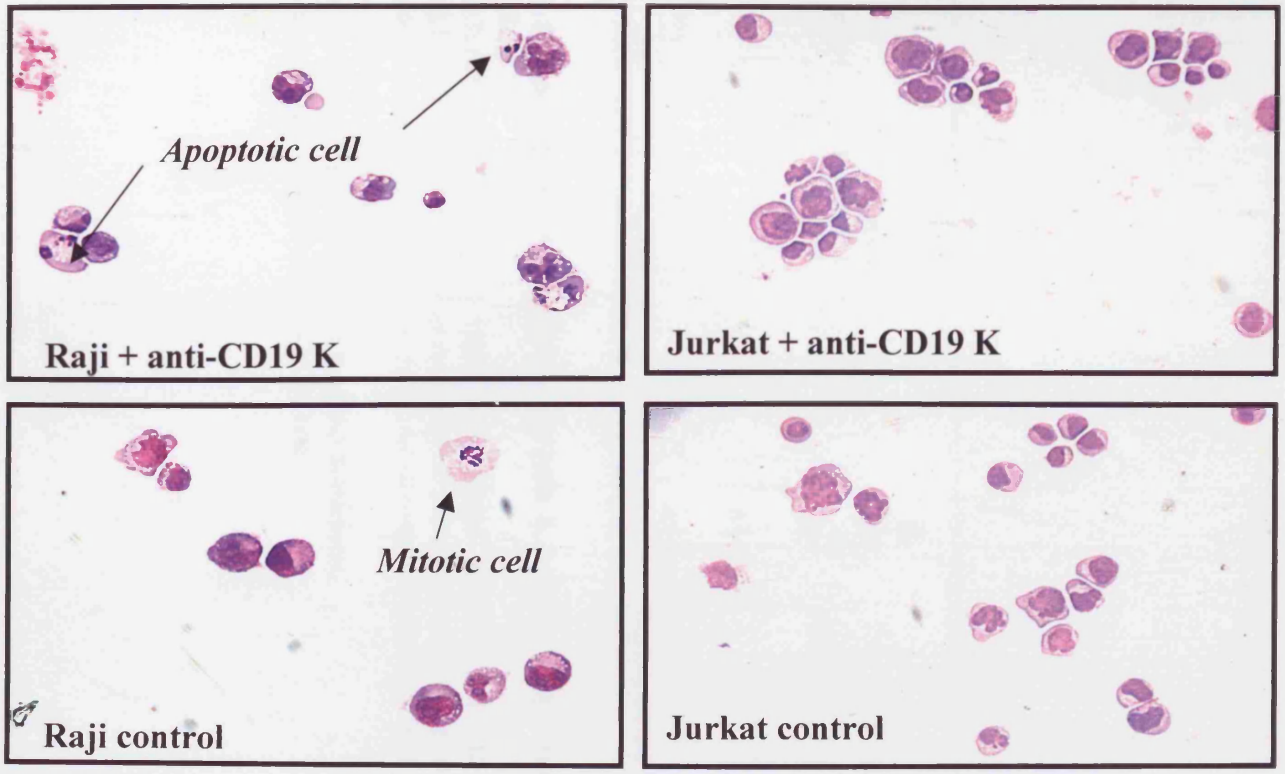


Fig.4.12 Morphology of cell lines incubated with anti-CD19 D(KLAKLAK)$_2$
Cell were incubated in the presence or absence of anti-CD19 D(KLAKLAK)$_2$ for 48 hours Cytospin preparations were stained with MGG and representative fields were photographed.

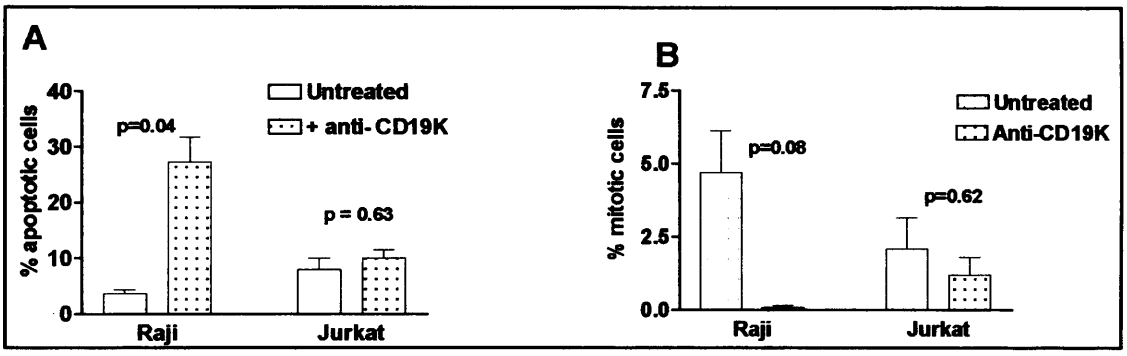


Fig 4.13 Percentage of cells on cytospins with A, apoptotic or B,mitotic morphology.

Raji and Jurkat cells were either untreated (control) or incubated with 10nM anti-CD19 D(KLAKLAK)$_2$ for 48 hours. Cytospin preparations were made and stained with MGG. The number of cells with either apoptotic or mitotic morphology was counted in three separate fields at low power.

Error bars represent SEM of triplicate determinations.

Statistical evaluation was by Student's t test.

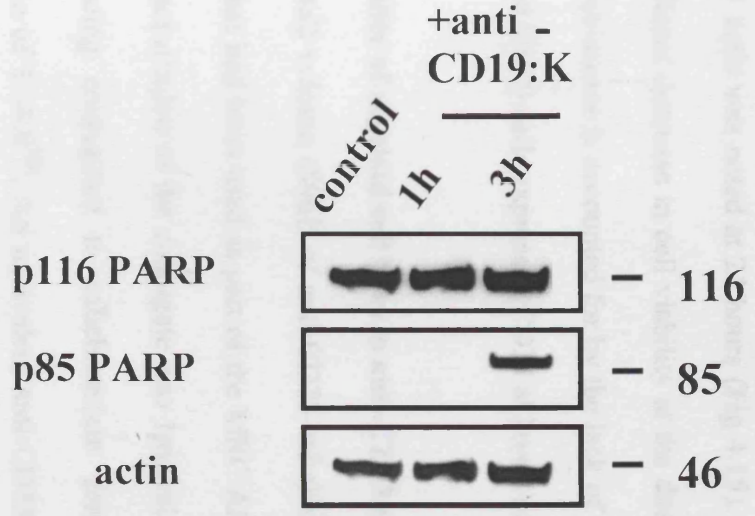


Fig 4.14 Western blot analysis of lysates of Raji cells treated with anti-CD19D(KLAKLAK)$_2$
Raji cells were incubated in the prescence or absence of anti-CD19 D(KLAKLAK)$_2$ for 1 or 3 hours. Lysates were analysed by Western blotting. Results are typical of four experiments.

4.2.3 Some CD33 positive cell lines are resistant to anti-CD33-D(KLAKLAK)$_2$ but are susceptible to other CD33 immunoconjugates

4.2.3.1 Resistance of myeloid cell lines to anti-CD33 D(KLAKLAK)$_2$

Four myeloid cell lines, (THP1, HL60 , U937 and K562) were incubated with increasing doses of anti-CD33-D(KLAKLAK)$_2$. CD33 expression was first assessed by immunophenotyping by flow cytometry (upper panel Fig 4.15). Sample for MTT assays were taken at 24, 48 and 72 hours. As before the maximal decrease in cell viability for THP1 cells was noted at 24 hours (Fig 4.15). None of the other cell lines showed any significant decrease in cell viability at the doses of antibody used. In the case of K562 the resistance is accounted for by the lack of surface expression of CD33. However U937 and HL60 cells expressed CD33 at levels comparable to THP1.

4.2.3.2 Susceptibility of myeloid cell lines to anti-CD33 calicheamicin

We obtained a small volume (50µl) of anti-CD33-calicheamicin. This was the excess drug from a vial that had been used as part of the MRC AML 15 trial[72]. The data sheet stated that the concentration of the conjugate was 1mg/ml with approximately 50% of the anti-CD33 being conjugated to calicheamicin derivatives with an antibody: calicheamicin ratio of 1: 4-6[224]. An equivalent anti-CD33-D(KLAKLAK)$_2$ conjugate was selected for comparison, with a concentration (estimated by biorad assay) of 1mg/ml and an antibody to peptide ratio of 1:6. Concentrations of anti-CD33-calicheamicin that had been used in published in-vitro cell line experiments[225] were noted to be in the same range as the concentration we had previously established for the use of anti-CD33-D(KLAKLAK)$_2$, with the majority of the molecular weight of both compounds being made up by the antibody portion. Two CD33 positive cell lines (HL60 and THP1) were incubated with either anti-CD33-calicheamicin or anti-CD33-

$D(KLAKLAK)_2$ at equivalent concentrations and samples for MTT assay were analysed at 24,48,72 and 96 hours (Fig.4.16). The results showed that THP1 cells were susceptible to the cytotoxic action of both agents, with maximal decrease in cell viability occurring by 24 hours with the anti-CD33-$D(KLAKLAK)_2$ conjugate and at 96 hours with anti-CD33-calicheamicin. HL60 cells showed a similar pattern of maximal decrease in cell viability at 96 hours with the anti-CD33-calicheamicin but were completely resistant to the cytotoxic effect of anti-CD33-$D(KLAKLAK)_2$.

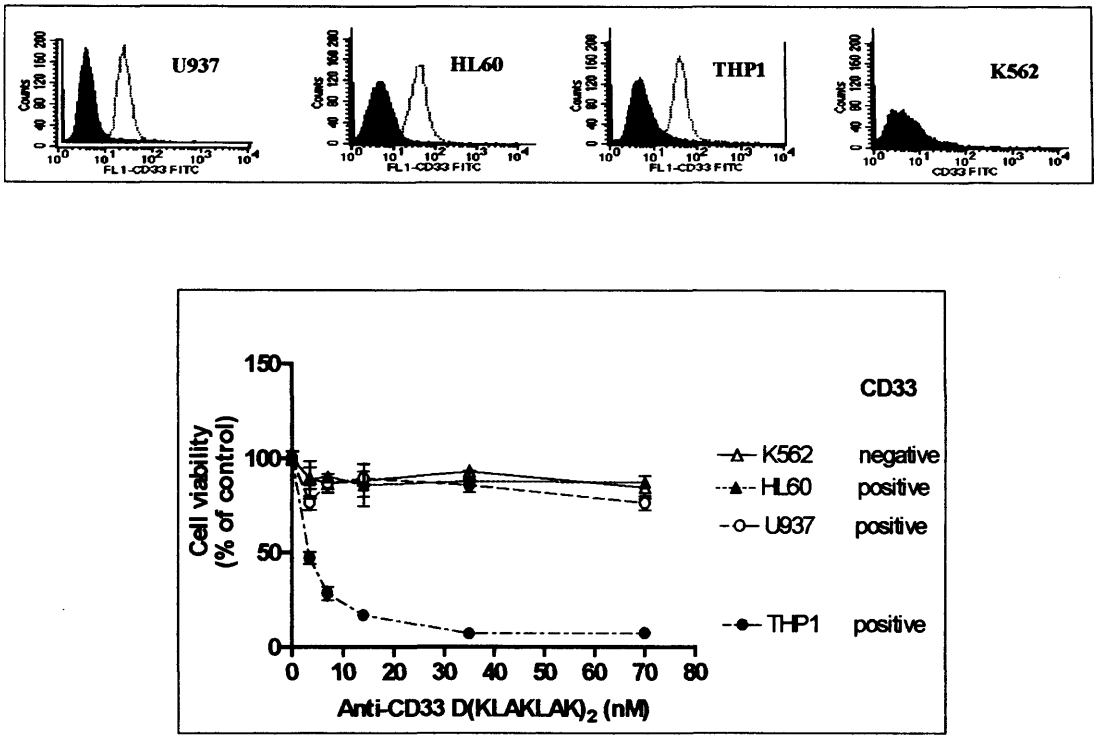


Fig 4.15 action of anti-CD33 D(KLAKLAK)$_2$ on four myeloid cell lines
Upper panel
FACScan analysis for the expression of CD33 by four myeloid cell lines
Lower panel
The same cell lines were incubated with increasing concentrations of anti-CD33 D(KLAKLAK)$_2$. Cell viability was assessed by MTT assay at 24 hours.
Results are representative of three independent experiments.
Error bars represent SEM of duplicate determinations.

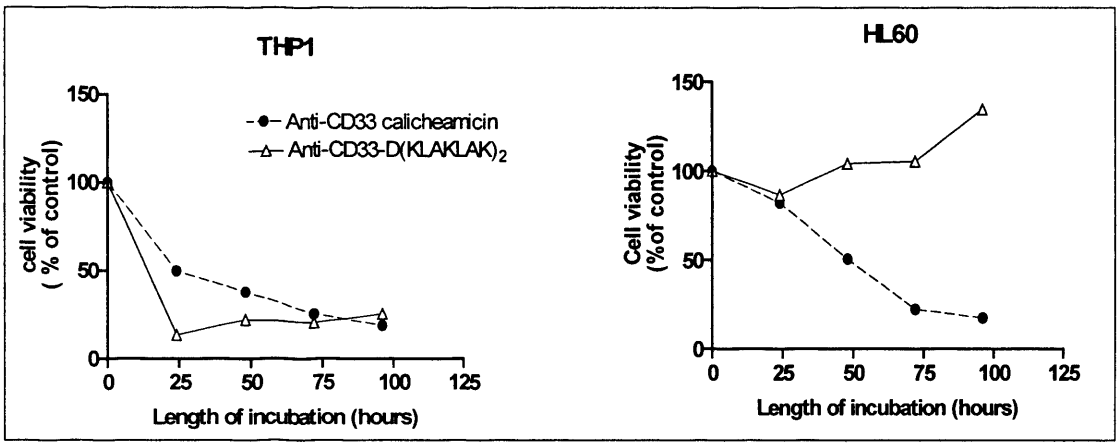


Fig 4.16 Action of anti-CD33-calicheamycin and anti-CD33-D(KLAKLAK)$_2$ on THP1 and HL60 cells.

THP1 and HL60 cells were incubated with equivalent concentrations of either anti-CD33 D(KLAKLAK)$_2$ or anti-CD33 calicheamicin. MTT assays were performed at 24,48,72 and 96 hours.

Only one experiment was performed with single MTT assays at each time point due to the limited availability of the anti-CD33 calicheamicin.

4.3 Discussion

We have demonstrated that the antibody-D(KLAKLAK)$_2$ conjugates synthesised as described in Chapter 3 have selective cytotoxic actions, at nanomolar concentrations, on many (but not all) of the cell lines tested, that express the relevant antigens. All cell lines were susceptible to the cyotoxic actions of the unconjugated peptide alone, but only at micromolar concentrations i.e. approximately one thousand times the concentration required with the targeted peptide. None of the cell lines showed any decrease in cell viability following incubation with unconjugated antibody in the concentration ranges used.

Internalisation of the antibody conjugates into cells expressing the relevant antigen was demonstrated by flow cytometry and by confocal microscopy using either FITC-streptavidin to label the biotinylated peptide portion of the conjugate or FITC- anti-mouse immunoglobulin to label the antibody moiety.

