Conditions for safe and effective
ADEPT treatment

Dr Duncan Keith Wilkins

MD (Res) Oncology Thesis

UCL

University College London
Declaration

I, Dr Duncan Keith Wilkins confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Duncan Keith Wilkins

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Abstract

Antibody directed enzyme prodrug therapy (ADEPT) is a drug delivery system developed for the treatment of cancer. ADEPT uses a systemically administered antibody, tethered to an enzyme, to localize enzyme in tumour deposits. When the antibody-enzyme has cleared from the circulation, a low-toxicity prodrug is given. The prodrug is converted by the tumour-bound enzyme into an active cytotoxic drug. The system has potential to generate a highly potent cytotoxic agent at the tumour site.

A clinical ADEPT system using MFECP1, a recombinant fusion protein consisting of an anti-carcinoembryonic antigen single chain Fv antibody and the bacterial enzyme carboxypeptidase G2, in combination with a bis-iodo phenol mustard prodrug (BIP) has been developed. A previous phase I/II clinical trial established the maximum tolerated dose of a single treatment cycle of this ADEPT system.

In-vivo models with human tumour xenografts indicate that repeated ADEPT treatment with MFECP1/BIP led to greater efficacy without increased toxicity. This thesis aims to establish conditions required for safe and effective ADEPT when using MFECP1/BIP in man. This was achieved by conducting a phase I/II clinical trial of repeat-treatment ADEPT and comparing and combining the results with data from the single-treatment trial. The combined dataset provided mechanistic and clinical information on 43 patients.

Multiple parameters were investigated to examine the likely cause of toxicity and clinical risk factors for its occurrence. Efficacy was evaluated using CT, FDG-PET and serum tumour markers. The nature of the immune response to MFECP1 was investigated and possible strategies to reduce immunogenicity were developed.

Results showed that repeated therapy was feasible in man and did not increase the risk of MFECP1 infusion reactions. At the maximum tolerated total prodrug dose for 2 ADEPT treatments, one of three patients experienced tumour response on FDG-PET imaging. This MD (Res) thesis significantly increases understanding of the conditions required for safe and effective ADEPT.

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List of abbreviations

ADA	anti-drug antibody
ADCC	antibody dependent cellular cytotoxicity
ADEPT	antibody directed enzyme prodrug therapy
ALT	alanine aminotransferase
AML	acute myeloid leukaemia
APC	antigen presenting cell
AST	aspartate aminotransferase
A5B7	CEA-specific murine monoclonal antibody
BIP prodrug	bis-iodo phenol mustard prodrug
CDC	complement dependent cytotoxicity
CDR	complementarity determining region
CEA	carcinoembryonic antigen
CPG2	carboxypeptidease G2
cpm	counts per minute
CRUK	Cancer Research UK
CT	computerized tomography
CTC	common toxicity criteria
DLT	dose limiting toxicity
DMSO	dimethyl sulfoxide
E.coli	Escherichia coli
EDTA	ethylenediaminetetra-acetic acid disodium salt
EGFR	epidermal growth factor receptor
ELS	erythrocyte lysing solution
FACS	fluorescence-activated cell sorting

List of abbreviations (continued)

Fc	fragment crystalizable
FcγR	Fc-gamma receptor
FDG	2-[^{18}F] fluoro-2-deoxy-D-glucose
GMP	Good Manufactoring Practice
HACPA	human anti-CPG2 antibody
HAMA	human anti-mouse antibody
His-tag	histamine tag
HLA	human leucocyte antigen
HPLC	high-performance liquid chromatography assay
IgG	immunoglobulin G
IFN-γ	interferon gamma
mAb	monoclonal antibody
MFECP1	recombinant fusion protein that combines an anti-CEA scFv and the enzyme CPG2
MHC	major histocompatibilty complex
MHRA	Medicines and Healthcare products Regulatory Agency
MTD	maximum tolerated dose
NAbs	neutralizing antibody
NCI-CTC	National Cancer Institute Common Toxicity Criteria
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD$_{PET}$	disease progression on FDG-PET by Green criteria
PEG	polyethylene glycol
PET	positron emission tomography

List of abbreviations (continued)

PI	principal investigator
PPD	tuberculin purified protein derivative
RECIST	Response Evaluation Criteria in Solid Tumours
ROI	region of interest
rpm	revolutions per minute
scFv	single chain Fv antibodies
SD_{PET}	stable diease on FDG-PET by Green criteria
SEB	Staphylococcal enterotoxin B
SI	stimulation index
SPECT	single photon emission computed tomography
SUV	standardized uptake value
TCR	T Cell Receptor
T_h lymphocyte	T-helper lymphocyte
TPD	total prodrug dose
ULN	upper limit of normal
VEGF	vascular endothelial growth factor
V_H	variable heavy chain
V_L	variable light chain
WHO	World Health Organization

Chapter 1

Introduction

1.1 Drug treatment of cancer

Improving cancer treatment is a formidable international challenge. In many cancer types the prognosis of patients with metastatic disease is measured in months rather than years and worldwide annual cancer mortality is estimated to be 7 million (Kamangar *et al.*, 2006).

Systemic drug therapy is an important component of the contemporary multi-modality treatment of cancer. Efficacy has been demonstrated in a number of tumour types both against early disease (i.e. adjuvant therapy) and against metastatic disease. Systemic treatment can be broadly divided into cytotoxic chemotherapy (principally acting by disrupting DNA function) and targeted biological therapy. In patients with metastatic solid tumours the efficacy of cytotoxic chemotherapy has varied markedly between tumour types. For example in patients with testicular germ cell tumours (which are usually very sensitive to cytotoxic chemotherapy) metastatic disease can frequently be eliminated (Honecker *et al*, 2009).

However in most solid tumours cytyotoxic drugs cannot eliminate metastatic disease and benefit is typically measured in modest improvements of median survival. In the most resistant tumour types e.g. hepatocellular carcinoma, cytotoxic chemotherapy has failed to improve median survival.

A major limitation to the effectiveness of conventional chemotherapy is their relative non-selectivity between malignant and non-malignant cells. Toxicity to non-malignant cells can cause significant side effects e.g. myelosuppression, stomatitis. Hence it may not be possible to deliver the

ideal drug concentration to a tumour because the systemic effects of such a concentration would cause excessive toxicity.

Targeted therapy aims to overcome these limitations by destroying tumour while sparing normal tissue. Therapeutic monoclonal antibodies (mAbs) and small molecule drugs (small non-protein compounds) have both demonstrated anti-tumour efficacy. For example small molecule drugs e.g. sunitinib, can be used to inhibit intracellular pathways that promote cancer cell survival (Motzer *et al*, 2007). Small molecule drugs are already playing an important role in oncology but their specificity can be limited by inhibition of multiple targets.

1.1.1 Antibody therapy of cancer

The exquisite specificity of antibodies provides an ideal mechanism for targeted cancer therapy. The earliest use of antibodies to treat human disease was developed by Emil Behring and Paul Ehrlich at the end of the 19th century. They developed a treatment for diphtheria by immunizing sheep and horses with an attenuated form of the causative agent of diphtheria. Serum from the animals was administered to patients in order to provide the patients with a passive immunization of polyclonal antibodies against diphtheria toxin. The success of this serum therapy led to Behring receiving the Nobel Prize for Physiology/Medicine in 1901. However the administration of non-human serum to patients caused some recipients to develop serum sickness characterised by joint pain, rash and enlarged lymph nodes (von Pirquet and Schick, 1905).

In 1975 Kohler and Milstein described an immortal clone of cells that produced antibodies with a single specificity i.e. a monoclonal antibody, produced by fusing an antibody generating mouse spleen cell with a myeloma cell (Kohler and Milstein, 1975). The ability to produce antibodies with a single specificity has produced a revolution in cancer therapy. The structure of immunoglobulin G (IgG), the most abundant form of antibody in serum, is shown in Figure 1.1.
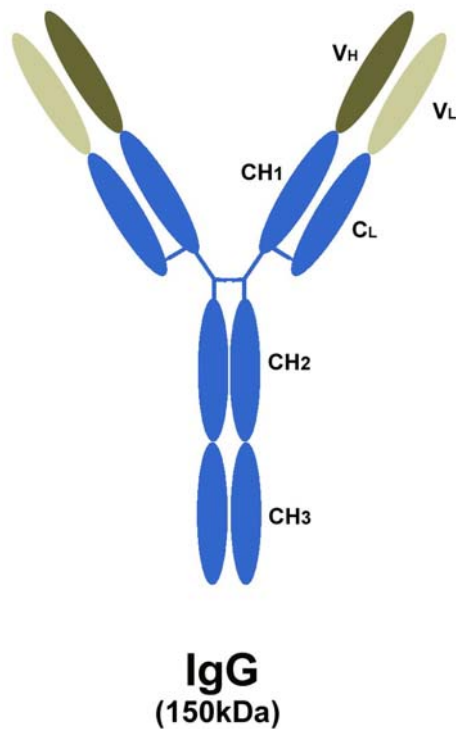
Figure 1.1

Schematic representation of intact immunoglobulin G (IgG). Each IgG molecule consists of 2 identical heavy chains and 2 identical light chains held together with disulfide bonds. Each chain consists of a variable region and a constant region (the heavy chain constant region is sub-divided into CH1, CH2 and CH3). Spatial complementarity between antibody and antigen allows non-covalent binding of the two molecules. V_H: variable heavy region, V_L: variable light region, C_H: constant heavy region, C_L: constant light region. For simplicity the variable and constant regions are labelled on one side only of the molecule.

1.1.2 The development of monoclonal antibodies for cancer therapy

The production of mAbs by Kohler and Milstein soon led to mAbs being used to target cancer cells. Bernstein and colleagues demonstrated that the survival of mice with leukaemia was increased by the administration of a murine mAb targeting thymus cell differentiation antigen on leukaemia cells (Bernstein *et al*, 1980). Similarly clinical trials demonstrated that mAbs could bring benefits to patients. For example administration of a murine IgG mAb targeting GD3 (a disialoganglioside expressed on melanomas) to 21 patients with metastatic melanoma,

produced a significant reduction in tumour size in 4 patients (Vadhan-Raj *et al*, 1988). However 14 patients developed an urticarial skin reaction and 20 of the 21 patients developed specific antibodies against the administered murine mAb i.e. human anti-mouse antibodies (HAMA). These HAMA are generated because foreign murine mAbs are immunogenic i.e. they induce a specific immune response by components of the acquired immune system. The generation of HAMA can increase the risk of anaphylactic reactions, alter mAb clearance and reduce or abolish efficacy. Hence the immunogenicity of murine mAbs significantly limits their therapeutic usefulness.

Over the last two decades recombinant technology has been used to progressively reduce immunogenicity by replacing murine sequences with human sequences, while retaining antigen specificity (Figure 1.2).

The first development in this process of humanization was the generation of chimeric mAbs which consist of a murine variable region combined with a human constant region (Morrison *et al*, 1984). Several antibodies licensed for the treatment of cancer have a chimeric structure e.g. rituximab and cetuximab. In a phase I study of cetuximab in patients with solid tumours anti-drug antibodies (ADAs) were produced in 5% of patients i.e. 1 of 19 (Baselga *et al*, 2000). In general, patients who receive a chimeric mAb are less likely to produce ADAs than patients who receive a murine mAb (Hwang and Foote, 2005).

The murine component of a mAb can be further reduced by the production of humanized mAbs in which the entire antibody has human sequences except for the complementarity determining regions (CDRs) which remain murine (Jones *et al*, 1986). Several humanized mAbs have been licensed for the treatment of cancer e.g. trastuzumab and bevacizumab. The percentage of patients producing ADAs to humanized antibodies is generally low. For example in a total of 37 patients treated with bevacizumab in two phase I clinical trials no patients produced ADAs (Gordon *et al*, 2001) and (Margolin *et al*, 2001). However humanized

mAbs can remain immunogenic. In patients with colon cancer who received the unconjugated humanized mAb A33, 20 of 28 (71%) produced ADAs (Ritter *et al,* 2001).
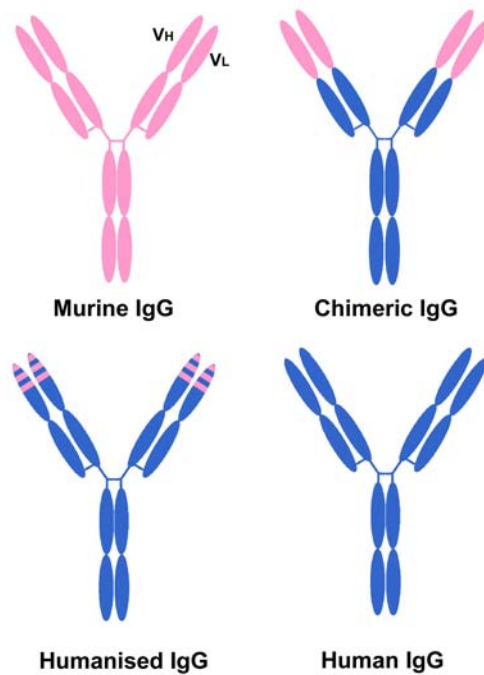
Figure 1.2

Schematic representation of murine, chimeric, humanized and fully human IgG. Murine sequences are shown in pink and human sequences are shown in blue. In chimeric IgG the variable regions are murine whereas in humanized IgG only the CDRs are murine. V_H: variable heavy region, V_L: variable light region

Two different approaches have successfully led to the development of entirely human mAbs. The first has been to create *in vitro* recombinant human antibody libraries (Marks *et al,* 1991). Libraries can be generated using light-chain variable domain (V_L) genes and heavy-chain variable domain (V_H) genes derived from human B-lymphocytes and incorporated into a selection platform such as phage display. Each phage particle expresses a unique antibody on its surface and recombinant antibody

libraries can contain billions of different antibodies. Phage display technology was used to generate adalimumab, a human antibody that targets tumour necrosis factor. Adalimumab is licensed for the treatment of rheumatoid arthritis and psoriatic arthritis (Weinblatt *et al*, 2003; Mease *et al.*, 2005). Human mAbs can also be produced using transgenic mice in which the mouse germ line has been manipulated by introducing human heavy and light chain genes while the endogenous murine antibody genes have been disrupted (Lonberg *et al*, 1994: Green *et al*, 1994). The transgenes function in a manner comparable to native immunoglobulin genes i.e. they can undergo V(D)J joining, heavy-chain class switching and somatic mutation. Panitumumab is a fully human anti-EGFR mAb generated from transgenic mice (Yang *et al*, 2001). Administration of panitumumab to 88 patients with advanced renal cell carcinoma did not cause any ADA formation or hypersensitivity reactions (Rowinsky *et al*, 2004).

The advent of recombinant technology has also allowed the production of a wide variety of antibody derived molecules (Figure 1.3). The structure of these molecules can be tailored to a particular clinical need. For example single chain Fv antibodies (scFv) are univalent recombinant antibody fragments consisting of a variable heavy (V_H) chain joined to a variable light (V_L) chain by a polypeptide linker (Chester *et al*, 1994; Huston *et al*, 1988; Begent *et al*, 1996).

Single chain Fv antibodies (27 kDa) are smaller than intact IgG (150 kDa). Dimers consisting of two scFv molecules (diabodies) can be produced by reducing the length of the polypeptide linker between V_H and V_L which inhibits intramolecular V_H and V_L interaction and instead favours dimer formation. Furthermore fusion proteins can be produced by joining the gene for a recombinant antibody fragment (e.g. scFv) with the gene for a second protein. Fusion proteins can create antibody-like molecules with multiple biological functions. Hence using hybridoma technology, antibody libraries, transgenic mice and recombinant technology high

affinity mAbs and antibody-derived molecules can be produced against an extensive range of targets.
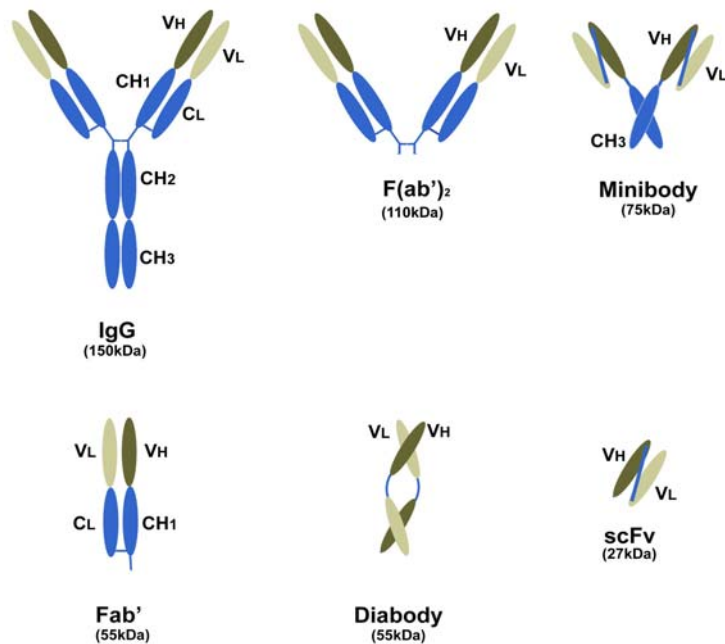
Figure 1.3

Schematic representation of IgG and smaller antibodies produced by enzymatic cleavage (e.g. F(ab')$_2$ and Fab') or recombinant technology (e.g. minibody, diabody and scFv). scFv: single chain fragment variable antibody, V_H: variable heavy region, V_L: variable light region, C_H: constant heavy region, C_L: constant light region.

1.1.3 Delivering a therapeutic antibody to cancer cells

A further consideration in the design of mAb therapy is ensuring that there is sufficient delivery of antibody to the tumour. Antibody delivery to the peripheral rim of the tumour mass is usually good because this region is well perfused by relatively normal blood vessels from adjacent tissue. However the centre of a tumour is frequently poorly perfused and this limits antibody delivery. Furthermore antibody is required to diffuse throughout the tumour mass. Several characteristics of the antibody can affect this process, e.g. size, affinity and valency. These factors affect the total quantity of antibody targeted to a tumour, the tumour to normal

tissue antibody ratio, the depth of tumour penetration and the time course of tumour targeting / antibody clearance. Yokota and colleagues demonstrated that small antibody formats e.g. scFv (27kDa) penetrate more deeply and rapidly into the tumour mass than larger antibody formats e.g. Fab' (55kDa), F(ab')$_2$ (110kDa) and 150kDa IgG (Yokota *et al*, 1992). Although small antibodies achieve rapid tumour penetration they are also cleared quickly from the systemic circulation. Single chain Fv antibodies for example, undergo first pass renal clearance. This fast clearance can reduce the proportion of injected antibody that reaches each gram of tumour tissue. For example Milenic and colleagues demonstrated that 24 hours after administration, the percentage of injected antibody per gram of tumour tissue (%IDg^{-1}) was less with scFv or Fab' than with IgG or F(ab')$_2$ (Milenic *et al*, 1991). The ideal affinity of an antibody appears to be complex since low affinity antibodies may achieve reduced tumour localization whereas very high affinity antibodies may fail to diffuse from the perivascular region (Adams *et al*, 1998; Adams *et al*, 2001). Adams and colleagues have also demonstrated that for antibodies of similar size increased valency increases tumour retention (Adams *et al*, 2006).

1.1.4 Unconjugated antibodies

There are two broad mechanisms by which an unconjugated mAb can inhibit or kill a cancer cell. The first is that antibody binding to antigen can disrupt the biological function of the antigen. The second is that bound antibody can interact with components of the immune system leading to an immunological assault on a targeted cell. These two mechanisms reflect the dual action of naturally occurring antibodies i.e. target recognition and interaction with complement and immune cells. The target antigen may be an antigen only found on cancer cells (tumour specific antigen) or an antigen that is over-expressed on malignant tissue but present to a lesser extent on normal tissue (tumour associated antigen). For example the mAb cetuximab targets the tumour associated antigen EGFR (epidermal growth factor receptor). An alternative strategy has been to target a soluble growth factor that promotes tumour survival.

Vascular endothelial growth factor (VEGF) is produced by many types of tumour and stimulates angiogenesis. Bevacizumab is a humanised mAb that binds to VEGF and inhibits angiogenesis. Bevacizumab was the first anti-angiogenesis mAb to increase survival in cancer patients (Hurwitz *et al*, 2004).

Mabs can also kill cancer cells by effects exerted through the Fc (fragment crystalizable) portion of the antibody. After binding to a target cell the CH2 and CH3 regions of the mAb can bind to Fc-gamma receptors (FcγR) on immune cells (e.g. natural killer cells, neutrophils, macrophages) leading to antibody dependent cellular cytotoxicity (ADCC). Similarly following mAb-antigen binding, the interaction of two or more CH2 regions with the complement component C1q can activate the complement cascade resulting in complement dependent cytotoxicity (CDC). Activation of ADCC and CDC appears to be important in the mechanism of action of the anti-CD20 mAb rituximab which is licensed for the treatment of lymphoma. The response rate and time to disease progression of patients receiving rituximab for follicular lymphoma are significantly higher in individuals with certain FcγRIIa and FcγRIIIa polymorphisms (Weng *et al*, 2003). Furthermore in mice injected with lymphoma cells the therapeutic effect of rituximab is dependent on the animals having a functioning complement system. In knockout mice that are deficient in the complement component C1q the activity of rituximab is completely abolished (Di Gaetano *et al*, 2003). ADCC also appears to be important in the mode of action of the anti-HER2 mAb trastuzumab. In mice bearing human breast cancer xenografts the efficacy of trastuzumab is enhanced in mice deficient in the inhibitory Fc receptor FcγRIIb and reduced in mice deficient in activating Fc receptors (Clynes *et al*, 2000).