Apoptotic morphology was noted on cytospin preparations of conjugate treated Raji cells, as well as a decrease in the mitotic index. Unconjugated anti-CD19 and anti-CD19-immunotoxins have also been noted to cause cell cycle arrest in in-vitro experiments with CD19 positive cell lines[226]. Further evidence of apoptosis induction was obtained by demonstrating the caspase-mediated cleavage of poly(ADP-ribose) polymerase in anti-CD19 D(KLAKLAK)$_2$ treated cells.

The principal measure of cell viability used in these experiments was the MTT assay. This assay is based on the ability of metabolically active cells to reduce a tetrazolium compound to a blue formazan product. As such it is specifically a test of mitochondrial oxidative phosphorylation. The clonogenic assay that measures the proliferative potential of cells and their ability to form colonies is a truer measure of cell viability and is generally regarded as the "gold standard" assay[227]. However clonogenic assays

are much more time consuming than MTT assays and require using larger amounts of drug. The correlation between MTT and clonogenic assay results vary depending on the drug being tested[228]. In the case of our antibody-D(KLAKLAK)$_2$ conjugates, we have demonstrated that both tests yield comparable results.

All of the CD19 positive cell lines tested were sensitive to anti-CD19D(KLAKLAK)$_2$. In contrast only one out of three of the CD33 positive cell lines tested (THP1) showed a significant decrease in cell viability after incubation with anti-CD33-peptide conjugate. Nevertheless we were able to demonstrate peptide internalisation into HL60 cells as well as THP1 cells, indicating that differences in sensitivity may reflect different molecular responses to the peptide by the two different cell lines, rather than a differential ability to internalise anti-CD33. We found that both HL60 and THP1 cells were sensitive to anti-CD33-calicheamicin, again implying that the resistance of HL60 cells to anti-CD33-D(KLAKLAK)$_2$ was not explained by an inability to internalise the conjugate. Amico et al also described decreases in cell viability of these two cell lines in response to anti-CD33-calicheamicin as measured by thymidine uptake. They demonstrated that the drug induced G2 cell cycle arrest in both cell lines but that this was only followed by apoptosis in the HL60 cells. They also demonstrated that another myeloid cell line (KG1) cells were resistant to the cytotoxic action of this conjugate[229]. They found no correlation between these responses and the expression of CD33 or multiple drug resistance proteins by these cell lines, although KG1 cells were shown to have a high cyclosporine inhibitable efflux activity, possibly explaining the resistance of this cell line to the calicheamicin conjugate. They postulated that these findings go some way to explaining the heterogeneity of response to anti-CD33-calicheamicin observed in AML patients.

In summary we have show that internalisation of the amphipathic peptide $D(KLAKLAK)_2$ into malignant B lymphoid cell lines efficiently induces killing via an apoptotic mechanism. However the strategy was less successful against myeloid cell lines.

Summary

We have demonstrated that the antibody- $D(KLAKLAK)_2$ conjugates synthesised in the previous chapter have selective cytotoxic activity against haemopoeitic cell lines. An anti-CD19 conjugate effectively killed three out of three lymphoid cell lines, however an anti-CD33 conjugate was cytotoxic towards only one out of three myeloid cell lines. Cell viability was assessed by MTT and clonogenic assays with IC_{50} in the low nanomolar range. Internalisation of the antibody conjugate into cells expressing the relevant antigen was demonstrated by confocal microscopy and flow cytometric methods. An apoptotic mode of cell death was inferred from the characteristic morphology of treated cells viewed by direct microscopy and Western blot analysis of PARP cleavage.

Chapter 5

Cytotoxicity of D(KLAKLAK)$_2$ antibody conjugates towards mononuclear cells isolated from patients with chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML)

5.1 Introduction

5.1.1 B-CLL

B-CLL is the most common leukaemia in the western hemisphere and has a peak incidence between 60 and 80 years of age. The aetiology is unknown. It is a disease characterised by the accumulation of relatively mature B-cells in the blood, bone marrow, liver, spleen and lymph nodes as a result of prolonged lifespan and impairment of normal apoptosis in vivo[230]. The circulating cells are characteristically CD19, CD5 and CD23 positive with weak expression of surface immunoglobulin.

Poor prognostic factors in B-CLL include Binet stage (B, C) (table 5.1), male sex, rapid lymphocyte doubling time, dysfunction of the p53 pathway[231], high expression of anti-apoptotic proteins especially Bcl-2[232] , chromosomal abnormalities (such as 13q del) the presence of unmutated VH immunoglobulin genes[233,234] and the elevated expression of ZAP70 protein tyrosine kinase[235,236].

Stage	Clinical features at diagnosis
A	Lymphocytosis and <3 areas of palpable lymphoid tissue enlargement.
B	Lymphocytosis and 3 or more areas of palpable lymphoid tissue enlargement.
C	As B but with anaemia or thrombocytopenia

Table 5.1 Binet Clinical Staging System

Although Bcl-2 gene rearrangements are rare in B-CLL, more than 85% of B-CLL express high levels of Bcl-2 protein[232], possibly associated with hypomethylation of BCl-2 gene[237]. In addition, elevated levels of Mcl-1 protien expression occurs in nearly half of CLL B-cells[238].However in vitro, B-CLL cells undergo spontaneous apoptosis which suggests that in B-CLL cells the apoptotic mechanism is influenced by interaction with the microenvironment[239] in vivo. In-vitro data suggest that several cytokines such as IL-4 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[240,241] .

B-cell VH genes undergo somatic hypermutation in germinal centres of lymph nodes. In CLL the VH genes are hypermutated in approximately 60% of cases suggesting origin from the post germinal follicle centre cells. These patients are more likely to be in stage A with stable disease and typical morphology with median survival of 25 years, whereas patients with unmutated VH genes are more likely to have an advanced stage, atypical morphology, progressive disease, and a poorer prognosis with a median survival of about 8 years[233,234]. Studies have shown a strong correlation between the presence of unmutated VH genes and elevated expression of ZAP70 protein kinase[235,236]. Malignant cells isolated from this group of poor risk patients are characteristically defective in the induction of the p53 dependent pathway in response to DNA damage, either due to mutation or deletion of the p53 genes themselves or to defects in the ATM protein kinase, which is an upstream mediator of this pro-apoptotic pathway[242,243].

Both defects in the p53 pathway and elevated expression of anti-apoptotic proteins (Bcl-2, Mcl-1 and XIAP) are thought to contribute to resistance of CLL cells to both conventional cytotoxic drugs[238,244-247] or to Rituximab[248].

CLL is not curable by conventional cytotoxic chemotherapy[230]. While cure may be achieved by allogeneic stem cell transplantation, this procedure is associated with a high rate of transplant related morbidity and mortality[249] and is usually reserved for younger patients. Standard therapy for CLL includes conservative management and drug therapy with alkylating agents, purine analogs, corticosteroids or combinations of these drugs. Therapy with unconjugated monoclonal antibodies such as Campath 1H (anti-CD52) and Rituximab (anti-CD20) also produce responses in a proportion of patients[250,251].

5.1.2 AML

Acute myeloid leukaemia is a clonal disorder of haemopoeitic tissue characterised by proliferation of abnormal blasts in the bone marrow and impaired production of normal blood cells, resulting in anaemia and thrombocytopenia and in some cases tissue infiltration with tumour cells. AML occurs in all ages and is the commonest form of acute leukaemia in adults; however it forms only a minor fraction (10 – 15%) of childhood leukaemias. Established causative agents in AML include high dose radiation, chronic benzene exposure and treatment with alkylating agents and other chemotherapeutic drugs. AML may also develop from myelodysplasia, chronic myeloproliferative disorders and other haematological diseases. Classification of AML is based on the World Health Organisation histological classification of AML[252] which incorporates and interrelates morphology, cytogenetics, molecular genetics and immunological markers. This has supplanted the still widely used French American

British (FAB) scheme in which the emphasis was more on morphological and immunophenotypic characteristics which were used to classify AML into eight variants (M0-M7). For the purposes of decription in this chapter the FAB scheme has been used. The typical AML immunophenotype is CD13+, CD33+ and TdT negative with additional antibodies being useful for the diagnosis of specific FAB subtypes (M0, M6 and M7). Cytogenetic abnormalities are present in about half of patients at presentation and have a major influence on prognosis. 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.

Treatment for AML includes supportive therapy and intensive chemotherapy with agents such as cytosine arabinoside, daunorubicin, idarubicin, 6-thioguanine, mitoxantrone or etoposide. Allogeneic stem cell transplantation is considered in younger patients with poor risk disease and is generally performed in first remission.

The place of targeted monoclonal antibody therapy with anti-CD33-calicheamicin is currently being investigated as part of the AML 15 trial[72], and may prove to be especially useful for patients over the age of 60 years old, who form a group who are particularly difficult to manage due to both primary disease resistance and poor toleration of intensive chemotherapy. 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) [354]. P-gp, which is a multidrug resistance protein and a product of the *mdr1* gene (multidrug resistance-1), has been shown to be involved in AML resistance against spontaneous and drug induced apoptosis.

5.2 Results

5.2.1 Assessment of cytotoxicity of anti-CD19-D(KLAKLAK)$_2$ against cells isolated from patients with CLL

CLL was diagnosed by established clinical criteria and confirmed by immunocytochemical analysis for the expression of CD5, CD19 and restricted cell-surface immunoglobulin.[253]

Mononuclear cells were isolated from the peripheral blood of 20 patients with CLL by Ficoll density gradient (see chapter 2 section 2.2.2). Three of these patients donated samples on more than one occasion. The characteristics of the patients are summarised in table 5.2. Of the 18 patients on whom ZAP70 expression data were available, 5 were classified as being in the poor risk category as defined by elevated ZAP expression[211,235,236]. For 12 of these patients, prognosis was additionally established by sequencing of immunoglobulin VH genes. Patients with less than 2% divergence from germline sequence were classified as poor risk[212,233,234].

5.2.1.1 Anti-CD19-D(KLAKLAK)$_2$ has cytotoxic activity against CD19 positive CLL cells as assessed by MTT assay

Cytotoxicity of anti-CD19-D(KLAKLAK)$_2$ (antibody: peptide ratio 1:6) was assessed by incubation of increasing concentrations of the conjugate with mononuclear cells isolated from the peripheral blood of 12 patients with CLL. Samples for MTT assay were taken at 24, 48 and 72 hours. Figure 5.1 illustrates the dose response curve of the action of anti-CD19 D(KLAKLAK)$_2$ on three representative CLL samples. All MTT assays were performed in duplicate. In each experiment a positive control of CD19 positive Raji cells and a negative control of CD19 negative Jurkat cells were used. As was noted in the previous chapter, the anti-CD19 conjugate had maximal effects after

48 hours incubation, with mean decreases in cell viability at 24 hours of 75.2% (range 40.3%-96.1%) and of 84.2% (range 40.3%- 97.5%) at 48 hours. At 72 hours incubation there was generally a high level of spontaneous cell death in the control wells making assessment of the cytotoxic action of the conjugate at this time difficult. As with the cell line experiments the IC_{50} values at 48 hours determined using cells from twelve patients were in the low nanomolar range with a mean value of 1.8nM (range 1.6 to 4.6 nM). The mean IC_{50} for the cells from four poor risk patients (2.3 ± 1.6 nM) was not significantly different from that for eight good prognosis patients (1.6 ± 0.8 nM; $p=0.36$; Student *t* test for unpaired samples).

Three of the patients donated samples of peripheral blood on more than one occasion, several months apart. This allowed us to assess the reproducibility of our assay over time. Figure 5.2 illustrates the dose response curve of the action of anti-CD19-$D(KLAKLAK)_2$ on cells isolated from the peripheral blood of a single patient ,taken six months apart. The patient had received no treatment during this time period. The two dose response curves are similar with both IC_{50}s (2.0 and 2.7nM) above the mean value for the sample population as a whole and at the upper end of the range.

Patient Number	Age/sex	Binet stage	Risk category	Treatment history
1	81/M	B	Poor*	chl
2	85/F	C	Good*	chl
3	73/M	B	Poor*	cyc
4	62/F	A	Good*	NA
5	63/F	C	Good*	Untreated
6	57/M	B	Good*	chl
7	98/M	A	Good*	Untreated
8	75/F	B	Good*	chl
9	43/M	C	Poor	chl
10	72/M	A	Good	chl
11	75/M	A	Good	Untreated
12	67/M	C	Poor*	chl
13	75/M	A	Good	Untreated
14	90/M	A	Good*	Untreated
15	77/M	A	Good	Untreated
16	69/M	NA	NA	chl
17	73/F	C	Good*	Untreated
18	72/M	A	NA	chl
19	49/M	A	NA	Untreated
20	67/M	C	Poor*	chl
21	62/F	NA	Good*	NA
22	74/M	A	NA	Untreated
23	82/M	A	NA	Untreated

Table 5.2 Clinical characteristics of CLL patients.
Risk categories were assigned on the basis of FACScan determination of ZAP70 expression. Asterisks denote that the risk category was additionally confirmed by immunoglobulin heavy chain sequencing. Previous treatment with chlorambucil (chl) or cyclophosphamide (cyc) is indicated. NA, data not available.
Patient number 10 and 18, 11 and 13, 12 and 20 samples come from the same patient taken several months apart.

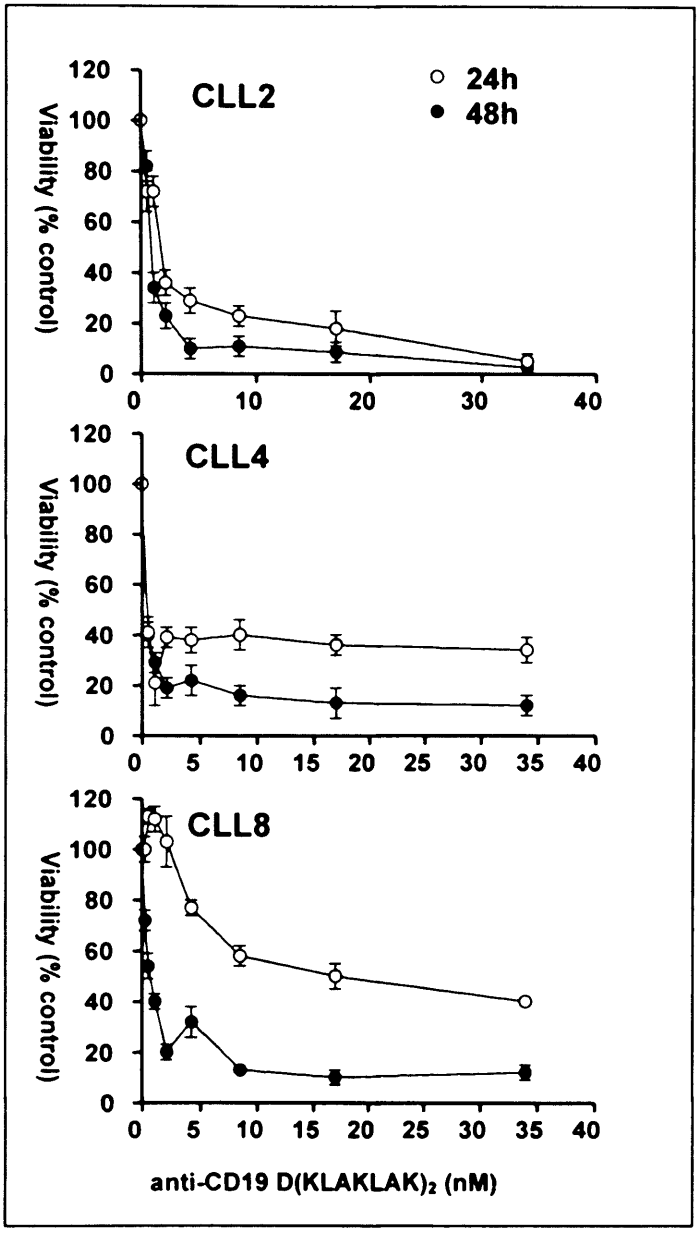


Fig 5.1 Cytotoxicity of anti-CD19 D(KLAKLAK)$_2$ towards CLL cells
Dose response curves for the action of anti-CD19 D(KLAKLAK)$_2$ on three representative CLL cell isolates.
Cell viability was determined by MTT assay following 24 and 48h incubation.
Error bars represent SEM of duplicate determinations.