1.1.5 Conjugated antibodies

It is possible to combine a mAb to a second entity so that the resulting conjugate mAb has a second biological function not present on naturally occurring antibodies e.g. radioactivity. Conjugated mAbs therefore consist

of an antibody component for tumour targeting and a payload that is responsible for cytotoxicity. Monoclonal antibodies can be conjugated to a variety of effectors including radioisotopes, cytotoxic drugs, enzymes or immunotoxins. Conjugation of the two entities can be achieved via a chemical bond or by production of a recombinant fusion protein. Two radioimmunotherapy conjugates are licensed for the treatment of lymphoma. Both use a β-emitting radioisotope and target the cell surface molecule CD20, which is expressed on normal and malignant B cells. Yttrium-90 ibritumomab tiuxetan (Zevalin®) consists of the murine anti-CD20 mAb ibritumomab (the murine version of rituximab) covalently bound via the chelator tiuxetan to ^{90}Y (Gordon *et al*, 2004; Witzig *et al*, 2002). Tositumomab (Bexxar®) is an ^{131}iodine labelled anti-CD20 murine IgG mAb (Kaminski *et al*, 2005). Cytotoxic drugs can also be conjugated to antibodies to selectively deliver the cytotoxic agent to a tumour. Gemtuzumab ozogamicin (Mylotarg®) is a humanised anti-CD33 mAb conjugated with the cytotoxic calicheamicin. When gemtuzumab ozogamicin binds to CD33 the resulting complex is internalized into the cell and the cytotoxic calicheamicin moiety is released by hydrolysis of the mAb-calicheamicin link (Larson *et al*, 2005). Several unconjugated and conjugated mAbs are licensed to treat haematological or solid tumours (Table 1.1).

1.2 ADEPT

Antibody Directed Enzyme Prodrug Therapy (ADEPT) uses an antibody-enzyme conjugate to deliver an enzyme to a tumour. When the antibody-enzyme conjugate has bound to the tumour and cleared from the circulation, a low-toxicity prodrug is given that is converted by the tumour-bound enzyme into an active cytotoxic (Figure 1.4). Many molecules of the active cytotoxic drug can be produced by each targeted enzyme.

				Table 1.1				
				Monoclonal antibodies approved for the treatment of cancer				
Antibody type	**Generic name**	**Trade name**	**Target**	**Murine/human composition**	**Oncology indication**	**Proposed mechanism of action**	**Year of FDA approval**	**Reference**
Unconjugated	Rituximab	MabThera, Rituxan	CD20	Chimeric	NHL, CLL	ADCC, CDC	1997	Coiffier *et al.*, 2002; Forstpointner *et al.*, 2004; Marcus *et al.*, 2005; Byrd *et al.*, 2003.
	Trastuzumab	Herceptin	HER-2	Humanized	Breast cancer	Interferes with receptor signal transduction, ADCC	1998	Slamon *et al.*, 2001; Piccart-Gebhart *et al.*, 2005 Romond *et al.*, 2005
	Alemtuzumab	Campath	CD52	Humanized	CLL	Uncertain: possibly CDC	2001	Elter *et al.*, 2005
	Bevacizumab	Avastin	VEGF	Humanized	CRC, Breast cancer, NSCLC, RCC	Inhibits angiogenesis	2004	Hurwitz *et al.*, 2004 Sandler *et al.*, 2006 Yang *et al.*, 2003
	Cetuximab	Erbitux	EGFR	Chimeric	CRC SCCHN	Interferes with receptor signal transduction	2004	Cunningham *et al.*, 2004 Bonner *et al.*, 2006
	Panitumumab	Vectibix	EGFR	Human	CRC	Interferes with receptor signal transduction	2006	Van Custem *et al.*, 2007
Conjugated	Gemtuzumab ozogamicin	Mylotarg	CD33	Humanized	AML	Conjugated to the toxin calicheamicin.	2000	Larson *et al.*, 2005
	Ibritumomab tiuxetan	Zevalin	CD20	Murine	NHL	Conjugated with [90]Yttrium.	2002	Gordon *et al.*, 2004 Witzig *et al.*, 2002
	Tositumomab	Bexxar	CD20	Murine	NHL	Conjugated with [131]Iodine.	2003	Kaminski *et al.*, 2005

VEGF: vascular endothelial growth factor, EGFR: epidermal growth factor receptor, CRC: colorectal carcinoma, NSCLC: non-small cell lung cancer, RCC: renal cell carcinoma, SCCHN: squamous cell carcinoma of head and neck, NHL: non-Hodgkins lymphoma, CLL: chronic lymphocytic leukaemia, AML: acute myeloid leukaemia, ADCC: Antibody Dependent Cellular Cytotoxicity, CDC: Complement-Dependent Cytotoxicity, FDA: United States food and drug administration.
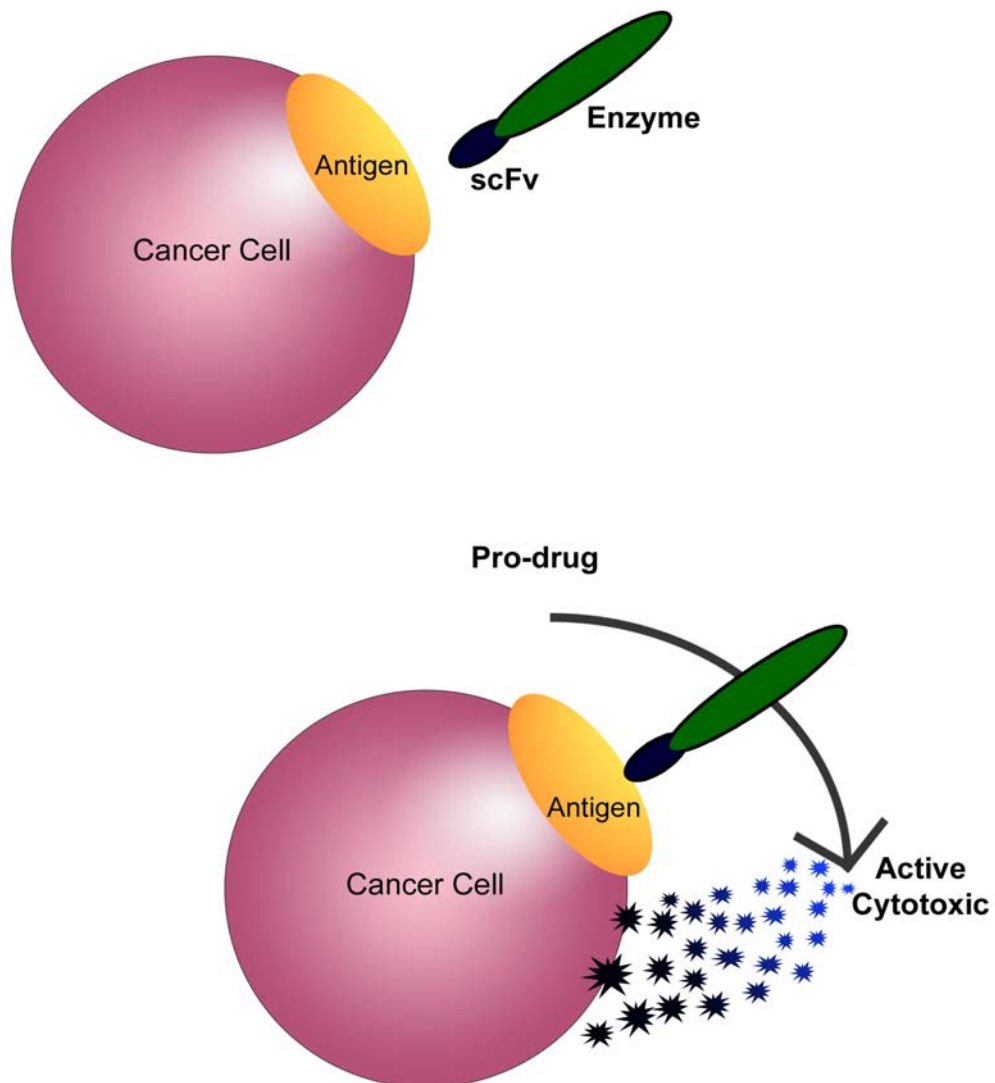
Figure 1.4

Antibody directed enzyme prodrug therapy (ADEPT). ADEPT delivers an antibody-enzyme conjugate to tumour. When the antibody-enzyme conjugate has cleared from the systemic circulation, a low toxicity prodrug is given that is converted by the tumour-bound enzyme into an active cytotoxic. The figure shows an antibody-enzyme composed of a single chain Fv antibody (scFv) however other antibody formats could in principle be used.

The aim of ADEPT is to maximize the dose of cytotoxic drug in tumour and to minimize the concentration of cytotoxic drug in non-tumour. The principle of ADEPT is generic and could be applied to a variety of tumour associated antigens using a number of possible enzyme/prodrug combinations.

Results from pre-clinical experiments and ADEPT clinical trials have explored the principal requirements of ADEPT. These include:

- **A suitable antigen target**
- **An antibody to selectively delivery antibody-enzyme to tumour**
- **Clearance of unbound antibody-enzyme from the circulation**
- **A prodrug that is converted by the enzyme into a cytotoxic drug**
- **An active drug that causes minimal systemic toxicity**
- **Patient safety and clinical efficacy**
- **Minimal immunogenicity of the antibody-enzyme so that treatment can be given repeatedly**

These requirements are discussed in more detail below, drawing particular attention to the results of ADEPT clinical trials. Details of ADEPT clinical trials are also shown in Table 1.2.

1.2.1 Antigen target for ADEPT

Pre-clinical *in vivo* ADEPT experiments have targeted a variety of tumour associated antigens including carcinoembryonic antigen (CEA) (Sharma *et al*, 2005), L6 antigen (Senter *et al*, 1989), CD20 (Senter *et al*, 1989), Ep-CAM1 (Wolfe *et al*, 1999), seminoprotein (Hao *et al*, 2006), β-human chorionic gonadotrophin (Springer *et al*, 1991) and TAG-72 (Alderson *et al*, 2006).

Table 1.2

Components of clinical ADEPT trials

Antigen Target	Antibody-enzyme product	Enzyme	Clearing antibody	Prodrug	Reference(s)
CEA	F(ab')$_2$ fragment of murine mAb (A5B7) chemically conjugated to CPG2	CPG2	Galactosylated murine mAb (SB43-gal) binds and inactivates CPG2	Benzoic acid mustard prodrug (CMDA)	Bagshawe *et al*, 1995. Bagshawe and Begent, 1996.
CEA	"	"	"	"	Napier *et al*, 2000
CEA	"	"	No	Bis-iodo phenol mustard prodrug (BIP prodrug)	Francis *et al*, 2002
CEA	Glycosylated recombinant fusion protein (MFECP1) composed of scFv and CPG2	"	No	Bis-iodo phenol mustard prodrug (BIP prodrug)	Mayer *et al*, 2006

ADEPT: antibody directed enzyme prodrug therapy, mAb: monoclonal antibody, CPG2: carboxypeptidaseG2, CEA: carcinoembryonic antigen, scFv: single chain variable antibody fragment

CEA has been the chosen target in clinical trials of ADEPT (Bagshawe *et al*, 1995; Bagshawe and Begent, 1996; Napier *et al*, 2000; Francis *et al*, 2002; Mayer *et al*, 2006). CEA is an oncofoetal antigen and is expressed by a variety of adenocarcinomas in particular colorectal carcinomas. Normal expression of CEA in adult tissue is confined to the luminal surface of the gut, where it is inaccessible to antibodies given into the circulation, provided that IgA is not used.