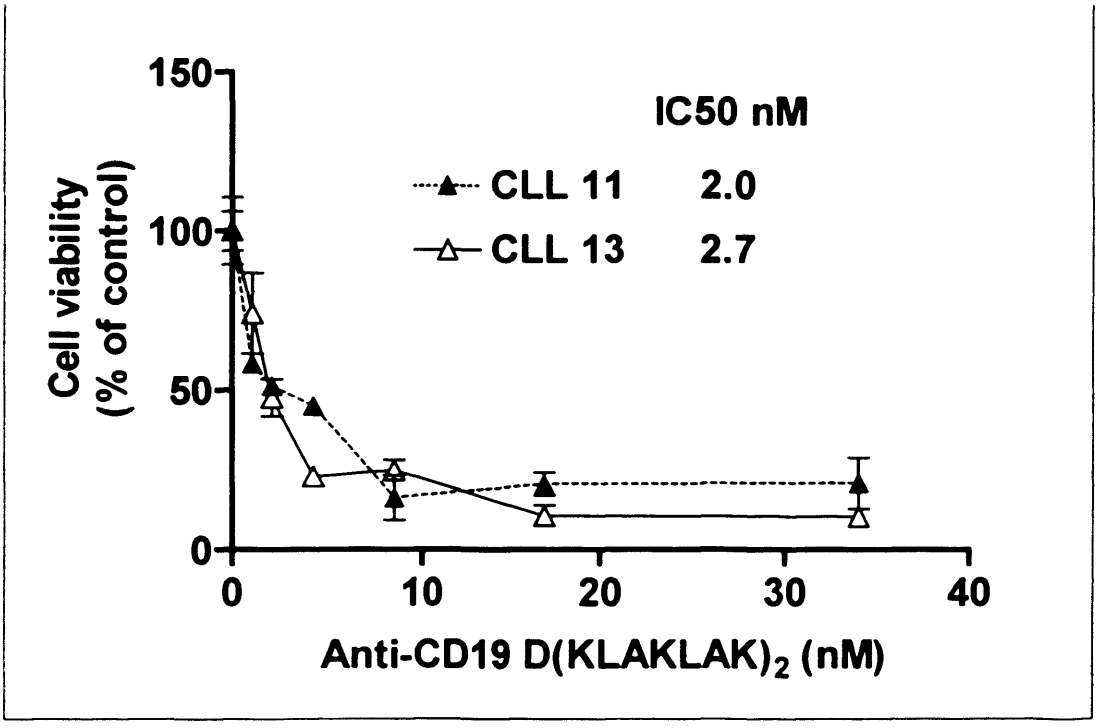


Fig 5.2 Action of anti-CD19 D(KLAKLAK)2 on cells isolated from a single CLL patient, taken 6 months apart

Dose response curves for the action of anti-CD19 $D(KLAKLAK)_2$ on cells isolated from a single CLL patient, taken 6 months apart. MTT assay at 48 hours.

Error bars represent SEM of duplicate determinations

5.2.1.2 CD2 positive T-cells are resistant to the cytotoxic action of anti-CD19-D(KLAKLAK)$_2$

The percentage of CD2 positive cells in the mononuclear preparation was assessed by flow cytometry prior to incubation with the cytotoxic agents. The range of CD2 positive cells was wide, ranging from 0.6% to 38.7%.

A strong positive correlation ($p=0.0004$) was noted between the percentage of cells that were resistant to the effects of the anti-CD19 conjugate and the percentage of cells that were CD2 positive (Fig.5.3), suggesting that the anti-CD19 conjugate had specific cytotoxic action on the CD19 positive B-cells in the samples and the CD19 negative, CD2 positive T-cells in the mononuclear preparation remained unaffected.

Further evidence for the sparing of CD2 positive T-cells came from flow cytometric analysis of CD2 count on a single patient, both pre and post treatment with anti-CD19-D(KLAKLAK)$_2$ (Fig 5.4) Pre-treatment flow cytometric analysis of mononuclear cells showed 92.4% of cells in the live cell gate defined by forward and side scatter as described[254] . Of these live cells 6.2% were CD2 positive. Following 24 hours incubation with 5nM anti-CD19-D(KLAKLAK)$_2$, a decrease in cell viability was noted , with only 48.1% of cells in the live cell gate. CD2-positive cells accounted for 14.8% of events within this gate, consistent with the resistance of T lymphocytes to the conjugate. The low yield of cells following 48h incubation with the conjugate precluded FACScan analysis at this later time point.

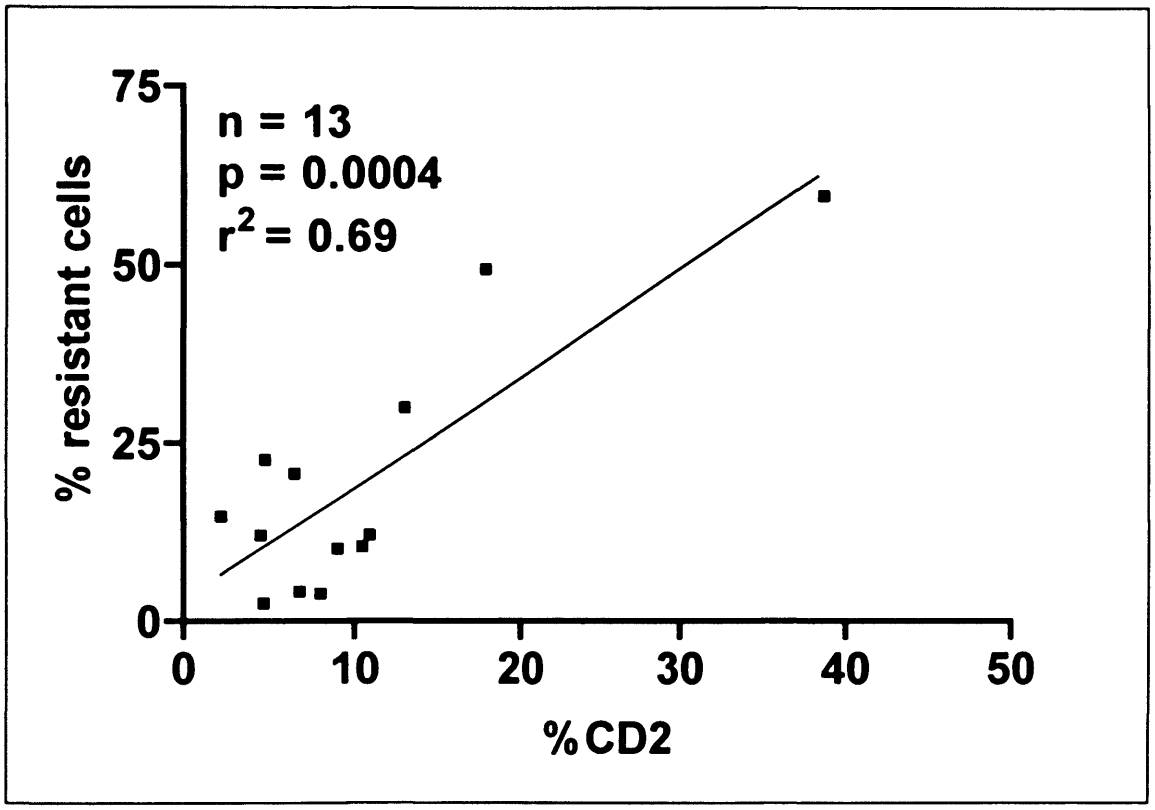


Fig 5.3 Relationship between anti-CD19 D(KLAKLAK)$_2$ resistance and CD2 count.
Correlation between percentage of cells isolated from a patient with CLL that are resistant to the cytotoxic effect of anti-CD19 D(KLAKLAK)$_2$ and the CD2 count as estimated by flow cytometry on the same samples prior to treatment.
Pearsons parametric test was used for statistical analysis since the data was normally distributed.

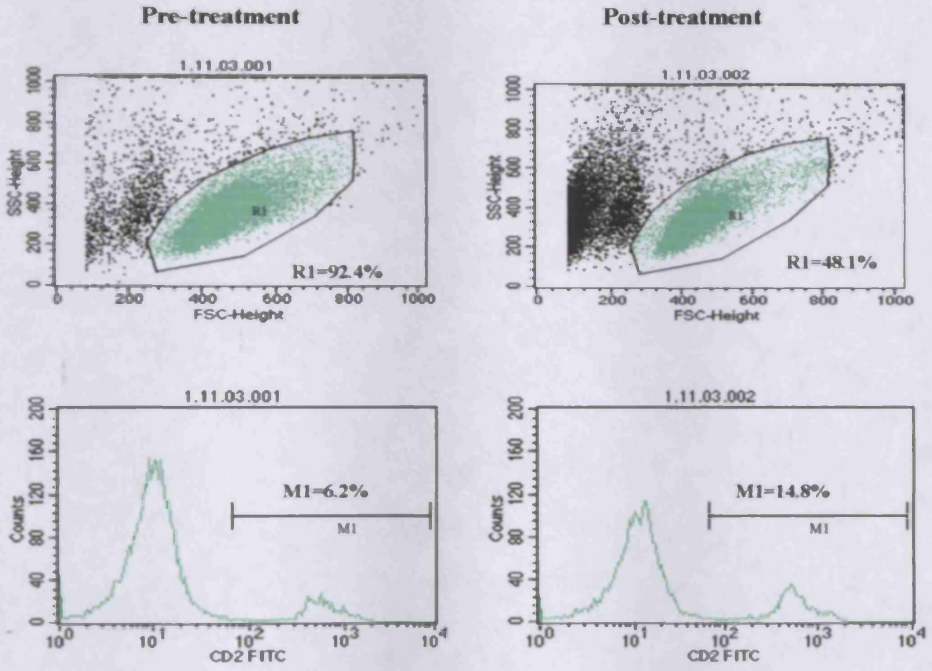


Fig 5.4 Enrichment of CD2-positive T lymphocytes in the anti-CD19 D(KLAKLAK)$_2$ -resistant population.

A CLL isolate was incubated with 5 nM anti-CD19 D(KLAKLAK)$_2$ for 24h. Untreated and treated cell populations were subjected to FACScan analysis. The percentage of cells within the live gate (R1) that were reactive with FITC-labelled anti-CD2 was then quantified M1.

5.2.1.3 Cytotoxicity of unconjugated anti-CD19

Mononuclear cells isolated from the peripheral blood of patients CLL1 and CLL2 were incubated with unconjugated anti-CD19 in the same dose range as was used in the initial experiment with the conjugated peptide (section 5.2.1.1). Samples for MTT assay were taken at 48 hours (Fig. 5.5A). A high concentration of the antibody (30 nM) resulted in approximately 30% loss of viability. The IC_{50}s of the conjugate towards these isolates were 1.6 and 2.0 nM respectively. These levels of the antibody alone resulted in no killing of the CLL2 isolate and 11% loss of viability of CLL1.

5.2.1.4 Cytotoxicity of uncongugated peptide

The same mononuclear CLL samples were incubated with increasing concentrations of unconjugated $D(KLAKLAK)_2$. Samples for MTT assay were taken at 48 hours (Fig. 5.5B). As was noted in the cell line experiments (section 4.2.1.5), both samples were sensitive to the peptide, with IC_{50}s in the micromolar range. In contrast to the cell lines, which all showed 100% decreases in cell viability when incubated with the peptide, a percentage of CLL cells (8.8-23.6 %.) remained resistant to the cytotoxic effect of the peptide. Although the sample size was only two, there was no apparent correlation between the percentage of resistant cells and the CD2 count as was noted with the anti-CD19 conjugated peptide.

5.2.1.5 Cyotoxicity of anti-CD33-$D(KLAKLAK)_2$ conjuagte

The same mononuclear CLL samples as were also incubated with increasing concentrations of anti-CD33-D $(KLAKLAK)_2$. As before samples for MTT assay were taken at 48 hours (Fig.5.5C). No decreases in cell viability were noted at the concentrations of anti-CD33 conjugate used.

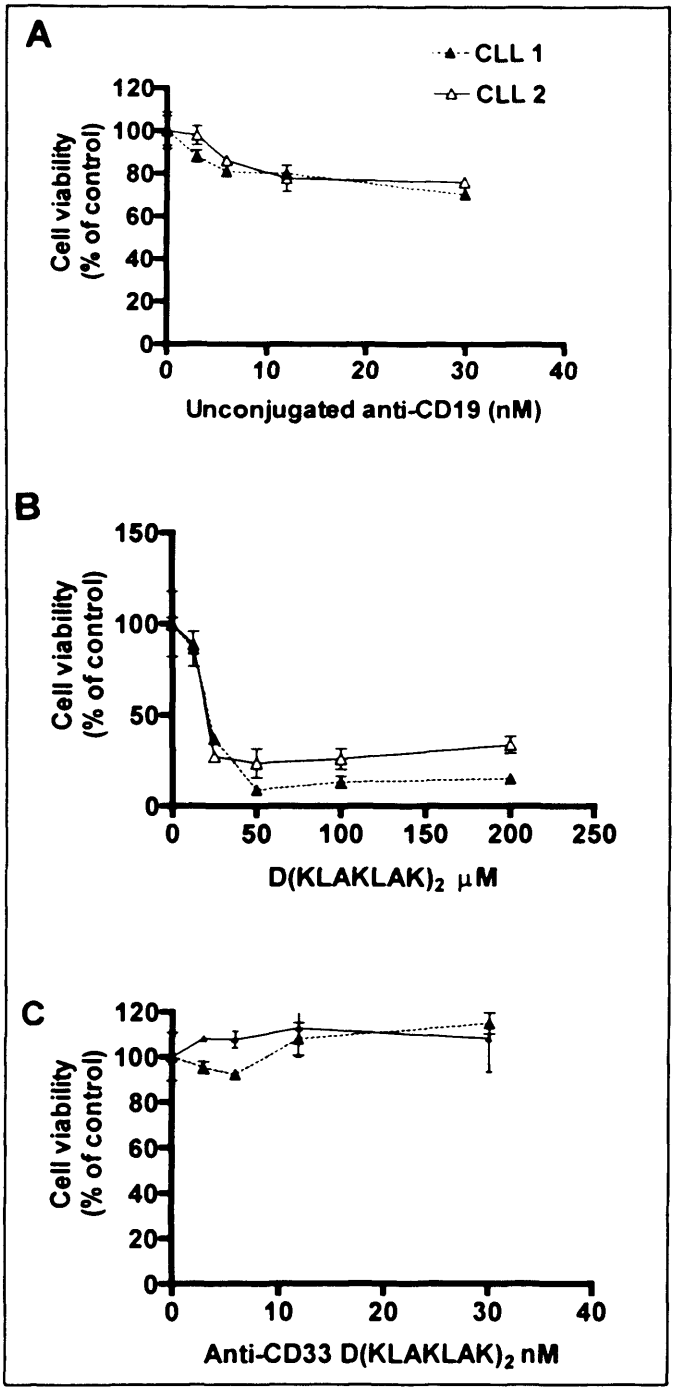


Fig 5.5 Actions of unconjugated anti-CD19 (A), unconjugated D-(KLAKLAK)$_2$ (B) and (C) anti-CD33-D-(KLAKLAK)2 on cells isolated from two CLL patients
Cells isolated from two patients with CLL were incubated with increasing concentrations of unconjugated anti-CD19 (A), unconjugated D-(KLAKLAK)$_2$ (B) and (C) anti-CD33-D-(KLAKLAK)$_2$.
Cell viability was determined by MTT assay following 48h incubation.
Error bars represent SEM of duplicate determinations.