A feature of ADEPT systems is that activated prodrug can kill not only the antigen positive cell to which the antibody-enzyme is bound, but also other nearby tumour cells. This is known as the bystander-effect. There is *in vivo* evidence for killing of antigen negative tumour cells following ADEPT treatment (Cheng *et al*, 1999). This effect may help overcome the challenges of heterogeneous antigen distribution and inadequate tumour penetration by therapeutic mAbs.

1.2.2 Antibody component of antibody-enzyme product

The antibody component of the antibody-enzyme product aims to selectively target all regions of the tumour. Initial *in vivo* ADEPT experiments used intact IgG (Senter *et al*, 1988) or a F(ab')$_2$ fragment (Bagshawe *et al*, 1988) chemically conjugated to an enzyme. A chemical conjugate of a F(ab')$_2$ and enzyme has been used in clinical trials (Bagshawe *et al*, 1995; Bagshawe and Begent, 1996; Napier *et al*, 2000; Francis *et al*, 2002). However chemical conjugation can create difficulties in producing a uniform product. This challenge can be overcome by creating a recombinant fusion protein produced by fusing the gene encoding a selected scFv with the gene for a chosen enzyme. The fusion protein allows production of a uniform therapeutic. Furthermore using recombinant technology the amino acid sequence can be modified to meet clinical requirements. A recombinant fusion protein (MFECP1) that combines an anti-CEA scFv and the enzyme carboxypeptidease G2 (CPG2), has been an important development in ADEPT clinical trials (Mayer *et al*, 2006). To produce MFECP1 a library of scFvs was produced from the spleen cells of mice immunised with CEA and high

affinity antibodies were selected from this library (Chester *et al*, 1994). The scFv MFE-23 was chosen for clinical use and successful tumour localisation of MFE-23 was demonstrated in patients by radioimmunoscinitgraphy (Begent *et al*, 1996) and a trial of radioimmuno-guided surgery (Mayer *et al*, 2000).

1.2.3 Clearance of antibody-enzyme from the circulation

At the time of prodrug administration it is necessary that there is adequate antibody-enzyme on the tumour (to achieve efficacy) and as little antibody-enzyme as possible in non-tumour tissue (to minimize off-target prodrug activation). The quantity of antibody-enzyme at any particular location varies with time. After intravenous administration of antibody-enzyme its concentration falls in the circulation as it is cleared. In the tumour, antibody-enzyme concentration rises following its administration (as the product diffuses into the tumour) and then subsequently falls. Hence the timing of prodrug administration is crucial; too soon and off-target activation may occur, too late and the absolute quantity of antibody-enzyme on the tumour may be inadequate.

One strategy to reduce antibody-enzyme in the circulation is to administer a clearing antibody. A clearing antibody is given after the antibody-enzyme and is used to neutralize antibody-enzyme that has not bound to tumour and remains in the bloodstream. This technique has been used in clinical trials (Bagshawe *et al*, 1995; Bagshawe and Begent, 1996; Napier *et al*, 2000). In one phase I clinical trial administration of antibody-enzyme (an anti-CEA F(ab')$_2$ chemically conjugated to CPG2) was followed by a clearing antibody capable of neutralizing the catalytic activity of CPG2 (Napier *et al*, 2000). Tumour biopsies were taken on 5 patients following administration of clearing antibody and immediately before prodrug administration. The median tumour to plasma ratio of enzyme activity was >10,000 to 1. The importance of the clearing antibody can be seen from the results of a subsequent phase I clinical trial in which the same antibody-enzyme was used but a clearing antibody was not used. In this trial the median tumour to plasma ratio of enzyme was 0.4 to 1 (Francis *et*

al, 2002). Hence a clearing antibody appeared to be an effective mechanism for achieving high tumour to non-tumour enzyme ratios. However the use of a clearing antibody also made ADEPT more complex.

An alternative strategy was the development of MFECP1 a novel antibody-enzyme fusion protein that cleared more rapidly. This was achieved by production of MFECP1 in the yeast *Pichia pastoris* which resulted in mannosylation of the fusion protein. Mannose structures on MFECP1 bind to mannose receptors on cells in the liver and spleen resulting in faster clearance of the fusion protein (Kogelberg *et al*, 2007). In mice bearing human xenografts mannosylated fusion protein achieved a greater tumour to plasma ratio than non-mannosylated fusion protein (Medzihradszky *et al*, 2004). Furthermore maximal tumour targeting occurred sooner with mannosylated fusion protein than non-mannosylated fusion protein i.e. 4hours versus 48 hours.

1.2.4 Enzyme in ADEPT systems

Enzymes from a variety of sources have been examined during *in vivo* testing of ADEPT systems. One approach is to construct an antibody-enzyme product that contains a human enzyme. Administration of the antibody-enzyme product is then followed by a prodrug that is activated by the human enzyme. The system aims to achieve a significantly higher concentration of the human enzyme in tumour compared to non-tumour tissue.

Several ADEPT systems using human enzymes have been examined *in vivo* i.e. alkaline phosphatase (Senter *et al*, 1988; Senter *et al*, 1989; Wallace *et al*, 1991; Haisma *et al*, 1992), carboxypeptidase-A (Hao *et al*, 2006) and β-glucuronidase (Bosslet *et al*, 1994; Houba *et al*, 1996; Houba *et al*, 2001; Biela *et al*, 2003). These systems have never been tested in patients. The concern with using a human enzyme is that a patient's endogenous enzyme could activate prodrug in non-tumour tissue. In theory this challenge can be overcome if the endogenous enzyme is confined intracellularly and the prodrug is incapable of

diffusing into the cell. However in the complex reality of the clinical environment it is difficult to be certain that the situation would be so absolute. Even low concentrations of the enzyme in blood could cause significant prodrug activation.

In ADEPT the ideal enzymatic scenario is one in which prodrug can only be catalysed by the tumour bound antibody-enzyme i.e. prodrug cannot be catalysed by the patient's naturally occurring enzymes. The most frequently applied method to achieve this is to use a bacterial enzyme with no mammalian homologue such as cytosine deaminase (Wallace *et al*, 1994), β-lactamase or CPG2.

The bacterial enzyme β-lactamase has been incorporated into several *in vivo* ADEPT systems (Meyer *et al*, 1993; Vrudhula *et al*, 1993; Rodrigues *et al*, 1995; Kerr *et al*, 1999; Cortez-Retamozo *et al*, 2004; Alderson *et al*, 2006). Alderson and colleagues used a fusion protein of a scFv (targeting the tumour associated antigen TAG-72) and β-lactamase (Alderson *et al*, 2006). Cleavage of a cephalosporin-melphalan prodrug by β-lactamase released the cytotoxic drug melphalan. In mice bearing human colorectal carcinoma xenografts (LS174T) tumour to plasma activity ratios of 1000 to 1 were achieved. In mice that received fusion protein followed by prodrug at 300mg/kg, tumour volume was significantly less than control mice and there was a reduction in tumour volume compared to pre-treatment. (In contrast minimal efficacy was observed following the systemic administration of the licensed cytotoxic drug irinotecan alone.) However the ADEPT system caused significant toxicity in the mice i.e. following a prodrug dose of 300mg/kg the mice lost 14% of their body mass. The extent of the weight loss was much greater following ADEPT than following prodrug alone and this suggested that leak-back of melphalan into non-tumour tissue was responsible for most of the toxicity. This system has not undergone clinical testing. It is possible that toxicity due to leak-back of melphalan could also occur in patients, since the terminal half life of melphalan in patients is 108 minutes +/- 21 minutes (melphalan summary of product characteristics, GlaxoSmithKline).

The bacterial enzyme CPG2 has been extensively investigated *in vivo* and is the only enzyme to have been used in ADEPT clinical trials. CPG2 is a metalloenzyme produced by the bacteria *Pseudomonas sp.* strain RS16. It is a homodimer and each subunit of the dimer has a catalytic domain containing two zinc ions (Rowsell *et al*, 1997). CPG2 cleaves the C-terminal glutamate from folic acid and its analogues e.g. the cytotoxic drug methotrexate. Recombinant CPG2 is used in oncology in two scenarios.

One use of CPG2 is the emergency treatment of excessively high methotrexate levels in patients receiving high-dose methotrexate. In this indication CPG2 (which is also known by the international nonproprietary name glucarpidase) is used to cleave methotrexate in patients with delayed methotrexate excretion (Buchen *et al*, 2005).

CPG2 has also been extensively used in ADEPT systems. CPG2 is useful in ADEPT for 2 reasons. Firstly it has no human enzyme equivalent with the same substrate specificity. Secondly it can cleave the C-terminal glutamate from benzyl nitrogen mustards prodrugs. CPG2 has been used in all ADEPT clinical trials. Initially it was used with the prodrug CMDA (Bagshawe *et al*, 1995; Bagshawe and Begent, 1996; Napier *et al*, 2000) and subsequently with a bis-iodo phenol mustard prodrug (BIP prodrug) (Francis *et al*, 2002; Mayer *et al*, 2006).

The principal challenge of using CPG2 in the clinic is that it frequently causes a specific immune response in patients. This is because CPG2 is a bacterial enzyme and it is recognized by the immune system of patients as being foreign. The immune response to foreign proteins is characterized by the activation of specific T-helper lymphocytes (T_h lymphocytes) and the production of human antibodies directed against the protein. ADAs to non-human therapeutic proteins can neutralize the biological activity of the protein, alter the clearance of the protein and increase the risk of adverse events. Immunological issues are an

important consideration in the development of ADEPT. The immune response to ADEPT using CPG2 is discussed in detail in Chapter 5.

1.2.5 Prodrug in ADEPT systems

There are a number of characteristics required in an ideal prodrug:

- **prodrug is stable *in vivo* and water soluble**
- **prodrug is non-toxic**
- **prodrug is readily activated into cytotoxic drug by the antibody-enzyme product**
- **activated prodrug is many times more toxic than the prodrug**
- **activated prodrug has a steep dose-cytotoxicity curve i.e. the increased dose of cytotoxic drug generated by ADEPT should lead to increased efficacy.**
- **minimal leak-back of activated prodrug into non-tumour tissue**

Many different prodrugs have been used in pre-clinical ADEPT testing. These prodrugs can be divided into two categories, prodrugs of licensed cytotoxic drugs and prodrugs of bespoke cytotoxic drugs developed for ADEPT. For example prodrugs of methrotrexate (Hao *et al*, 2006), doxorubicin (Biela *et al*, 2003) and melphalan (Alderson *et al*, 2006) have been investigated *in vivo*. However prodrugs of licensed cytotoxic drugs have never been used in ADEPT clinical trials. The relatively long half lives of licensed cytotoxic drugs may increase the risk of side-effects caused by drug leaking back into non-tumour tissue.

ADEPT clinical trials have used prodrugs of bespoke alkylating agents. Alkylating agents were chosen because *in vitro* they maintain a log-linear tumour cell kill over a wide range of doses, whereas with non-alkylating agents there was a plateau of cell kill at higher doses (Frei *et al*, 1988). Hence ADEPT is designed to produce tumour-localized high-dose chemotherapy. Whether the ADEPT approach to high-dose

chemotherapy can increase efficacy is unknown and it should be noted that non-ADEPT high-dose chemotherapy regimes have not increased survival in common solid epithelial cancers (Hortobagyi, 2004).

The first prodrug used in ADEPT clinical trials was CMDA, a benzoic acid mustard prodrug that was converted by CPG2 into the alkylating agent CJS11 (Bagshawe *et al*, 1995; Bagshawe and Begent, 1996; Napier *et al*, 2000). Napier and colleagues reported on 10 patients with advanced CEA expressing malignancy who were treated with a chemical conjugate (anti-CEA F(ab')$_2$ combined with CPG2) followed by a clearing antibody and subsequently CMDA prodrug (Napier *et al*, 2000). Although no active enzyme was found in plasma at the time of prodrug administration, 40% of patients experienced grade 3/4 neutropaenia and thrombocytopaenia. The half life of the activated prodrug was 36 minutes +/- 14 minutes (Martin *et al*, 1997). It is likely that myelosuppression was caused by leak-back of activated prodrug into non-tumour tissues.

To address these challenges a new prodrug for ADEPT clinical trials was selected. This BIP prodrug (previously referred to as ZD2767P) was chosen based on effective conversion of BIP prodrug by CPG2 and the very short half life of the activated prodrug (Springer *et al*, 1995).

BIP prodrug has since been used in 2 ADEPT clinical trials (Francis *et al*, 2002; Mayer *et al*, 2006). Unactivated BIP prodrug appears to cause little toxicity since when it was given to 3 patients (without antibody-enzyme) only grade 1 adverse events occurred (Francis *et al*, 2002).

1.3 Single ADEPT treatment with MFECP1 and BIP prodrug
It was hypothesized that MFECP1 could produce a high tumour to plasma enzyme activity ratio without the use of a clearing antibody, due to its rapid clearance from the circulation. Furthermore by combining MFECP1 with BIP prodrug it was hypothesized that adverse events could be reduced and efficacy increased.

The *in vivo* characteristics of ADEPT using a glycosylated fusion protein (anti-CEA scFv and CPG2) and BIP prodrug were investigated in nude mice bearing human colon carcinoma xenografts (Sharma *et al*, 2005). The half life of the fusion protein in mice bearing LS174T xenografts was 30 minutes. Six hours after administration of fusion protein the tumour to plasma ratios of CPG2 activity in LS174T and SW1222 xenografts were 1400 to 1 and 339 to 1 respectively. The lowest tumour to non-tumour CPG2 activity ratio at 6 hours was 43 to 1 (tumour versus spleen in mice with SW1222 xenografts). Selective localization of CPG2 in tumour tissue was also demonstrated by immunohistochemistry 6 hours after fusion protein administration. A single ADEPT treatment consisting of intravenous fusion protein followed by BIP prodrug (given at 6, 7 and 8 hours intra-peritoneally) significantly delayed growth of LS174T and SW1222 xenografts compared to no treatment. Administration of fusion protein alone or BIP prodrug alone did not slow tumour growth. The mean percentage weight loss following a single ADEPT treatment was <2%.

1.3.1 Phase I clinical trial of a single ADEPT treatment with MFECP1 and BIP prodrug

Following these encouraging pre-clinical results a phase I clinical trial of single ADEPT treatments, using MFECP1 and BIP prodrug, was conducted (Mayer *et al*, 2006). The trial recruited 31 patients with advanced CEA expressing malignancy; all had received previous chemotherapy. Gamma camera imaging of patients who received MFECP1 labelled with ^{123}I or ^{131}I showed that MFECP1 cleared rapidly through the liver. The pharmacokinetics of MFECP1 was measured after administration of $3000U/m^2$ (α half life 0.44h, β half life 1.96h) and $5000U/m^2$ (α half life 0.52h, β half life 4.6h). Adverse events related to MFECP1 were mild i.e. grade 1 only. Analysis of tumour biopsies by immunohistochemistry with anti-CPG2 antibodies showed MFECP1 up to 15hours after $3000U/m^2$ and up to 19hours after $5000U/m^2$. The median half life of the BIP prodrug was 10 minutes. Details of treatment received are shown in Table 1.3.

Number of treatments	MFECP1 dose per treatment (U/ml)	Total prodrug dose (mg/m^2)	Number of patients treated
		\<center\>Table 1.3\</center\>	

Table 1.3

Treatment received in phase I trial of single-treatment ADEPT with MFECP1 and BIP prodrug

Number of treatments	MFECP1 dose per treatment (U/ml)	Total prodrug dose (mg/m^2)	Number of patients treated
1	5000	37	6
		48	3
		63	2
		101	2
		202	2
		403	1
		806	4
		1613	2
		3226	1
	3000	806	3
		1613	2
	1500	600	3

Data from Mayer A *et al.*, 2006 and CRUK database

Three dose-levels of MFECP1 were evaluated: 5000U/m^2, 3000U/m^2 and 1500U/m^2. At an MFECP1 dose of 5000U/m^2 and with prodrug administered when serum CPG2 activity ≤ 0.005 U/ml, total prodrug dose (TPD) was escalated from 37.26mg/m^2 to 3225.6 mg/m^2. DLT occurred in a patient who received a TPD of 3225.6 mg/m^2 (grade 4 elevation of AST,

ALT and creatinine) and grade 3 thrombocytopaenia. These toxicities were reversible but temporary haemodialysis was required.

One patient (patient 3) received prodrug when serum CPG2 activity was > 0.005 U/ml (serum CPG2 activity 0.0167 U/ml). The patient received a total prodrug dose of 37mg/m^2 and developed grade 3 neutropaenia and thrombocytopaenia. (Initial patients had been permitted to receive prodrug when serum CPG2 activity was <0.05U/ml.) Following this toxicity at the lowest prodrug dose level, the serum CPG2 activity that permitted prodrug administration was lowered from 0.05U/ml to 0.005U/ml.

In patients who received MFECP1 5000U/m^2 and prodrug when serum CPG2 activity was ≤ 0.005U/ml the median time interval between end of MFECP1 infusion and start of prodrug was 19.1 hours (range 4.9 to 37.2 hours). However in mice studies favourable tumour to normal tissue MFECP1 ratios were seen 6 hours after administration of MFECP1. In order to investigate whether a reduced dose of MFECP1 would permit earlier prodrug administration, MFECP1 doses of 3000U/m^2 and 1500U/m^2 were evaluated.

When MFECP1 was given at 3000U/m^2 and prodrug was given at a serum CPG2 activity ≤ 0.005U/ml the median time interval between end of MFECP1 infusion and start of prodrug was reduced to 13.0 hours (range 11.6 to 14.9 hours). At a TPD of 806mg/m^2 no grade 3 or 4 toxicities occurred. At a TPD of 1613mg/m^2, DLT occurred (grade 4 elevations of ALT and AST, grade 3 fatigue).

MFECP1 at 1500U/m^2 was investigated at a TPD of 600mg/m^2 in 3 patients. Prodrug was given at a median of 8.4 hours (range 7.9 to 23.8 hours) after the end of the MFECP1 infusion. Grade 3 thrombocytopaenia and fatigue occurred in 1 of the 3 treated patients.

The maximum tolerated dose (MTD) of a single ADEPT treatment was determined as MFECP1 $3000U/m^2$ followed by a TPD of $806mg/m^2$, given when serum CPG2 activity ≤ 0.005U/ml. With this regime no grade 3 or 4 toxicities occurred and tumour biopsies showed evidence of MFECP1 localization and cross-linking of tumour DNA consistent with exposure to activated prodrug (Mayer *et al*, 2006).

The presence of human anti-CPG2 antibodies (HACPA) was measured before and during follow up. Complete HACPA data was available on 30 patients (no follow up HACPA data was available on patient 31 due to clinical deterioration because of disease progression). Twelve of 30 patients became HACPA positive (40%).

No complete or partial responses were seen on computerized tomography (CT) imaging by RECIST criteria (Response Evaluation Criteria in Solid Tumours; Therasse *et al*, 2000). One patient with metastatic peritoneal carcinoma had a 9% reduction in tumour size by RECIST methods on Day 29 on CT imaging.

1.4 Repeat ADEPT treatment using MFECP1 and BIP prodrug

How can the efficacy of ADEPT using MFECP1 and BIP prodrug be increased? One possibility is to give repeat ADEPT treatments. Evidence to support the technique of repeatedly treating a cancer with a cytotoxic drug comes from *in vitro* experiments using murine L1210 leukaemia cells (Skipper, 1971). The results demonstrate that a cytotoxic drug displays logarithmic cell kill kinetics i.e. when cells receive repeated chemotherapy treatments a constant percentage of tumour cells are killed per treatment, rather than a constant number of cells. In a drug-sensitive cell line all the cancer cells can be eliminated following repeat treatments. The model simplifies a complex *in vivo* system where there may be heterogeneity of drug delivery and heterogeneity of cell sensitivity. However it emphasizes the principle that when a cytotoxic system is unable to kill all cancer cells with a single treatment, repeat treatments are necessary to achieve the

maximum reduction in cancer cell numbers. This principle lies behind the dominance of repeat treatment schedules in oncology.

1.4.1 Pre-clinical investigation of repeat ADEPT treatment

Pre-clinical *in vivo* testing of repeat treatments of ADEPT was performed in nude mice bearing human colon carcinoma xenografts (Sharma *et al*, 2005). Each treatment consisted of intravenous glycosylated fusion protein followed by BIP prodrug given at 6, 7 and 8 hours intra-peritoneally. Mice bearing LS174T xenografts received 9 ADEPT treatments over 3 weeks and mice bearing SW1222 xenografts received 10 ADEPT treatments over 4 weeks. Control mice received repeat treatment of prodrug alone, repeat treatment of fusion protein alone, a single ADEPT treatment or no treatment. Therapeutic efficacy was significantly greater with repeat treatments compared to a single treatment in both types of xenograft. Repeat ADEPT treatments induced growth delay in LS174T xenografts and tumour regression in SW1222 xenografts. Weight loss following repeat ADEPT treatments was <2% in both xenograft types.

1.4.2 Phase I/II clinical trial of repeat ADEPT treatment with MFECP1 and BIP prodrug

It is hypothesized that in patients, repeat ADEPT treatments will also deliver greater efficacy compared to a single ADEPT treatment. However to date, no patient has ever received more than one treatment of ADEPT with MFECP1 and BIP prodrug.

This MD (Res) thesis aims to investigate repeat ADEPT treatments and to increase understanding of the immune response to MFECP1. Hence at the centre of this thesis is a phase I/II clinical trial of repeat ADEPT treatments using MFECP1 and BIP prodrug. In addition laboratory experiments were conducted to increase understanding of the immune response to MFECP1.