5.2.1.6 Killing of CLL cells by anti-CD19 D(KLAKLAK)$_2$ is via an apoptotic mechanism.

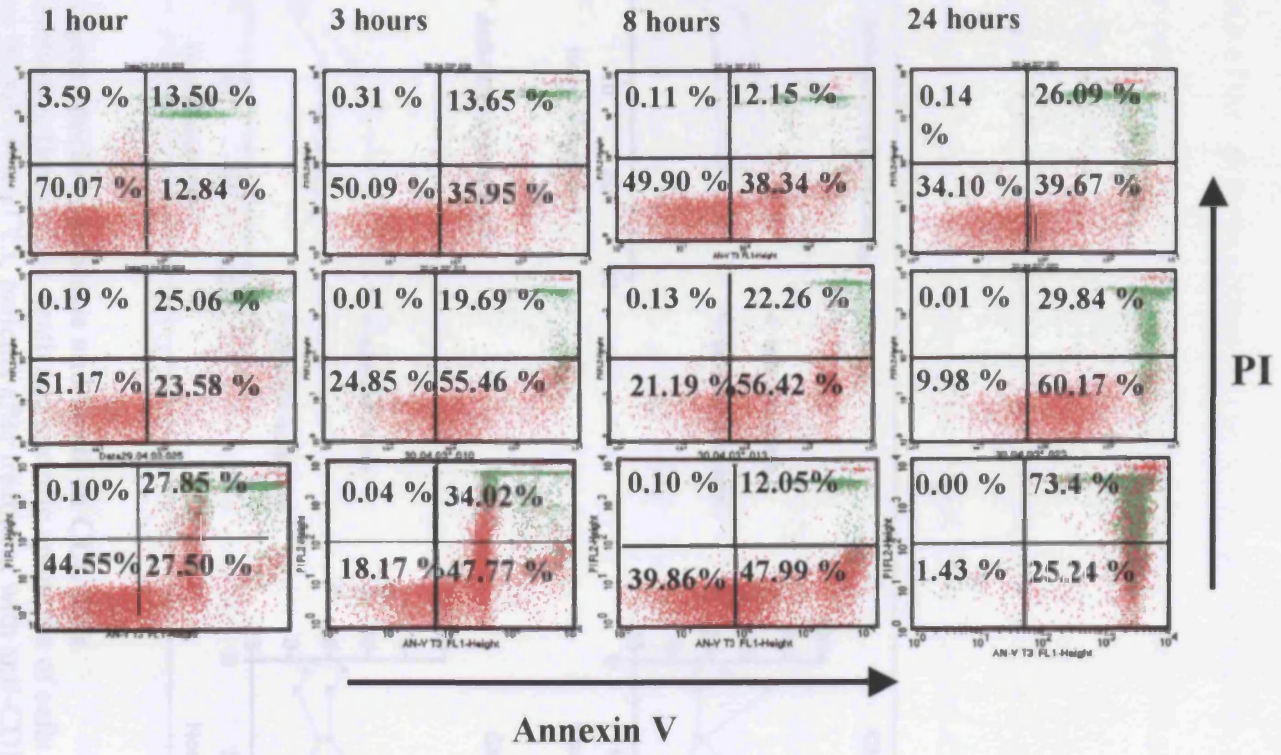
Apoptotic cell death is associated with the induced expression of cell-surface signals which mediate recognition and engulfment of the dying cell by phagocytes. These signals include the externalization of phosphatidylserine[156]. Since engulfment of apoptotic cells circumvents the release of cellular contents and consequent inflammatory reactions, it was important to demonstrate that anti-CD19 D(KLAKLAK)$_2$- induced killing of CLL cells was by an apoptotic mechanism.

Mononuclear cells isolated from the peripheral blood of two CLL patients were treated with either nothing (negative control), 5nM anti-CD19-D(KLAKLAK)$_2$ or 20μg/ml chlorambucil (positive control). At various time points samples were stained with annexin V and propidium iodide and analysed by flow cytometry. The flow cytometry plots for one patient (CLL 17) are illustrated in Fig 5.6. The control samples shown in the left hand panel of this figure illustrate the levels of background spontaneous apoptosis occurring in the untreated CLL cells, with a baseline level of live cells (annexin v/PI negative) of 70% at 1 hour decreasing to only 34% viable cells at 24 hours. The treated cells (both anti-CD19-conjugate and chlorambucil) showed decreased levels of viable cells and increased levels of both apoptotic cells (annexin V positive,PI negative) and secondary necrotic cells (annexin V positive , PI positive) at every time point as compared to the untreated controls.

Results were more clearly expressed in graphical form (Fig 5.7), employing a calculation modified from one used by Posovsky et al [255] which quantifies specific apoptosis and specific cell death that is attributable solely to the cytotoxic agent and that factors out the effects of background levels of spontaneous apoptotic cell death

$$\text{Specific apoptosis} = \frac{\%\text{ apoptosis in treated sample} - \%\text{of apoptosis in untreated control}}{100 - \%\text{ of apoptosis in untreated control}}$$

These plots demonstrated that although the action of both drugs led to levels of apoptotic cell death above background levels, the dynamics of cell death appeared to be different. The antibody conjugate reached a peak activity at between one to three hours and then maintained a fairly constant level of specific apoptosis and cell death up to 24 hours. In contrast the plots for the action of chlorambucil showed that most of the cytotoxic activity of the drug occurred between 8 and 24 hours, with a steeper curve and a more complete cytotoxic affect at this time (almost 100% cell death). These results would be consistent with the different mechanisms of action of the two drugs. Chlorambucil is able to enter cells rapidly by free diffusion, where its active metabolite leads to DNA strand breaks and cross-linking. In contrast, entry of anti-CD19 $D(KLAKLAK)_2$ into cells depends upon the level of expression of the CD19 antigen and the efficiency of CD19 internalisation ,which is a dynamic process with only a proportion of cells expressing the required levels of antigen at any one time.



KEY

	Secondary necrosis
Live	*Apoptotic*

Fig 5.6 Induction of apoptosis by anti-CD19 D(KLAKLAK)$_2$
Flow cytometric analysis of annexin V/propidium iodide stained CLL cells, at various time points after being treated with anti-CD19-D(KLAKLAK)$_2$ or chlorambucil.
No gating was used in the FACScan analysis.

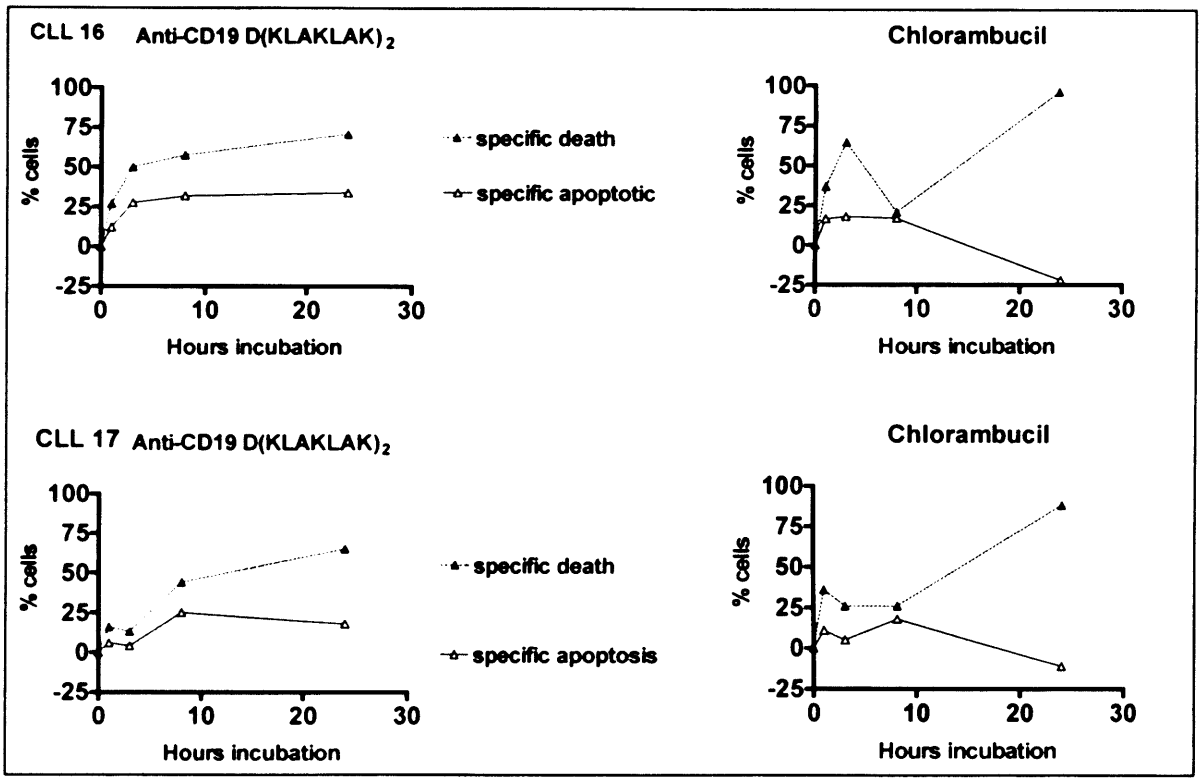


Fig.5.7 Agent specific apoptosis and death in CLL cells
Graph illustrating the specific death and specific apoptosis of cells isolated from the peripheral blood of two CLL patients after treatment with anti-CD19 D(KLAKLAK)2 or chlorambucil.

Activation of the apoptotic protease caspase 3 results in cleavage of the 116 kDa PARP with the consequent generation of an 85 kDa fragment, providing a stringent molecular criterion for apoptotic cell death[223].

Mononuclear cells isolated from the peripheral blood of a CLL patient were incubated for 24 hours with either nothing (negative control), 5nM of anti-CD19-D(KLAKLAK)$_2$ or 20μg/ml chlorambucil (positive control).At this time, protein lysates were prepared and fractionated by SDS-PAGE. The blots were probed with anti-P116 PARP- HRP, anti-P85 PARP -HRP and anti-actin-HRP to ensure equal loading (Fig5.8). Results were expressed as the proportion of total PARP that was represented by the cleaved p85 fragment.

$$\text{i.e.:} \quad \frac{\text{p85PARP}}{(\text{p85 PARP} + \text{p116 PARP})} \times 100$$

In this patient very little spontaneous apoptosis was noted, with the untreated negative control showing only 10% cleavage of the p116 PARP fragment at 24 hours. Both treated samples showed significant P116 PARP cleavage at 24 hours compared to the untreated control, with the chlorambucil treated sample demonstrating 92% PARP cleavage and the anti-CD19-D(KLAKLAK)$_2$ treated sample having an intermediate value of 44%. This intermediate value may be partly explained by the observation that anti-CD19-conjugate does not have its maximal effects until 48 hours, and also by the fact that this particular sample had a relatively high CD2 count of 13% and these T-cells would have been resistant to the cytotoxic effects of the conjugate.

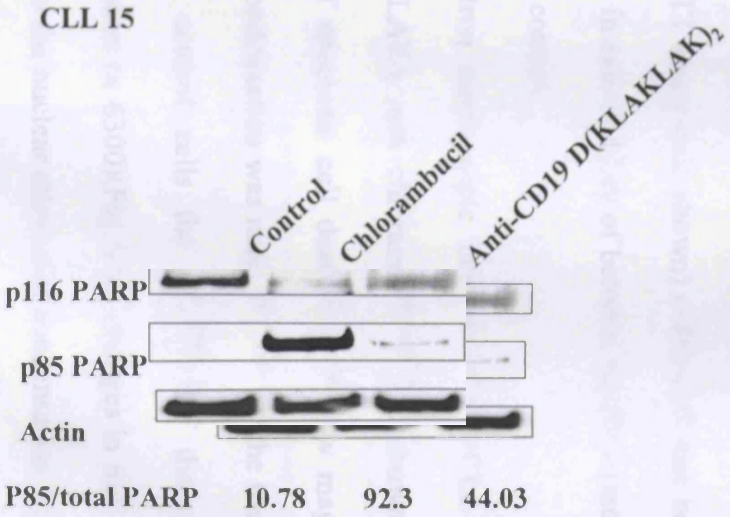


Fig 5.8 Western blot analysis of lysates of CLL cells
CLL cells were incubated in the presence or absence of of anti-CD19 D(KLAKLAK)$_2$ or chlorambucil. Lysates were analysed by Western blotting

Electron microscopic analysis of glutaraldehyde-fixed osmium tetroxide-stained cells also established that anti-CD19 D(KLAKLAK)$_2$ induced apoptotic death of CLL cells. Mononuclear cells isolated from the peripheral blood of a patient with CLL (CLL14) were treated with either nothing (negative control) , 5nM anti-CD19-D(KLAKLAK)$_2$ or 20μg/ml chlorambucil (positive control). After 24 hours incubation, 100μl of cells were taken for MTT cell viability assay (in duplicate), and the rest were fixed in 0.5ml of gluteraldehyde. Electron microscopy of the samples was kindly performed by Jackie Lewin of the Electron Microscopy department of the Royal Free Hospital. The results of the MTT assay (not shown) confirmed that both the treated CLL samples showed decreases in cell viability of between 68-72% (mean 70%) at 24 hours compared to the untreated control.

The electron microscopic images of the CLL cells treated with both anti-CD19 D(KLAKLAK)$_2$ and chlorambucil (not shown) illustrated morphological features typical of apoptotic cell death. In the low magnification images (x1400) (fig 5.9) nuclear condensation was noted in 50% of the treated cells in this field, whereas in the untreated control cells the majority of the nuclei appeared normal. At higher magnification (x 6300)(Fig 5.10) changes in mitochondrial morphology were noted in addition to the nuclear chromatin condensation. Many of the mitochondria in the treated cells showed abnormal morphological features such as prominent cristae and swelling (green arrow), with a lesser proportion showing more advanced changes with vacuolisation of two mitochondria seen in the cell (pink arrow).