The thesis will focus on 4 questions:

1)   Can repeat ADEPT be given safely?

2)   Is there preliminary evidence that repeat ADEPT has efficacy and how is this best measured?

3)   What clinical factors affect toxicity and efficacy?

4)   What is the nature of the immune response to MFECP1 and is this affected by repeat administration of ADEPT?

1.5 Safety of repeat ADEPT treatment

The principal aim of the phase I/II clinical trial is to investigate whether repeat ADEPT treatments can be given safely. At the MTD of a single ADEPT treatment ($3000U/m^2$ MFECP1 with a TPD of $806mg/m^2$) the most severe grade of adverse event was grade 2 (Table 1.4).

Table 1.4	
Most severe adverse events (all grade 2) at the maximum tolerated dose of a single ADEPT treatment with MFECP1 and BIP prodrug	
Adverse event	**Number of patients (%)**
Fatigue	2 (66%)
Nausea	1 (33%)
Vomiting	1 (33%)
Leucocytes (total WBC)	1 (33%)
Neutrophils / granulocytes	1 (33%)
Haemoglobin	1 (33%)
Data on patients 26, 27 and 28. Data from CRUK database	

It is not known what adverse events and what severity of adverse events may occur after repeat ADEPT treatments. It is possible that repeat treatment could increase the severity or frequency of the toxicities seen following a single treatment. In addition new adverse events, not seen with single treatment, could emerge.

This MD (Res) thesis aims to use safety data from the repeat-treatment ADEPT clinical trial to answer important clinical questions: What is the maximum number of tolerated treatments? What is the maximum tolerated total prodrug dose?

Understanding ADEPT toxicity is likely to be more complex than understanding toxicity in a conventional single-agent phase I clinical trial. This is due to two reasons. Firstly more than one drug is administered i.e. MFECP1 and BIP prodrug. Furthermore a third drug (activated prodrug) is generated. Hence any toxicity could be due to MFECP1, BIP prodrug or activated prodrug.

Secondly the use of more than one drug creates multiple variables, all of which could affect toxicity:

1. Dose of MFECP1
2. Serum CPG2 activity at time of prodrug administration
3. Time interval between MFECP1 and prodrug
4. Total prodrug dose
5. Number of ADEPT treatments

In addition toxicity may also be dependent on patient characteristics e.g. organ function.

Hence it is desirable to identify principles underlying ADEPT toxicity. This would be very useful clinically and would allow the design of safer treatment schedules. In view of the large number of variables it is desirable to analyse data from as many patients as possible. Hence this

MD (Res) aims to perform a combined analysis on *all* available safety data i.e. single-treatment and repeat-treatment patients. Combined analysis aims to answer important clinical questions: Which dose schedules predispose to hepatic toxicity? What factors increase the risk of myelosuppression?

1.6 Efficacy of repeat ADEPT treatment

The principle aims of a phase I trial are to establish a safe dose, the toxicity profile and the pharmacokinetic profile. In addition it is also desirable to confirm that the drug is having the desired biological effect on target tissue.

If a novel investigational agent can satisfy the requirements of a phase I trial then the drug may progress to a phase II trial. The principal aim of the phase II trial is to investigate efficacy in a larger number of patients, using the dose and schedule established in the phase I trial.

Although efficacy is formally assessed in a phase II trial it is desirable to detect some evidence of efficacy in a phase I trial since it may provide an early indication of which dose/schedule, tumour types or patient characteristics are associated with efficacy.

1.6.1 CT imaging

Perhaps the simplest method to assess efficacy is to measure the size of tumours before and after treatment. In a meta-analysis of data from over 3000 patients who received cytotoxic chemotherapy for metastatic colorectal cancer, tumour response (based on reduction in tumour size) was a highly significant predictor of survival (Buyse *et al*, 2000). This effect was independent of the patient's performance status.

CT imaging is the most widely used technique to measure tumour size. The international use of RECIST criteria has provided a common language to consistently describe efficacy assessment. Since the activated prodrug in ADEPT is a cytotoxic agent it is appropriate to

measure tumour size because production of activated prodrug in tumour may cause tumour shrinkage. Hence CT imaging and RECIST criteria will be used to assess efficacy in the current trial of repeat ADEPT treatments.

1.6.2 FDG-PET imaging

Whereas CT measures the size of tumour positron emission tomography (PET) is designed to examine the activity of tumour. The most frequently used positron emitting isotope is ^{18}F which can be incorporated into the glucose analogue 2-[^{18}F] fluoro-2-deoxy-D-glucose (FDG). FDG is taken into cells and phosphorylated by hexokinase. However unlike glucose FDG is not significantly further metabolized and hence becomes trapped in the cell. Many tumours have increased rates of aerobic glycolysis compared to non-tumour tissue and hence tumour tissue can be identified by a region of increased FDG uptake. FDG-PET imaging can be used to measure response to systemic anti-cancer drugs. In these situations the reduction in FDG uptake in tumour following treatment, can be caused by a reduction in the number of viable tumour cells or by a reduction in the uptake/metabolism of glucose per cell.

Unike CT anatomical imaging, there is still wide variation between investigators regarding the methods used to quantify tumour activity and the criteria used to assess response to therapy. The most frequently used method to quantify FDG activity in tumour is to calculate the tumour's standardized uptake value (SUV). This is the FDG uptake relative to the injected dose of radioactivity. The injected dose is normalized to body mass, lean body mass or body surface area. There is no standard technique for defining the region of interest (ROI) of the tumour i.e. the region where the SUV is measured. A variety of different methods are currently used e.g. hand-drawing, fixed dimension ROI including the pixel with highest activity, volume-growing algorithms that define ROI based on the increased uptake of tumour compared to normal tissue.

Despite variation in methodology between studies there is evidence that FDG-PET can be useful in measuring the response of gastrointestinal carcinomas to chemotherapy. For example a clinical trial evaluated the usefulness of FDG-PET imaging in patients with adenocarcinoma of the oesophago-gastric junction undergoing chemotherapy followed by resection (Ott *et al*, 2006). FDG-PET imaging was performed before chemotherapy and 14 days after starting chemotherapy. Patients subsequently underwent surgical resection. ROI was defined as a circle of fixed diameter placed onto the tumour on the slice of maximal FDG uptake. FDG-PET response was defined *a priori* as a > 35% reduction in tumour SUV. A histopathological response occurred in 44% of patients who had a FDG-PET response and only 5% of patients who did not have a FDG-PET response (p=0.001). Median survival was significantly longer in patients who had a FDG-PET response compared to those who did not.

Other groups have developed volume-growing algorithms to attempt to measure total activity in the tumour volume i.e. total lesion glycolysis. For example Green and colleagues have developed a technique which begins with the investigator selecting a point on a visualized tumour lesion (Green *et al*, 2008). Computer software then runs a volume-growing algorithm to define the volume of interest and calculate the total number of counts in the volume. The technique can be applied to multiple lesions within a patient so that the total number of counts in the target lesions can be calculated. The clinical validity of this semi-automatic drawing method was investigated in 20 patients who underwent 1st or 2nd line standard chemotherapy for metastatic or locally advanced solid tumours. Fourteen of the 20 patients had gastrointestinal carcinomas. Tumour response was assessed by CT and FDG-PET prior to treatment. Following treatment patients underwent FDG-PET (at 2 - 4 weeks and 9 – 12 weeks) and CT (at 9 - 12 weeks). Response on FDG-PET was defined as a reduction of >15% in the total number of counts or disappearance of all target lesions, disease progression (PD_{PET}) was an increase of >10% in the total number of counts and stable diease (SD_{PET}) was defined as

values between response and disease progression. Response on CT images was assessed by RECIST criteria. Patients with a response by FDG-PET at 2-4 weeks or 9-12 weeks lived significantly longer than patients with (SD_{PET}) or (PD_{PET}). In the 9 patients who had SD on CT imaging 4 patients had a response on FDG-PET. Hence in this small group of patients response based on the volume-growing algorithm of Green and colleagues was positively associated with an important clinical end-point i.e. survival.

Francis and colleagues have used the same algorithm to assess FDG-PET response after one cycle of chemotherapy in patients with mesothelioma (Francis *et al*, 2007). Risk of death was significantly less in patients in whom there was a ≥ 10% reduction in total number of counts in the volume of interest.

Hence in addition to efficacy assessment by CT (which will be performed in all patients), assessment by FDG-PET will be offered to patients. FDG-PET images will be quantified using the semi-automatic drawing method described by Green and colleagues.

1.6.3 Serum tumour markers

Elevation of serum tumour-associated antigen is associated with a number of solid tumours. For example more than 80% of patients with metastatic colorectal cancer have an elevated value of CEA. In addition serum CA 19-9 can be elevated in carcinomas of the gastrointestinal tract and biliary region. Measurement of serum tumour markers can provide clinically relevant information in patients with gastrointestinal carcinoma. For example the serum CEA kinetic (a measure of the change of serum CEA over at least 3 CEA values) was measured on patients with metastatic colorectal cancer undergoing chemotherapy (Iwanicki-Caron *et al*, 2008). The serum CEA kinetic values for patients with progressive disease, stable disease and responsive disease (measured by CT imaging) were significantly different. Furthermore high CEA kinetic values were significantly correlated with reduced disease free survival.

Since the ADEPT clinical trial uses an anti-CEA antibody, which reduces serum CEA, serum CA19-9 is examined rather than serum CEA.

1.7 Immune response to MFECP1

The fusion protein MFECP1 combines targeting and enzymatic functions. The bacterial enzyme CPG2 was chosen since it has no human enzyme equivalent with the same substrate specificity. However the use of non-human proteins can also have disadvantages. This is because the administration of a non-human protein to a patient can induce a specific immune response directed against the therapeutic protein i.e. the protein is immunogenic. One important consequence of immunogenicity is the production of ADAs.

1.7.1 Clinical relevance of immune response to therapeutic proteins derived from non-human proteins

Studying the immune response to therapeutic proteins derived from non-human proteins is important because the immune response can cause significant clinical effects. Immunological responses (including the formation of ADAs) can cause serious adverse events (e.g. serum sickness and anaphylaxis), reduce clinical effectiveness and alter clearance.

Serum sickness is characterised by joint pain, rash and enlarged lymph nodes. The mechanism is believed to be due to antibody-drug complexes lodging in small blood vessels. Serum sickness can occur after the first dose of a drug, (when it typically presents 8-13 days after drug administration) or after repeated doses. Serum sickness has been reported in patients receiving the chimeric mAb rituximab (Todd and Helfgott, 2007), the murine radiolabelled mAb Zevalin® (DeMonaco and Jacobs, 2007) and the bacterial protein streptokinase (Proctor *et al*, 1994). The clinical symptoms and signs are usually self-limiting provided that administration of the causative drug stops.