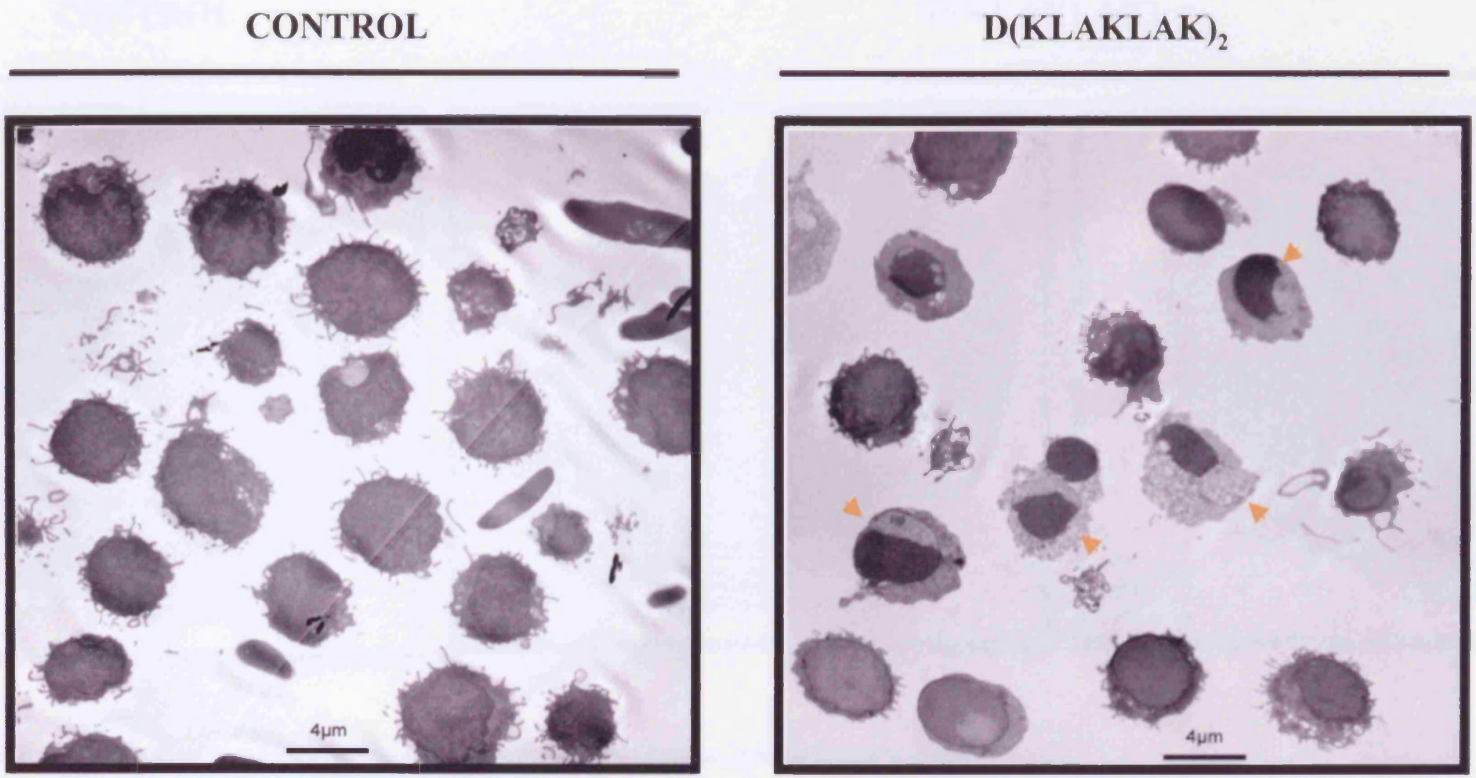


Fig 5.9 Electron microscopic analysis of CLL cells (lower magnification x2000)
Orange arrow – apoptotic cells with nuclear condensation

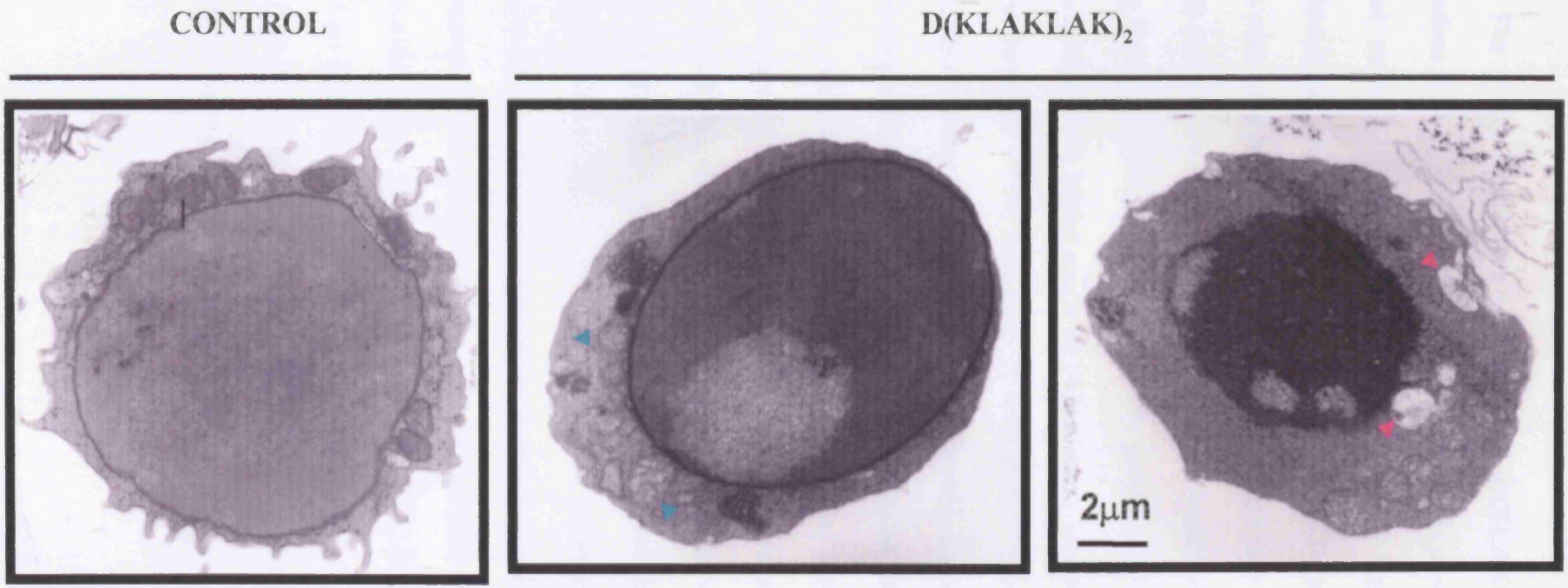


Fig 5.10 Electron microscopic analysis of CLL cells (higher magnification x 6300)
Changes in mitochondrial morphology in treated cells
Green arrow – swollen mitochondria with prominent cristea
Pink arrow – vacuolated mitochondria

5.2.1.7 The cytotoxic activity of anti-CD19 D(KLAKLAK)$_2$ on CLL cells is independent of the cell's expression of anti- apoptotic proteins

Increased expression of various proteins has been implicated in resistance to chemotherapeutic agents[245-247,256]. Members of the pro-survival Bcl-2 subfamily of proteins such as Bcl-2 and Mcl-1 are thought to exert their effects by modulating the apoptotic pathway by forming inactive heterodimers with pro-apoptotic proteins of the BH-only family[170] (section 1.4.4.2) .Similarly members of the inhibitors of apoptotic protease family (IAPs) inhibit apoptosis by binding to downstream effector caspases[257]. To assess the effect of the expression of anti-apoptotic proteins by CLL cells on the cytotoxic action of anti-CD19 D(KLAKLAK)$_2$, protein lysates were prepared from the untreated mononuclear cells isolated from the peripheral blood of nine CLL patients. The lysates were fractionated by SDS-PAGE and the blots were then probed with antibodies against actin (to ensure equal loading) and the anti-apoptotic proteins XIAP, Mcl-1and Bcl-2. The level of expression of these proteins was assessed after probing with the appropriate secondary antibodies. A representative western blot is shown in Figure 5.11A. Intensities of individual bands were determined by laser densitometric scanning and normalised with respect to the intensity of actin bands determined for the same samples. These expression ratios were plotted against the IC_{50} values determined by incubation of the CLL isolates with anti-CD19 D(KLAKLAK)$_2$ (Fig 5.1). We observed no correlation between the expression of Bcl-2, Mcl-1 or XIAP and the IC_{50} (Figure 5.11 B, C, D), suggesting that killing of CLL cells by the conjugate was not modulated by expression of these anti-apoptotic proteins.

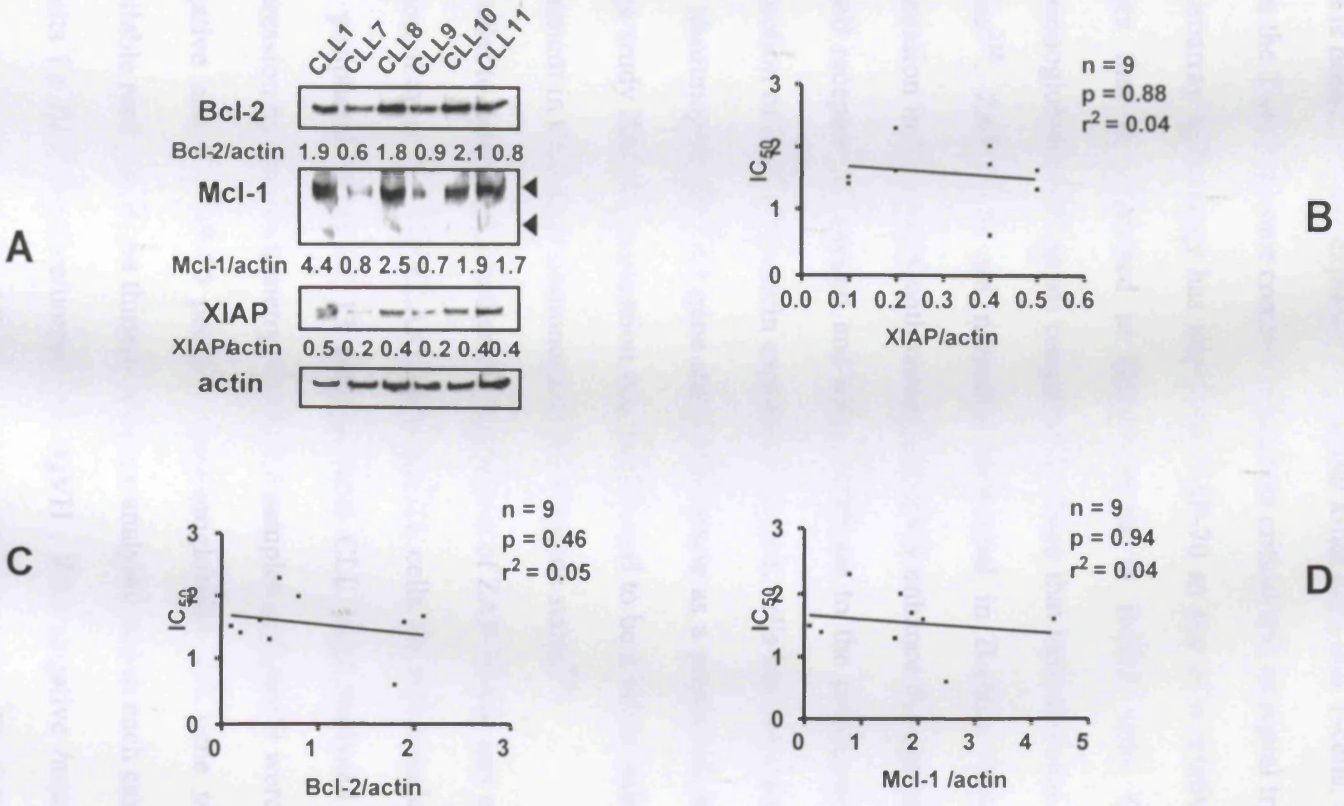


Fig 5.11 Relationship between IC_{50} for anti-CD19 $D(KLAKLAK)_2$ and the expression of anti-apoptotic proteins. Expression of anti-apoptotic proteins was determined by western blot analysis (A). The band intensity for each protein was normalized with respect to actin (B-D). The resulting ratios were plotted against IC_{50} values determined as in Fig 5.1 Statistical analysis was by Spearman rank correlation.

5.2.1.8 The cytotoxic activity of anti-CD19 D(KLAKLAK)$_2$ on CLL cells is independent of the cell's ZAP-70 status

Zeta-Associated Protein 70 (ZAP-70) is a 70kD cytoplasmic protein tyrosine kinase that is normally only expressed in natural killer cells and T-cells, where it is associated with the T-cell receptor complex and plays critical role in signal transduction pathways. Microarray technology has identified ZAP-70 as one of a relatively small number of genes that is expressed at higher levels in B-CLL cells that have unmutated immunoglobulin VH genes compared to those that have mutated immunoglobulin VH genes[258]. ZAP-70 is not normally functional in B-cells, however it's anomalous expression in CLL cells with unmutated IgVH enhance the signaling process when the B-cell receptor is engaged and may contribute to the pathogenesis of the disease[259]. Detection of ZAP-70 protein expression in CLL cells has been used a surrogate marker for immunoglobulin VH gene status and hence as a prognostic indicator[235,236]. In one large study ZAP-70 expression was even found to be a better indicator for the need for treatment in CLL than immunoglobulin VH gene status[260].

In order to determine whether the expression of ZAP-70 had any effect on the cytotoxic action of anti-CD19-D(KLAKLAK)$_2$ on CLL cells, the mononuclear cells isolated from the peripheral blood of 13 patients with CLL were analysed for ZAP-70 protein expression by flow cytometry. Of the 13 samples analysed 9 were found to be ZAP-70 negative and 4 ZAP-70 positive. Immunoglobulin VH gene mutational status was available nine out of the thirteen samples analysed and in each case showed concordant results i.e ZAP positive/unmutated IgVH , ZAP negative /mutated IgVH. Fig 5.12 illustrates the IC_{50} derived from dose response curves (see Fig 5.1) of the action of anti-CD19-D(KLAKLAK)$_2$ on the 13 CLL samples in which ZAP status was assessed. No

significant difference was noted between the median IC_{50} in the ZAP positive group compared to the ZAP negative group.

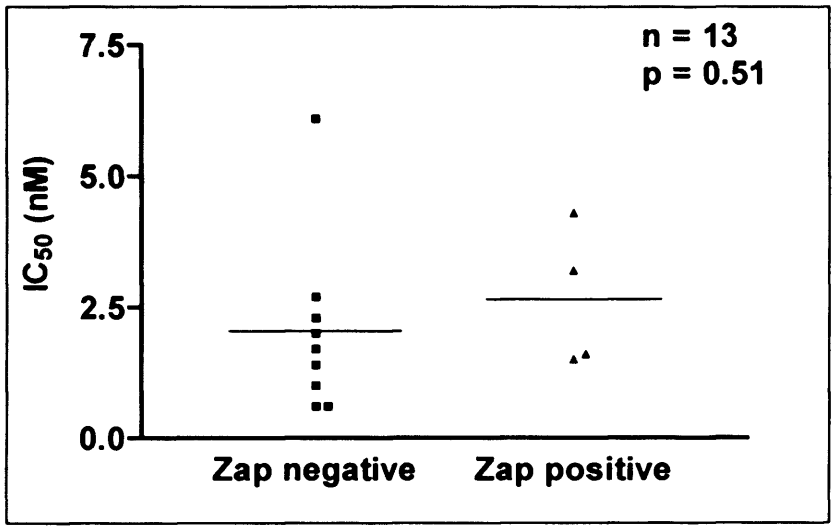


Fig 5.12 Relationship between IC_{50} for anti-CD19 D(KLAKLAK)$_2$ and ZAP-70 expression
ZAP-70 expression of thirteen CLL samples was determined by flow cytometry (section 2.3.9)
IC_{50} values were determined as in Fig 5.1
Statistical analysis was by unpaired t-test with Welch's correction

5.2.1.9 Anti-CD19 D(KLAKLAK)$_2$ and chlorambucil have synergistic cytotoxic effects on CLL cells

In order to assess whether D(KLAKLAK)$_2$ and chlorambucil had synergistic, additive or antagonistic cytotoxic effects on CLL cells, mononuclear cells isolated from the peripheral blood of 6 patients with CLL (CLLs 18-23 Table 5.2) were incubated with increasing concentrations of chlorambucil , anti-CD19 D(KLAKLAK)$_2$ and with serial dilutions of the two drugs in a fixed molar ratio of 20 000 : 1 (chlorambucil: conjugate). 100μl of cells were taken for MTT assay (in duplicate) after 48 hours incubation. Data were analysed using Calcusyn software (Biosoft, Fergusson Missouri[261])

Median effect plots were created for all 6 samples. Two representative examples are shown in Fig 5.13. In these plots the movement of the combination line (in red) slanting upwards from the chlorambucil alone line (in blue) gives a graphical indication of synergy. In Fig 5.13 the plot for CLL18 demonstrates drug synergy, whereas the plot for CLL22 does not. Overall strong synergy was demonstrated in 4 out of the 6 of the samples tested.