Anaphylaxis occurs in patients who have previously been exposed to the drug or its epitopes, and is characterized by an excessive secondary immunological response. The reaction begins with drug molecules binding to IgE on the surface of mast cells. This leads to cross-linking of surface IgE resulting in the release of pre-formed granules (containing a variety molecules including histamine) and the synthesis of arachidonic acid metabolites. The clinical features of severe anaphylaxis are the very rapid onset of urticarial rash, bronchospasm and hypotension. Severe anaphylaxis can be fatal within minutes of drug administration. For example retreatment with the murine mAb muromonab-CD3 (Orthoclone OKT®3), can cause life-threatening anaphylaxis due to the presence of anti-muromanab-CD3 IgE in serum (Abramowicz *et al*, 1992). In patients with rheumatoid arthritis receiving the mAb infliximab, ADA levels were significantly higher in patients who discontinued treatment due to an infusion reaction, than patients whose therapy was not stopped due to adverse events (Bendtzen *et al*, 2006).

Anti-drug antibodies can also reduce drug efficacy if the antibodies neutralize the active site of the drug i.e. neutralizing antibodies (NAbs). For example ADAs against the therapeutic enzyme L-asparaginase, derived from *Escherichia coli (E.coli)* can neutralize the enzyme activity of L-asparaginase (Avramis *et al*, 2002).

Furthermore there is some evidence that neutralization of a drug can affect clinical effectiveness. The most convincing evidence comes from longitudinal studies in which the efficacy of a drug in *individual* patients is examined during antibody negative and antibody positive periods i.e. each patient acts as their own control. Sorensen and colleagues examined the presence of NAbs in 541 multiple sclerosis patients who were treated with recombinant human interferon beta (Sorensen *et al*, 2003). The odds ratio for risk of relapse was significantly higher ($p < 0.03$) during NAb positive periods than during NAb negative periods.

1.7.2 Mechanism of immune response to therapeutics derived from non-human proteins

The immunological reaction to a therapeutic derived from a foreign protein is modelled on the immune response to a pathogen or vaccination. The most fundamental determinant of neo-antigen immunogenicity is whether a therapeutic protein can activate B-lymphocytes and CD4+ T_h lymphocytes. B-lymphocytes and T-lymphocytes recognize antigen in different ways.

Each B-lymphocyte produces antibody with a single specificity and many copies of this antibody are expressed on its cell surface. When antigen encounters a B-lymphocyte with surface antibody that is complimentary to it, the antigen binds the surface antibody causing activation of the B-lymphocyte. This induces clonal proliferation and differentiation leading to the production of plasma cells capable of making antibody specific to that particular antigen.

In contrast T-lymphocytes do not recognize antigen directly. T-lymphocytes require antigen to be presented to them by specialized antigen presenting cells (APCs). APCs e.g. dendritic cells, take up antigen, process it into short peptide fragments and display it with cell-surface major histocompatibilty complex (MHC) molecules. A naïve T-lymphocyte can be activated if there is complimentarity between the T-cell receptor (TCR) and the presented peptide-MHC. Each T-lymphocyte expresses a TCR with a single specificity. Activation of T-lymphoctytes leads to clonal proliferation and maturation. Non-naive lymphocytes can be activated by peptide-MHC on other cell types e.g. macrophages and B-lymphocytes.

Hence B-lymphocytes recognize surface epitopes whereas T-lymphocytes recognize linear peptide sequences.

Although antibodies are produced by plasma cells derived from activated B-lymphocytes, T_h lymphocytes are essential in the production of an

effective antibody response. In particular activated T_h lymphocytes are necessary for the production of high affinity, class switched antibodies and memory responses, which are all characteristics of ADA immune responses.

For example a TCR on a T_h lymphocyte can bind to a MHC class II-peptide complex on the surface of B-lymphocyte. This interaction leads to release of cytokines from T_h lymphocyte and upregulation of IL-4 receptors on the B-lymphocytes. These signals promote clonal proliferation of the B-lymphocytes and allow immunoglobulin class switching e.g. change of production from IgM to IgG. In addition activation of the B-lymphocyte leads to somatic hypermutation of immunoglobulin variable regions, which is essential for the production of novel immunoglobulins with higher antigen affinity i.e. affinity maturation.

1.7.3 ADEPT and immunogenicity

Early ADEPT clinical trials used the antibody-enzyme A5B7-CPG2. This chemical conjugate was immunogenic since it combined a $F(ab')_2$ fragment of a murine mAb (A5B7) and the bacterial enzyme CPG2. Hence patients frequently produced HAMA against the murine $F(ab')_2$ fragment and HACPA against CPG2.

For example in a phase I trial of a single ADEPT treatment using A5B7-CPG2, 100% of patients developed HAMA and 96% developed HACPA (Francis *et al*, 2002). The median time to develop HACPA was 15 days (range 7 to 50).

Subsequently the chemical conjugate A5B7-CPG2 has been replaced by MFECP1. Following a single infusion of MFECP1 40% of patients became HACPA positive (CRUK ADEPT trials database). Adverse events considered related to the infusion were mild i.e. Common Toxicity Criteria (CTC) grade 1 only.

After a single MFECP1 infusion the median day of becoming HACPA positive was day 14 (range 7 to 42). Since these antibodies formed *after* the treatment period they would not have affected the efficacy of single ADEPT treatment.

1.7.4 Repeat ADEPT treatment and immunogenicity

Repeated infusions of MFECP1 may cause more undesirable immunological consequences. For example if a patient who developed HACPA received a further MFECP1 infusion, HACPA could bind to MFECP1 and alter clearance. There is evidence from pre-clinical ADEPT systems that clearance can become accelerated after repeat treatments. Sharma and colleagues investigated clearance of the antibody-enzyme A5B7-CPG2 in mice (Sharma *et al*, 1996). Clearance was measured in treatment-naive mice and in mice who had received a dose of A5B7-CPG2 10 days before. Clearance was faster in mice that have previously been exposed to A5B7-CPG2 compared to mice who had never previously received A5B7-CPG2. It was demonstrated that following exposure to A5B7-CPG2 mice produced anti-CPG2 antibodies. The authors hypothesized that the rapid clearance of A5B7-CPG2 in pre-treated mice was caused by the presence of anti-CPG2 antibodies. Similarly it is possible that HACPA production in patients could neutralize the enzymatic activity of CPG2.

These issues could significantly affect the repeat treatment trial since the current evidence (from the single treatment trial) indicates that HACPA production is common i.e. 40% of patients. Furthermore the repeat administration of MFECP1 may increase the percentage of patients who become HACPA positive.

Importantly, increasing the number of MFECP1 infusions could also increase the incidence of symptomatic adverse events, since the immune response to a foreign protein may be significantly greater on the 2nd (or subsequent) exposure compared with the first exposure. An example of this can be seen with the drug streptokinase, a protein produced by the

bacterium beta-haemolytic streptococcus and used in the treatment of acute myocardial infarction. When a single treatment of streptokinase is given the frequency of allergic reactions is <5% (ISIS-2 collaborative group, 1988; GISSI-1 collaborative group, 1986). However when 8 patients received re-treatment with streptokinase, 4 patients (50%) experienced allergic reactions (White *et al*, 1990). The streptokinase re-treatment was given a median of 4 days (range 3 to 7) after the first treatment.

1.7.5 Managing the immunogenicity of repeat ADEPT treatment

Patient safety is of paramount importance in a phase I clinical trial. It is likely that the risk of an immunological adverse event would be significantly higher in HACPA positive patients compared to HACPA negative patients. Furthermore HACPA could significantly alter MFECP1 pharmacokinetics (e.g. accelerated clearance) and/or reduce the biological activity of CPG2. To reduce the risk of such problems patients in the repeat-treatment ADEPT phase I/II trial that became HACPA positive will be ineligible for further ADEPT treatment.

An important consequence of this is that for many patients i.e. those who become HACPA positive, there is likely to be a relatively short time during which repeat ADEPT treatments can be given. This may limit the number of treatments that can be given and hence could compromise efficacy. It is therefore desirable to reduce the immunological response to MFECP1.

There are a variety of methods that could in principle be used to reduce the probability of ADA formation against MFECP1. Some of these are already performed i.e. vigorous quality control of MFECP1 production to Good Manufactoring Practice (GMP) standards and administration by the intravenous route. Early clinical trials of ADEPT (using the chemical conjugate A5B7-CPG2) used immunosupression with ciclosporin. Although ciclosporin appeared to delay HACPA formation ciclosporin produced significant adverse events in some patients (Bagshawe *et al*, 1995; Napier *et al,* 2000).

The most direct approach is to reduce the immunogenicty of MFECP1 itself either by removing B-lymphocyte epitopes (B-cell epitopes) or by removing amino acid sequences that are recognized by T-lymphocytes (T-cell epitopes).

1.7.6 B-cell epitopes

An important determinant of immunogenicity is whether a drug can activate B-lymphocytes. Attempts to disrupt B-cell epitopes can be divided into techniques that mask the epitope and techniques that remove the epitope. The aim of masking is to physically shield an epitope from B-lymphocytes without disrupting the therapeutic active site of the drug. Masking can be achieved by coating the surface of the molecule, via covalent bonds, with polyethylene glycol (PEG). In a randomized clinical trial pegylated asparaginase caused asparagine depletion as effectively as unpegylated asparaginase. Furthermore the pegylated drug induced ADAs in significantly fewer patients than the unpegylated drug (Avramis *et al*, 2002). However pegylation can have additional effects on the drug, such as altering absorption and serum half life.

An alternative is to identify and selectively remove B-cell epitopes. This approach has been demonstrated with the therapeutic enzyme staphylokinase which is derived from *E.coli* and induces NAbs in the majority of patients. Interaction analysis with a panel of murine mAbs identified three immunodominant epitopes (Collen *et al*, 1996). Epitopes were then disrupted by substitution of charged amino acids with alanine to form staphylokinase mutants. ADAs from patients treated with wild-type staphylokinase bound to the mutant enzyme less than wild-type enzyme. However the enzyme activity of mutants was significantly reduced. Subsequently comprehensive site-directed mutagenesis produced a mutant with retained enzyme activity and reduced Nab production when given to patients (Laroche *et al*, 2000).

Spencer and colleagues have investigated surface B-cell epitopes on CPG2 in ADEPT (Spencer *et al*, 2002). Using a phage display library of

scFvs, surface enhanced laser desorption/ionization affinity mass spectrometry and the crystal structure of CPG2 a discontinuous epitope at the C-terminus of CPG2 was identified. A novel antibody-enzyme fusion protein was produced and mutants synthesized containing site-specific mutations to eliminate the identified epitope. ADAs from patients who had received A5B7-CPG2, bound significantly less to the mutant fusion protein compared to wild-type fusion protein. Subsequently, it has been demonstrated that a C-terminal histamine-tag (His-tag) on MFECP1 (which was added to MFECP1 for immobilized metal affinity chromatography purification) also significantly reduces the binding of ADAs from ADEPT patients to the epitope (Mayer *et al*, 2004). It was hypothesized that the His-tag masks a B-cell epitope.