The IC_{50} values towards chlorambucil alone were decreased approximately two-fold when combined with anti-CD19 D(KLAKLAK)$_2$ (Table 5.3). Synergistic interactions were rigorously assessed by computing the combination index (CI) for each of the six CLL samples. . These figures were derived using an equation of Chou and Talalay that determines the additive effect of multiple drugs[262], with synergism being defined as a more than expected additive effect, and antagonism as less than expected additive effect.

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + ((D)_1(D)_2/(Dx)_1(Dx)_2$$

Where $(D)_1$ = the concentration of drug 1 that results in a fixed percentage of cell killing when used in combination and $(Dx)_1$ = the concentration of drug 1 that gives the same percentage of killing when used alone.

CI = 1 indicates an additive effect.

CI > 1 indicates an antagonistic effect.

CI < 1 indicates a synergistic effect, with lower values indicating stronger synergistic effect.

Table 5.3 shows the CI values for each of the six CLL samples calculated at various levels of killing. Five of the six isolates showed evidence of synergistic interaction between chlorambucil and anti-CD19 $D(KLAKLAK)_2$. In general, the CI values decreased under conditions of increasing cell killing, indicative of progressively increasing synergy. However, for the CLL22 isolate, the CI values remained close to unity at all levels of cell killing, indicative of an essentially additive interaction.

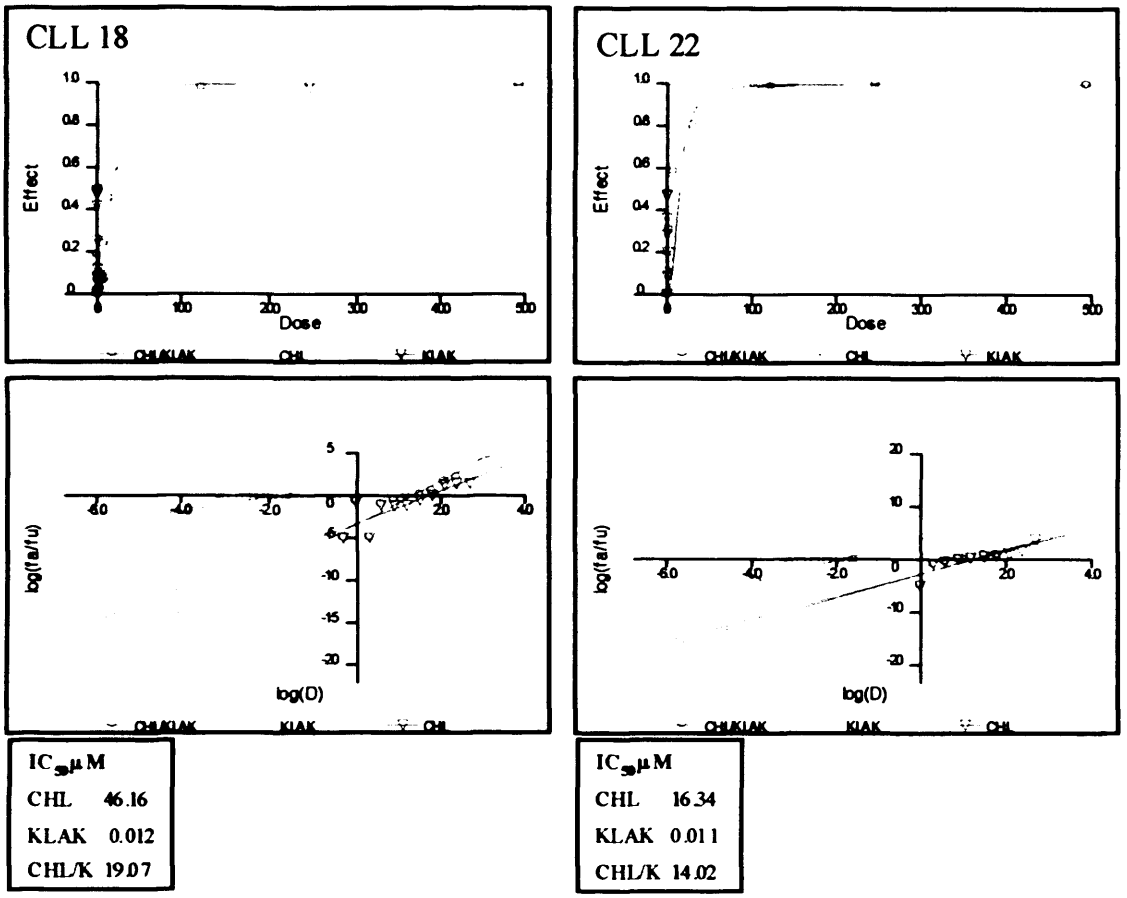


Fig 5.13 Analysis of interaction between anti-CD19 D(KLAKLAK)$_2$ and chlorambucil

Dose effect curves (upper panel) and median effect plots (lower panel) of the synergistic action of chlorambucil and anti-CD19 D(KLAKLAK)$_2$ on CLL samples CLL18 and 22

Median effect: [log (fa/fu) vs. log D]

fa: fraction affected, fu: fraction unaffected, D:dose

IC_{50} CHL: chlorambucil KLAK: anti-CD19 D(KLAKLAK)$_2$

CHL/KLAK: chlorambucil in the presence of anti-CD19 D(KLAKLAK)$_2$

Patient number	IC50 (μM)		Combination Index at:		
	Chl alone	Chl + anti-CD19:K	IC_{50}	IC_{75}	IC_{90}
18	46.2	19.1	0.53	0.41	0.34
19	11.8	5.4	0.47	0.33	0.23
20	3.6	2.8	0.84	0.69	0.58
21	35.3	13.9	0.56	0.51	0.47
22	16.3	14.0	0.98	0.94	0.91
23	14.9	6.7	0.51	0.58	0.64

Table 5.3 Interactions between chlorambucil and anti-CD19D(KLAKLAK)$_2$.
Chl chlorambucil
anti-CD19K anti-CD19 D(KLAKLAK)$_2$
Chl: conjugate was at a 20,000: 1 molar ratio

5.2.2 Assessment of cytotoxicty of anti-CD33-D(KLAKLAK)$_2$ against AML cells

Mononuclear cells were isolated from the bone marrow or peripheral blood of 5 patients with AML by Ficoll density gradient. The characteristics of the patients are summarised in table 5.5

	Age	Sex	Tissue	FAB type	CD33 expression
AML 1		59 M	BM	M1	3%
AML 2		54 M	BM	M2	76%
AML 3		63 M	BM	M4	76%
AML 4		39 M	PB*	M4	90%
AML 5		44 M	BM	M1	94%

* *Indicates leuopheresis sample*

Table 5.4 Characteristics of patients and AML samples used in Chapter 5.

5.2.2.1 Anti-CD33-D(KLAKLAK)$_2$ has cytotoxic activity against CD33 positive AML cells as assessed by MTT assay

Cytotoxicity of anti-CD33-D(KLAKLAK)$_2$ (antibody: peptide ratio 1:6) was assessed by incubation of increasing concentrations of the conjugate with mononuclear cells isolated from the bone marrow or peripheral blood of patients with AML. Samples for MTT assay were taken at 24 and 48 hours. All MTT assays were performed in duplicate. In each experiment a positive control of CD33 positive THP1 cells and a negative control of CD33 negative Jurkat cells were used. .

The cumulative results of the MTT assays at 24 are shown in Fig5.14. In contrast to the anti-CD19 conjugate, the anti-CD33 conjugate had maximal effects at 24 hours,

with mean decreases in cell viability at 24 hours of 45.84% (range 5.3%-65.1%). As with the cell line experiments the IC_{50} values at 24 hours were in the nanomolar range, with a mean value of 26.69nM (range 6.8nM-45.5nM).

In section 5.2.1.2, the specificity of the anti-CD19 conjugate was demonstrated indirectly by showing a strong correlation between the percentage of CD2 positive T-cell in the samples and the proportion of cells that were resistant to the anti-CD19 conjugate. In the case of the AML samples, CD33 expression was estimated directly by flow cytometry before exposing the cell samples to anti-CD33 conjugate. There was a wide variation in CD33 expression (Table 5.4), with one sample AML1 demonstrating only 3% of positive cells. Although negative CD33 expression in AML is a rare finding it has been reported in literature to occur in approximately 3% of AML cases[263]. As this was an unexpected result, the phenotype was kindly confirmed by APAP staining by Faith Wright in the Royal Free immunophenotyping laboratory.

Unsurprisingly this AML sample was resistant to the cytotoxic effects of the anti-CD33 conjugate.

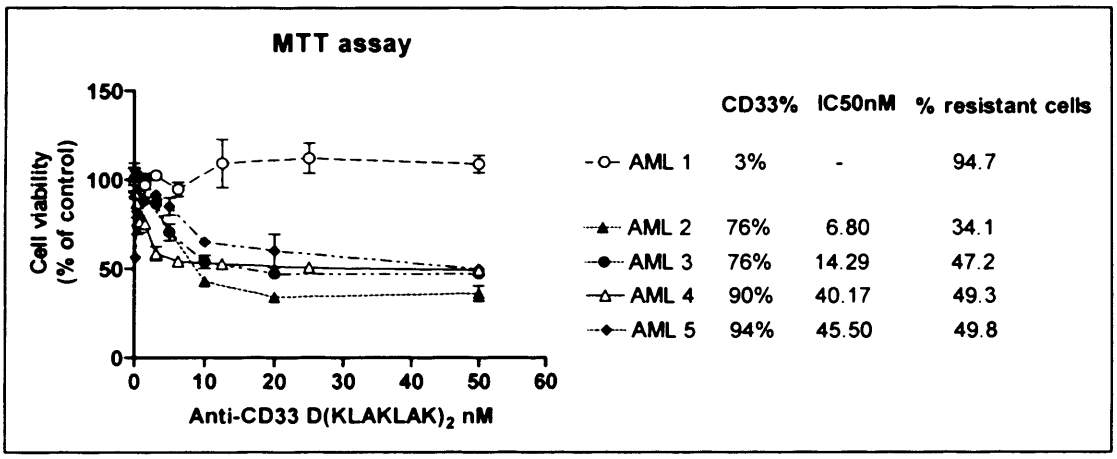


Fig 5.14 Cytotoxicity of anti-CD33 D(KLAKLAK)$_2$ towards AML cells
Dose response curve for the action of anti-CD33 D(KLAKLAK)$_2$ on cells isolated from the bone marrow or peripheral blood of five patients with AML.
MTT assays were at 24 hours.
Error bars represent SEM of duplicate determinations.

5.3 Discussion

We have demonstrated that anti-CD19 D(KLAKLAK)$_2$ and anti-CD33 D(KLAKLAK)$_2$ conjugates have potent and specific activity against CD19 positive CLL cells and CD33 positive AML cells respectively. In the case of the anti-CD19 conjugate, an apoptotic mechanism of cell killing was inferred on the basis of morphological nuclear features of affected cells demonstrated by electron microscopy, by PARP cleavage analysis and by annexin V /propidium iodide staining of treated cells. In addition the data suggests that the cytotoxic effect of the drug on CLL cells is independent of baseline expression levels of the anti-apoptotic proteins implicated in chemoresistance (Bcl_2, Mcl, XIAP) and of ZAP-70 expression.

We have also demonstrated that the anti-CD19 D(KLAKLAK)$_2$ conjugate acts synergistically with chlorambucil against CLL cells.

CLL has certain features that make it an ideal candidate for this type of immunoconjugate therapy. The relative resistance to cytotoxic drug induced programmed cell death that occurs secondary to abnormalities of anti-apoptotic protein expression and the characteristically quiescent nature of CLL cells arrested in G_0/G_1 phase of the cell cycle[264] leads to resistance to many conventional chemotherapeutic agents which are targeted towards rapidly dividing cells. CLL cells also have a characteristic phenotype providing many potential antigens for targeted therapy.

Therapy with unconjugated antibodies targeted to non-internalising antigens such as CD52 (Campath 1-H ,Alemtuzumab) and CD20 (Rituximab) have shown some benefit in CLL. Alemtuzumab has an overall response rate of 33% in patients with relapsed or refractory disease[63] and is clinically effective in patients with abnormalities of the p53 pathway[265]. Response rates with Rituximab used in previously treated patients vary from 0-45%, with better response rates when used in combination with other

chemotherapeutic agents[59,67]. In addition Rituximab in combination with CHOP chemotherapy may bypass Bcl-2 associated chemorsistance[266]. Other unconjugated antibodies currently in development for CLL therapy include antibodies directed against CD22, CD23, HLA class II, TRAIL and CD80[67].

The ideal surface antigen for the targeted delivery of toxins to CLL cells would be expressed reliably and selectively on CLL cells and would internalise efficiently on bonding to the ligand. Although CD19 is the most reliably expressed target antigen expressed in CLL cells, internalisation may not be as efficient as that of some other targets[113,267]. Anti-CD19 antibodies have however been used effectively to target various toxins[77,104,268] and immunoliposomes[269] to other B-cell malignancies. CD22 is only very weakly expressed in CLL[112], however it has been shown to internalise particularly well in CLL cells [267] and has also proved to be a suitable target for the delivery of immunotoxins in this disease[143]. The IL-2 receptor complex (including CD25,CD122 and CD132) which is expressed in approximately 50% of CLL patients [270] is the target for an IL-2 /diphtheria toxin fusion protein (Ontak), which has shown some responses in Phase II trials[271]. As well as levels of cell surface expression and degree of internalisation the therapeutic efficacy of these immunoconjugates may also be affected by other factors such as signalling events triggered by ligand binding and differences in intracellular trafficking of the toxins once internalised.

Our results indicate that the anti-CD19 antibody used internalises to a sufficient extent to effectively deliver toxic doses of $(KLAKLAK)_2$ peptide to the CD19 positive CLL cells while sparing CD2 positive T-cells. The presumed mechanism of action of $(KLAKLAK)_2$ is disruption of the mitochondrial membrane and triggering of the apoptotic cascade. We have demonstrated apoptotic morphology in treated cells as well as evidence of PARP cleavage and characteristic patterns of Annexin V and Propidium

iodide staining. Other groups who have targeted this peptide, mainly to target vascular endothelial cells in various malignancies, have similarly demonstrated apoptotic morphology and shown evidence of caspase 3 processing in treated cells[127,203]. In addition the Ellerby group have demonstrated that the peptide alone causes mitochondrial swelling and caspase 3 processing in a mitochondria- dependent cell free system of apoptosis[203,272].

The observation that the cytotoxic action of the anti-CD19 conjugate appears to be independent of the baseline expression levels of anti-apoptotic proteins involved in chemoresistance or of ZAP-70 status and that it acts synergistically with chlorambucil would further suggest that this immunoconjugate may be a useful addition to currently available therapeutic agents for CLL.

Summary

We have demonstrated that the antibody- D(KLAKLAK)$_2$ conjugates synthesised in chapter 3 have selective cytotoxic activity against cells isolated from patients with CLL and AML. An anti-CD19 conjugate had cytotoxic activity towards cells isolated from ptients with CLL, with IC_{50s} in the low nanomolar range. Cell killing was by an apoptotic mechanism as demonstrated by flow cytometrric analysis of annexin V and propidium iodide stained cells, Western blot of analysis of PARP cleavage and characteristic morphology on electron microscopy. The cytotoxic activity the anti-CD19 conjugate was independent of the level of expression of anti-apoptotic proteins and ZAP-70. In the majority of cases studied, combined treatment with the antibody conjugate and chlorambucil resulted in highly synergistic cell killing. In addition, an anti-CD33 conjugate was cytotoxic towards cells isolated from patients with AML

Chapter 6 Selection of CD33 binding peptides from a phage display library

6.1 Introduction

In the previous results chapters, we described a reliable method for the conjugation of the amphipathic peptide $D(KLAKLAK)_2$ to monoclonal antibodies and demonstrated that the resulting immunoconjugates have a potent and selective cytotoxic action against targeted cells. These results provide a proof of principle that $D(KLAKLAK)_2$ can be used effectively as targeted therapy for haematological malignancies.

The results in this section describe our attempts to isolate a peptide that binds to antigens expressed on the surface of malignant haematological cells and thereby create a multifunctional peptide reagent which combines the cytotoxic and targeting functions and would therefore have selective cytotoxic actions against leukaemia/lymphoma cells.

There are potential advantages of using peptides rather than antibodies for targeting: They can be easily and economically synthesized. Purification is also easier as they are less likely to be contaminated by DNA and viruses. They are less immunogenic than antibodies and due to their small size may have better tumour penetration than larger antibody conjugates.

Short peptides that bind to tumour vasculature have been selected from phage display libraries and been used to successfully target cytotoxic peptides which inhibit angiogenesis in various solid tumours[127,203,273]. Although ScFv that bind to target antigen expressed on haematological malignancies have been isolated[129], peptide sequences, which bind to CD19 or CD33, have not as yet been described.

6.2 Results

Despite the fact that our results in the previous chapters indicate that the anti-CD19 $D(KLAKLAK)_2$ conjugate was more effective at killing CLL cells than the anti-CD33 conjugate was at killing AML cells, we chose to attempt to isolate a CD33 binding peptide.This decision was made on the basis of the availability of a CD33-Fc secreting Chinese hamster ovary (CHO) cell line which was kindly supplied Paul Crocker at Dundee University .

6.2.1 Isolation and purification of a CD33-Fc construct from the supernatant of a CD33-Fc secreting CHO cell line

The construct secreted by the CHO cell line consisted of four Ig domains (two domains from CD33 and two from the Fc portion of IgG1[120]. The cell line was cultured for six weeks in medium supplemented with immunoglobulin-depleted FCS, with supernatant being removed weekly and frozen in aliquots.

To ensure that the CHO cell lines were secreting CD33-Fc, an ELISA assay of the supernatant was performed fortnightly, using murine anti-human CD33 as the primary antibody and horseradish peroxidase linked goat anti-mouse immunoglobulin as the secondary antibody. In each ELISA negative controls of the immunoglobulin depleted culture medium and PBS were used, with a positive control of mouse immunoglobulin. Fig 6.1a illustrates the results of one such assay, confirming that the cell line was secreting the CD33-Fc construct. Fig 6.1b shows the results of the three fortnightly ELISAs and demonstrates that the secretion of the construct remained essentially stable over the culture period.

After six weeks the aliquots were pooled and the CD33-Fc construct was isolated by high performance liquid chromatography using a protein A column.

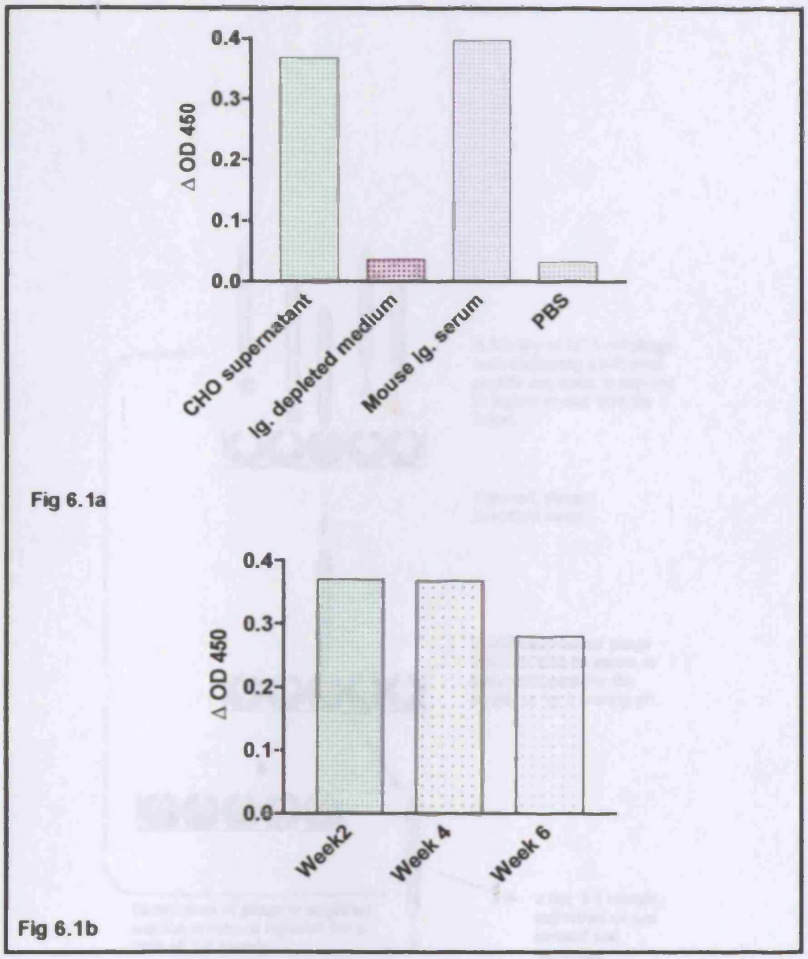


Fig 6.1 ELISA of CHO supernatant

ELISA assays were performed at fortnightly intervals on the supernatant of the CD33-Fc secreting CHO cell line using murine anti-human CD33 as the primary antibody and HRP-goat anti-mouse as the secondary antibody. Fig 6.1a, positive controls of mouse immunoglobulin and negative controls of Ig-depleted culture medium and PBS were used in each experiment.
Fig 6.1b shows the the results of three fortnightly ELISAs

6.2.2 Screening of bacteriophage library to select phage that bind to CD33-Fc construct and sequencing of the selected phage

A phage library consisting of random peptide 7-mers fused to a minor coat protein (pIII) of the M13 phage was used. Biopanning was carried out as illustrated in Fig 6.2 ,according to manufacturer's instructions[136].

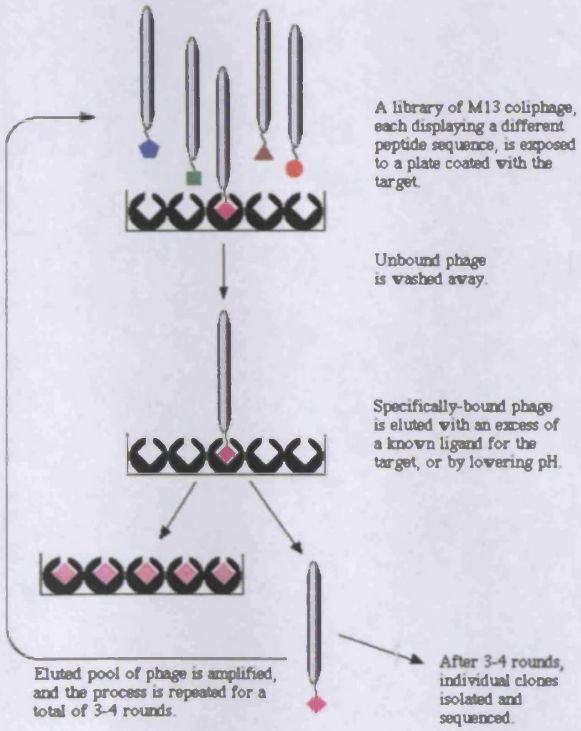


Fig 6.2 Schematic representation of biopanning a phage display library[136]

Briefly 150µl of CD33 supernatant was added to a single well of a 96 well flat bottom plate and incubated overnight at 4°C. After removing the supernatant, 10µl of the phage library containing approximately $2x10^{11}$ phage were added to the well. Human immunoglobulin was added to the phage media in order to decrease the likelihood of selecting phage that bound the immunoglobulin portion of the construct rather than the CD33 portion. Unbound phage were removed by extensive washing. Bound phage were eluted with 0.2M glycine HCL pH2.2 and then neutralized with 1M Tris HCL pH9.1. The eluted phage were propagated in E.coli and carried onto the next panning round. Ten phage clones were selected for sequencing from the fourth round of panning. The sequence of the random peptide inserts was deduced from positive clones by BigDye terminator cycle sequencing.

Results of the phage sequencing are shown in Fig 6.3. Two amino acid sequences appeared multiple times. One sequence was identical in five out of the ten sequenced clones: FHENWPS (in green) with one additional clone M5 sharing only the WPS sequence. Another sequence LPPQTLI (in red) was seen in three out of the ten clones.

M1.	TTG	CCT	CCG	TAG	ACT	TTG	ATT
	L	P	P	Q	T	L	I
M2.	TTG	CCT	CCG	TAG	ACT	TTG	ATT
	L	P	P	Q	T	L	I
M3.	TTT	CAT	GAG	AAT	TGG	CCT	TCG
	F	H	E	N	W	P	S
M4.	TTT	CAT	GAG	AAT	TGG	CCT	TCG
	F	H	E	N	W	P	S
M5.	TAN	GGC	ATN	AAA	TGG	CCT	TCG
	Y/Q	?	I/M	?	W	P	S
M6.	TTG	CCT	CCG	TAG	ACT	TTG	ATT
	L	P	P	Q	T	L	I
M7.	TTT	CAT	GAG	AAT	TGG	CCT	TC-
	F	H	E	N	W	P	S
M8.	TTT	CAT	GAG	AAT	TGG	CCT	TC-
	F	H	E	N	W	P	S
M9.	AGT	CTG	CCG	ACT	TAT	ACT	CAT
	S	L	P	T	Y	T	H
M10.	TTT	CAT	GAG	AAT	TGG	CCT	TC-
	F	H	E	N	W	P	S

Fig 6.3 Results of biopanning experiments
Genetic code and amino acid sequence of 10 phage clones selected after 4 rounds of biopanning on the CD33-Fc construct.

6.2.3 Binding of peptide sequences to CD33

The two amino acid sequences derived from the biopanning of the phage display library on the CD33-Fc construct were custom synthesised (Alta Biosciences) with a biotin residue at the N-terminus, separated from the peptide by two glycine residues, as follows:

Peptide 1 JGGLPPQTLI

Peptide 2 JGGFHENWPS

Where J=biotin

Binding of the peptides to CD33 was assessed by an ELISA assay and by flow cytometry.

Fig 6.4 illustrates the results of an ELISA assay in which binding of the biotinylated peptides to immobilised CD33-Fc and human immunoglobulin was compared using streptavidin-HRP as the second layer. Both peptides demonstrated a slight increase in binding to CD33 as compared to the immunoglobulin control.

For the flow cytometry experiment, THP1 and Raji cells were incubated at 4°C with each biotinylated peptide for 30 minutes, followed by incubation with streptavidin-APC for a further 30 minutes at 4°C. Neither peptide bound more streptavidin-APC than the control CD33 negative Raji cell line. Peptide 2, however, showed a slight shift possibly indicating increase in binding to CD33 positive THP1 cells as compared to both peptide 1 and the control (Fig.6.5).

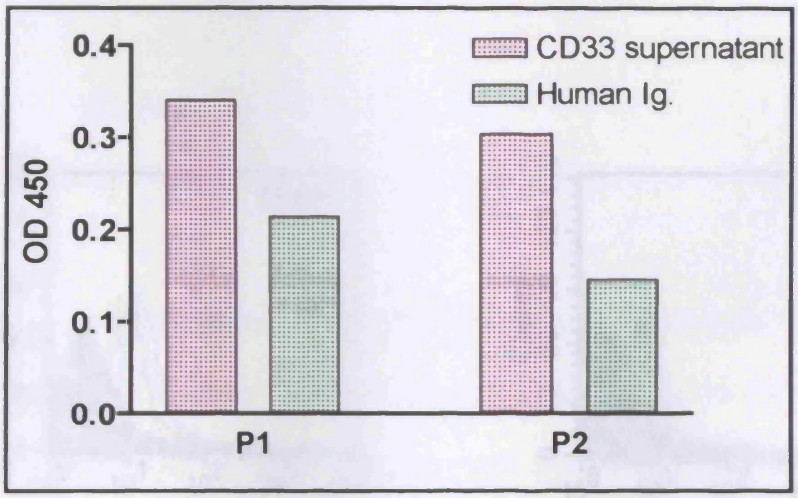


Fig.6.4 ELISA of binding of peptides 1 and 2 to CD33-Fc containing supernatant
Binding of the two biotinylated peptides 1 and 2 to immobilised CD33-Fc and human immunoglobulin was assessed by ELISA assay using streptavidin-HRP as the second layer.

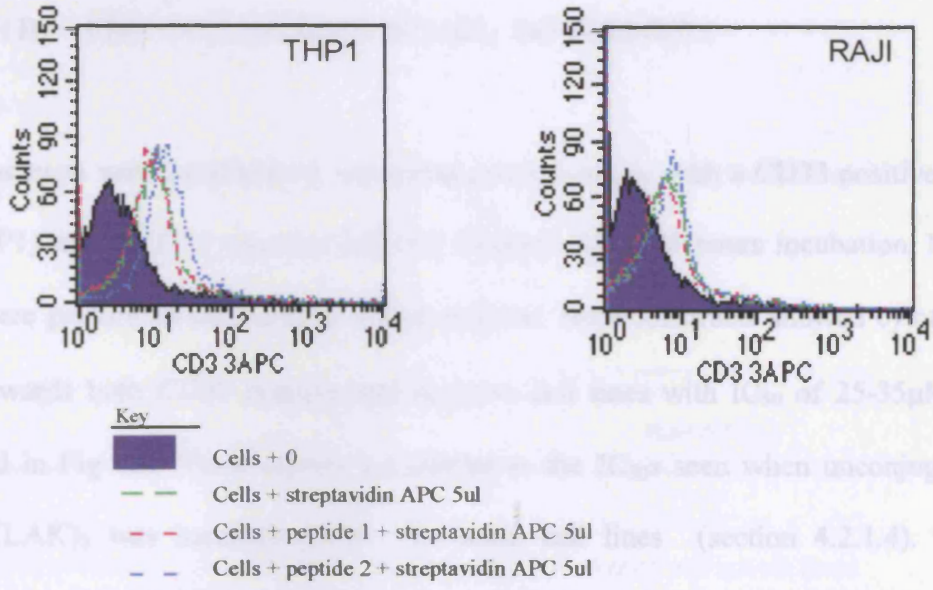


Fig 6.5 Flow cytometric analysis of binding of peptides 1 and 2 to cell lines
FACScan analysis of binding of biotinylated peptides 1 and 2 to THP1 and Raji cells, with 2nd layer of streptavidin APC.

6.2.4 Cyotoxicity of peptide constructs based on the sequence of peptide 2

Two peptide constructs based on the sequence of peptide 2 were custom synthesised (Alta Biosciences). The first construct (A) consisted of the D(KLAKLAK)$_2$ sequence linked to the sequence of peptide 2 by two glycine residues. The second construct (B) consisted of the D(KLAKLAK)$_2$ sequence flanked on either side by the peptide 2 sequence linked by two glycine residues.

Construct A FHENWPS GG D(KLAKLAK)$_2$

Construct B FHENWPS GG D(KLAKLAK)$_2$ GG FHENWPS

Both constructs were incubated at increasing concentrations with a CD33 positive cell line (THP1) and a CD33 negative cell line (Jurkat). After 48 hours incubation, MTT assays were performed on duplicate 100µl aliquots. Both constructs showed cytotoxic action towards both CD33 positive and negative cell lines with IC_{50} of 25-35µM as illustrated in Fig 6.6. These results are similar to the IC_{50}s seen when unconjugated D(KLAKLAK)$_2$ was incubated with the same cell lines (section 4.2.1.4). This observation and the lack of specificity of the toxic effects of the constructs suggest that neither construct enabled the selective internalisation of the cytotoxic peptide into CD33 positive cells.

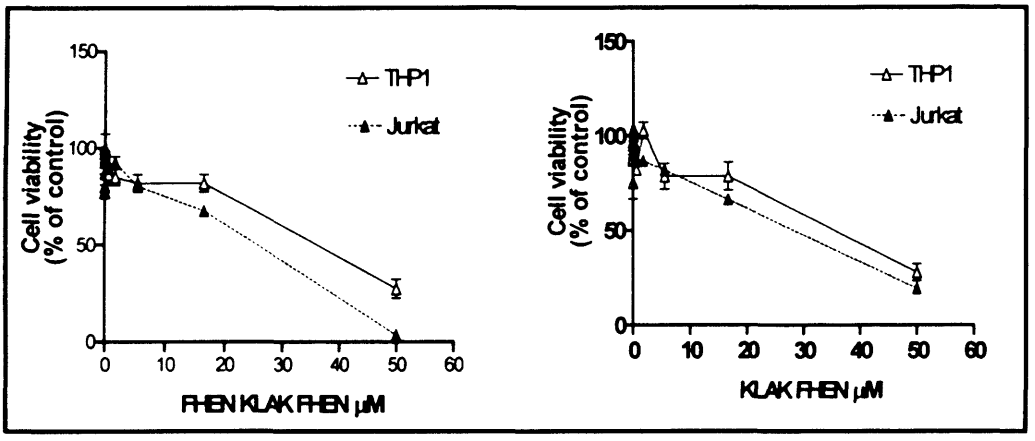


Fig 6.6 Cytotoxicity of peptide constructs towards haemopoeitic cell lines
Dose response curve of the action of 2 peptide constructs consisting of a CD33 binding motif and $D(KLAKLAK)_2$ on THP1 and Jurkat cell lines. Cell viability was assessed by MTT assay at 48 hours.
Results are representative of three independent experiments.
Error bars represent SEM of duplicate determinations.

6.3 Discussion

By using a phage display library to pan a CD33-Fc construct we identified two consensus peptide sequences. Only one of these (peptide 2) appeared to bind to the CD33 positive cell line THP1. In addition neither of the two peptide constructs based on the sequence of peptide 2 and the $D(KLAKLAK)_2$ peptide had any cytotoxic action over and above the cytotoxicity of the $D(KLAKLAK)_2$ peptide alone (demonstrated in previous chapters). These results indicate that the peptide sequences derived from the phage display biopanning experiments failed to selectively internalise the cytotoxic peptide into CD33 positive cells.

There are numerous possible reasons why we were unable to identify selective internalising phage in these experiments. Firstly the construct on which the phage library was panned was a CD33-Fc construct rather than pure CD33. Although care was taken to avoid selecting phage that bound to the immunoglobulin moiety of the construct by adding human immunoglobulin to the phage containing media, it is possible that the phage selected bound to some other part of the construct rather than the CD33 portion. Equally phage may have been selected that did not bind to the construct at all but to some material in the experimental system such as the plastic wells or eppendorphs. It is also possible that the phage bound to an epitope of CD33 that did not facilitate endocytosis of the CD33 antigen. Secondly, the experiments were not specifically designed to select internalising phage. This can be achieved in protocols where biopanning is done on whole cells, and internalised phage are recovered from lysed cells before the amplification stage[274-276]. Thirdly the addition of the two glycine linker peptides to the sequences derived from the selected phage may have altered the peptides' binding characteristics and decreased their affinity for the CD33 target.

Unfortunately due to time constraints we were unable to investigate any of these possibilities or to repeat the biopanning experiments under different conditions.

Summary

We used a phage display library to pan a CD33-Fc construct in an attempt to find peptide sequences that bind to and internalise into CD33 expressing myeloid cells. Two consensus sequences were identified but neither bound convincingly to CD33 as demonstrated by ELISA or flow cytometric experiments. Peptide constructs base on the consensus sequences and D(KLAKLAK)$_2$ did not show any specific cytotoxic activity towards CD33 expressing cells. The possible reasons why this strategy was not sucessful are discussed.

Chapter 7 Discussion

We have described a simple, reproducible procedure for coupling an amphipathic cytotoxic peptide to antibodies directed against lineage-restricted cell surface antigens expressed by B -lymphoid and myeloid cells. Conjugation of the peptide to anti-CD19 generated a reagent which efficiently killed 3/3 malignant B lymphoid cell lines tested. The killing of one of these lines (Raji) was shown to be by an apoptotic mechanism. However, a peptide-anti-CD33 conjugate killed only 1/4 myeloid cell lines. The resistance of one of these lines (K562) could be accounted for by the lack of expression of cell-surface CD33. However, the remaining two resistant lines (HL60 and U937) expressed abundant CD33, suggesting that resistance to killing resulted from a cell-type dependent inability of internalised peptide to access and/or activate the cell death machinery. This interpretation was corroborated by our demonstration that the resistant HL60 cell line nevertheless internalised a conjugate containing a biotinylated peptide and was also susceptible to anti-CD33 conjugated to a different toxin, calicheamycin.

While micromolar levels of the peptide itself was uniformly toxic to all of the cell lines tested here, linkage to antibodies resulted in at least a 100-fold increase in efficiency of killing of the targeted cells. The reagents generated by our protocol showed IC_{50} values in the low nanomolar range. The increased efficiency was accompanied by highly selective killing, since neither conjugate tested here killed cells which did not express the appropriate cell-surface target molecule.

We also show that conjugation of the amphipathic cytotoxic peptide D-$(KLAKLAK)_2$ to an anti-CD19 monoclonal antibody results in a reagent showing potent and highly targeted toxicity towards malignant cells isolated from patients with CLL . Cells from untreated or previously treated patients showed very similar susceptibility to the

conjugate, suggesting that it may be of value in treating patients who have become resistant to conventional therapeutic agents. Cell killing was by an apoptotic mechanism and was independent of level of expression of anti-apoptotic proteins and ZAP-70. In the majority of cases studied, combined treatment with the antibody conjugate and chlorambucil resulted in highly synergistic cell killing.

The mechanism by which internalised $D(KLAKLAK)_2$ is thought to engage the cell's apoptotic machinery is by disruption of mitochondrial membrane. We have demonstrated an apoptotic mode of cell death by PARP cleavage analysis and by annexin V /propidium iodide staining of treated cells as well as demonstating characteristic morphological features on light and electron microscopy. Ellerby et al demonstrated that $D(KLAKLAK)_2$ induced mitochondrial swelling and caspase 3 processing in a mitochondria- dependent cell free system of apoptosis[203,272]. Another group has demonstrated rapid release of cytochrome *c* from mitochondria in Jurkat cells which were temporarily transfected with $(KLAKLAK)_2$ linked to a protein transduction domain[277].

Mitochondrial membranes differ from eukarytic cell membranes in their lipid composition. Eukaryotic cell membranes are rich in cholesterol whereas mitochondrial membranes contain very little. The main phospholipids present in eukarytoic cell membranes are the amino-glycerophospholipids (phosphatidylcholine and phosphatidylethanolamine). The outer mitochondrial membrane also contains high levels of phosphatidylcholine; the inner mitochondrial membrane however is rich in phosphatidylglycerol and its derivative cardiolipin[278] (lesser amounts of cardiolipin are also found in the outer mitochondrial membrane[279]). Cardiolipin is a unique phospholipid having a dimeric structure with four acyl groups and the potential to carry two negative charges. Cardiolipin is also unique in that its biosynthesis is restricted to

mitochondria[278] presumably reflecting a common ancestry with bacteria, given that cardiolipin is also present in aerobic bacteria.

The majority of mitochondrial proteins are encoded by nuclear DNA and targeted to mitochondria by temporary amino terminal extentions called pre-sequences. These sequences have an amphipathic character containing both positively charged and neutral amino acids and have been predicted to adopt an α helical conformation in which the cationic residues lie on one side of the helix and the non-polar amino acids on the other. It is suggested that the non-polar face of the helix may penetrate into the hydrophobic region of the outer membrane of mitochondria, while the basic residues on the polar side of the helix would interact with the headgroup of the negatively charged phospholipids. Nicolay *et al* synthesised three such amphipathic peptides based on the pre-sequence of cytochrome *c* oxidase subunit IV to study the effect of these peptides on the structural and functional integrity of rat liver mitochondrial membranes[280]. They demonstrated that at low peptide to mitochondrial ratios peptide binding to the outer mitochondrial membranes occurred, outer membrane permeabilisation was demonstrated at higher ratios and at still higher concentrations the inner membrane permeability was increased.

Another group of amphipathic α helix forming peptides are the host defence antimicrobial peptides such as magainins. These peptides have the ability to kill bacteria by permeabilising their cell membranes but do not lyse erytherocytes[281]. The absence of acidic phospholipids on the outer monolayer and the abundant presence of sterols cells were thought to account for the reduced suseptibilty of the red blood cells to these agents[281].

These models of the mechanism of action of amphipathic peptides have been cited by Ellerby *et al*[203] as the likely explanation for apoptosis induction following

internalisation of the amphipathic peptide $D(KLAKLAK)_2$. However, this hypothesis has not been directly proven and it is possible that in haemopoeitic cells apoptosis is induced by an entirely different pathway. We have demonstrated antibody conjugate internalisation into haemopoeitic cells by confocal microscopy. However further tracking of the intracellular pathway of the conjugate proved problematic. The initial strategy of tracking a biotinylated version of the peptide was hampered by high levels of a constitutively expressed biotin containing protein in the Raji cells leading to high levels of background staining. Using FITC labelled anti-mouse immunoglobulin we were able to demonstrate internalisation into vesicular like structures within Raji cells, however it is likely that the peptide would be cleaved from the antibody portion of the conjugate within the acidic environment of the endosome so further tracking of the active peptide portion would not be possible by this method. One way of overcoming this problem would be to use an alternative tag for the amphipathic peptide alternatively an antibody could be generated towards the peptide itself. Unfortunately due to time constraints we were unable to investigate any of these possibilities.

Rituximab and alemtuzumab, which target CD20 and CD52 respectively, are the principal antibodies currently used in the treatment of CLL. However, treatment with these agents alone rarely results in complete remissions in patients who are refractory to conventional cytotoxic drugs[59,63,67,265]. Rituximab in combination CHOP chemotherapy appears to bypass Bcl-2 associated chemoresistance[266] but its cytotoxcity has been demonstrated to be compromised by elevated expression of Mcl-1 and other anti-apoptotic proteins[248]. The targeted killing of T lymphocytes by alemtuzumab can result in immunosuppression and fatal infections[67]. The ability of CD19 $D(KLAKLAK)_2$ to kill CLL cells irrespective of treatment history and of anti-apoptotic protein expression

together with the lack of killing of T lymphoid cells may offer significant advantages compared with currently available therapeutic antibodies.

Various natural toxins, including ricin and pseudomonas exotoxin, have been used in targeting strategies[141]. However, amphipathic peptides may offer some potential advantages over these natural agents. First, construction using D-amino acids would render these species resistant to proteolytic degradation, in both extra- and intracellular compartments. Second, they can be synthesized economically by purely chemical procedures, circumventing the risk of contamination by potentially hazardous biomolecules. Third, induction of vascular leak syndrome is a key dose-limiting factor in the therapeutic use of many natural toxins. A conserved three amino acid sequence has been identified as a cause of this syndrome[147,282].

The absence of this motif from the amphipathic peptide suggests that its use in targeted strategies may circumvent limitations resulting from vascular leakage.

Our attempts to identify peptides which bound to CD33 by biopanning a phage display library were unsuccessful. (The possible reasons for this have been discussed in the relevant chapter.) However peptides which bind selectively to specific immunoglobulins[132] or other unidentified cell-surface structures[134] of malignant B lymphoid cells have been identified by other groups. Our demonstration that targeted D-$(KLAKLAK)_2$ is toxic towards CLL cells offers the prospect of constructing completely synthetic reagents consisting of the amphipathic peptide linked to a targeting sequence. This type of multifunctional peptide reagent has previously been designed for targeting the neovasculature of solid tumors.[127,203] Adaptation of this concept to leukemias/lymphomas may therefore result in the generation of highly elective targeted agents for the treatment of hematopoietic malignancies.

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Publications relating to this thesis

Selective killing of leukaemia cell lines by antibody mediated targeting of cytotoxic amphipathic peptides.

AJ Marks, M Cooper, K Orchard, G Hales, HG Prentice, AB Mehta and RG Wickremasinghe

Oral presentation at the American Society of Hematology '01

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Targeted therapy with an amphipathic peptide results in selective apoptotic cell death in CD19 positive chronic lymphocytic leukemia cells.

AJ Marks, M Cooper, K Orchard, NI Folarin, K Ganashaguru, AV Hoffbrand, AB Mehta and RG Wickremasinghe

Oral presentation at the American Society of Hematology '03

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Selective apoptotic killing of malignant hemopoietic cells by antibody-targeted delivery of an amphipathic peptide.

AJ Marks, M Cooper, RJ Anderson, KH Orchard, G Hale, JM North, K Ganeshaguru, AJ Steele, AB Mehta, MW Lowdell and RG Wickremasinghe

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Selective toxicity of an anti-CD19/amphipathic peptide conjugate towards chronic lymphocytic leukemia cells.

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