1.7.7 T-cell epitopes

Despite the presence of the C-terminal His-tag on MFECP1, a single treatment of ADEPT using MFECP1 produced HACPA in 40% of patients. Since the development of an antibody response to foreign proteins is driven by T_h lymphocytes examining the T_h lymphocyte response to MFECP1 is clinically important. Furthermore identifying and removing immunodominant T_h lymphocyte epitopes appears to be a promising approach to reducing MFECP1 immunogenicity.

Identification of T_h lymphocyte epitopes requires identification of the amino acid sequences within the molecule that are responsible for T_h lymphocyte activation. One approach is to investigate whether a peptide is likely to bind to a given class II MHC molecule. This can be done using *in-silico* prediction methods that are available for a limited number of class II MHC molecules. Alternatively *in vitro* class II MHC binding assays can be used to measure peptide binding. These techniques are frequently used in combination (Warmerdam *et al.*, 2002). However both these techniques do not take into account the role of antigen processing. Furthermore they may over-estimate epitopes since not all peptides that bind class II MHC can bind the TCR.

Several investigators have identified T_h lymphocytes epitopes in therapeutic proteins. For example Tangri and colleagues used short peptides (15mers, overlapping by 10 amino acids) spanning the entire sequence of erythropoietin to investigate the location of T_h lymphocyte epitopes on erythropoietin (Tangri *et al.*, 2005). They identified 2 peptides that bound to a large number of human leucocyte antigen (HLA) types *in vitro* and caused activation of peripheral blood mononuclear cells (PBMCs) from healthy donors. Mutation of these peptides was used to produce two variant erythropoietin proteins which retained function and stimulated T_h lymphocytes significantly less than wild type erythropoietin.

The phase I/II clinical trial of repeat ADEPT patients also offers an opportunity to investigate T_h lymphocyte response in blood from treated patients. Are untreated patients truly naive to the epitopes on MFECP1 or have patients been exposed to CPG2 epitopes naturally e.g. through contact with CPG2 from *Pseudomonas*? Although ADEPT patients are confirmed as being HACPA negative before treatment it is possible that patients have been exposed to CPG2 sufficiently long ago that HACPA levels have fallen to undetectable. These patients would retain specific memory T-lymphocytes capable of stimulating a rapid secondary response.

Due to limited availability of PBMCs from healthy volunteers and very limited availability of patient blood, it is desirable to use a model system using animal T-lymphocytes. However the use of laboratory animals for pre-clinical immunogenicity testing is of limited value due to differences in MHC molecules between animals and humans. Hence a T_h lymphocyte response in mice may not reflect the response in humans.

The development of mice that are transgenic for human MHC (and do not express murine MHC) may provide a suitable model in which to conduct pre-clinical immunogenicity investigation. Altmann and colleagues developed HLA-DR1 transgenic mice by inserting genomic constructs for the α-chain of HLA-DR (HLA-DRA1*0101) and a β-chain of HLA-DR

(HLA-DRB1*0101) into mice that were deficient in murine MHC class II molecules (Altmann *et al.*, 1995). Responses in these HLA-DR1 transgenic mice to HLA II restricted peptides were consistent with conventional experimental tools e.g. class II MHC binding assays (Depil *et al.*, 2006). Mice transgenic for human MHC are used in vaccine development and may be suitable for pre-clinical investigation of protein therapeutics (De Groot *et al.*, 2008).

In **chapter 5** the nature of the immune response to MFECP1 is examined. Firstly the clinical aspects of MFECP1 administration is investigated, including the incidence of MFECP1 related adverse events. Data on all patients (single-treatment and repeat treatment patients) is analysed to examine whether repeat infusions of MFECP1 increase the risk of an infusion reaction. The frequency of HACPA production is measured and the effect of repeat infusions on HACPA production assessed.

Secondly the *in vitro* response of T_h lymphocytes from healthy volunteers and an ADEPT patient to MFECP1 is examined. T-cell epitope mapping is performed on CPG2 using PBMCs from 20 healthy volunteers. Furthermore the suitability of mice transgenic for human MHC as a possible source of lymphocytes for immunogenicity experiments is examined.

1.8 Aims of MD (Res) thesis

The overall aim of this MD thesis is to improve the safety and efficacy of ADEPT. The phase I/II clinical trial of repeat ADEPT treatments, described in this MD thesis, aims to investigate the safety of repeat treatments, obtain early evidence of efficacy and increase understanding of the immune response to MFECP1. A phase I/II clinical trial of repeat ADEPT has never been conducted before and therefore this MD (Res) thesis aims to significantly increase understanding of ADEPT.

The specific aims of this MD (Res) thesis are:

1) Investigate the safety of repeat ADEPT treatments

In **Chapter 3** the safety profile of repeat ADEPT treatment is examined. The phase I/II trial aims to establish the maximum number of tolerated treatments and the maximum tolerated TPD. Analysis of data from all patients who have received MFECP1 (during the single and repeat treatment phase I trials) aims to identify risks factors for toxicity.

2) Investigate whether there is preliminary evidence of efficacy with repeat ADEPT treatments

In **Chapter 4** evidence of efficacy is examined using CT, FDG-PET and serum CA19-9. Combined data from single and repeat treatment patients is used to examine the relationship between efficacy and the dose/schedule of ADEPT.

3) Investigate the nature of the immune response to MFECP1

Chapter 5 aims to examine the immunological response to MFECP1. The effect of repeat administration of MFECP1 on infusion reactions and HACPA production is examined. The mechanisms leading to the clinical immune response are examined. Lymphocytes from healthy volunteers, an ADEPT patient and mice transgenic for human MHC are used to examine the *in vitro* response to MFECP1 and the possible identity of T-cell epitopes.

Chapter 2

Materials and Methods

2.1 ADEPT phase I/II clinical trial

2.1.1 Trial conduct and regulation

The study was sponsored and monitored by Cancer Research UK (CRUK) Drug Development Office. The principal investigator (PI) was Professor RHJ Begent (UCL Cancer Institute, The Paul O'Gorman Building, University College London, Gower Street, London WC1E 6BT, UK). All drug administration, in-patient and out-patient activities were performed at the Royal Free Hospital, Pond Street, London NW3 2QG. The study was conducted in compliance with the protocol, the European Union Clinical Trials Directive, principles of the International Conference on Harmonization of Good Clinical Practice guidelines and the Declaration of Helsinki. The study was conducted under a Clinical Trials Authorisation with approval from the Medicines and Healthcare products Regulatory Agency (MHRA) and the appropriate research ethics committee. A license for the administration of radioactive substances was obtained from the Administration of Radioactive Substances Advisory Committee.

2.1.2 Study drugs

MFECP1 was produced in *Pichia pastoris* in the GMP facility in the Department of Oncology at the Hampstead Site of University College London. BIP prodrug was manufactured and supplied by Astra Zeneca (Macclesfield, UK). Each vial of BIP prodrug contained 610mg of crystalline BIP prodrug hydroiodide (equivalent to 500mg of BIP prodrug free base).

2.1.3 Study design

Each ADEPT treatment consisted of an infusion of MFECP1 followed by 3 bolus administrations of BIP prodrug. MFECP1 was given in the evening and prodrug given the following day, provided that serum CPG2 activity was < 0.002 U/ml. The starting prodrug dose was 200mg/m^2 and the first cohort of patients received 2 treatments i.e. TPD of 1200mg/m^2. The number of treatments was increased by one per cohort. The first cohort consisted of 3 patients and subsequent cohorts consisted of a minimum of one patient. In the event that grade 2 or higher toxicity occurred (ADEPT-related) the cohort would be expanded to 3 patients.

In the event of a dose limiting toxicity (DLT) when less than 8 treatments per patient had been given, the cohort was to be expanded to 6 patients. Absence of a second patient experiencing a DLT allowed a new cohort to open. In the event of a second patient experiencing a DLT then the dose of BIP prodrug in the next cohort was reduced by 25%. In the event of a DLT when 8 or more treatments per patient were given, the cohort was to be expanded to 6 patients but a new cohort was not opened. The daily treatment schedule for patients receiving 2 or 3 treatments is shown in Table 2.1. The treatment schedule for additional ADEPT treatments is shown in Appendix 1.

For patients in the same cohort an interval of 3 weeks was required between a patient completing scheduled treatment and the next patient starting treatment. This interval was increased to 4 weeks when the next patient was in a new cohort.

Treatment with MFECP1 and BIP prodrug could be repeated on a 4-6 weekly basis after the final scheduled treatment if there was no evidence of progression and the patient remained HAMA and HACPA negative. Patients could be given ciclosporin immunosupression to attempt to reduce HACPA formation. Intravenous ciclosporin (5mg/kg) was started 2 days before MFECP1 and given for 7 days. (The dose of intravenous ciclosporin was adjusted to maintain blood levels between 150-350ng/ml.)

Patients were then switched to oral ciclosporin at 15mg/kg per day in divided doses for 7 further days.

Day	Treatment number	Study drug	
		Cohort 1	Cohort 2
1	1	MFECP1	MFECP1
2		Prodrug x3	Prodrug x3
3	2	MFECP1	MFECP1
4		Prodrug x3	Prodrug x3
5			
6			
7			
8	3		MFECP1
9			Prodrug x3

Table 2.1

Treatment schedule for patients that received 2 or 3 ADEPT treatments

2.1.4 Eligibility criteria

Eligible patients had metastatic, unresectable or locally recurrent histologically proven CEA expressing carcinoma and had been offered standard treatment. The other inclusion criteria were a plasma CEA level between 10µg/L and 1000µg/L (or if serum CEA < 10µg/l then CEA had to be demonstrated by immunohistochemistry on tumour blocks), measurable disease on RECIST criteria, an age of ≥ 18 years, life expectancy ≥ 4 months, World Health Organization (WHO) performance status of 0, 1 or 2, adequate haematological function (neutrophil count ≥ $2x10^9$/l, platelet count ≥ $100x10^9$/l and haemoglobin ≥ 10g/dl), adequate renal function (plasma creatinine ≤ 120 µmol/l or EDTA/urine creatinine

clearance $\geq$ 50ml/min), adequate hepatic function (plasma bilirubin $\leq$ 30µmol/l, ALT/AST $\leq$ 2x upper limit of normal (ULN), (5x ULN in the presence of liver metastases), INR $\leq$ 1.5 (or within the therapeutic range for patients on anti-coagulation) and written informed consent. Patients were excluded if they had received radiotherapy (except for palliative reasons to non-target lesions), chemotherapy, endocrine therapy, or immunotherapy during the 4 weeks prior to study treatment, were pregnant or lactating, serologically positive for Hepatitis B, C or HIV, were considered poor medical risk because of non-malignant systemic disease, had a second current malignancy, had undergone major surgery within 4 weeks, were HAMA or HACPA positive or had a history of allergy to iodine or mouse proteins. All toxic manifestations of previous treatment must have resolved (excluding alopecia or grade 1 toxicities that in the opinion of the PI and CRUK should not exclude the patient). All patients were treated in the Department of Oncology at the Royal Free Hampstead NHS Trust, London.

2.1.5 Pre-treatment evaluation

Before beginning ADEPT all patients underwent complete history, performance status assessment, physical examination, ECG, full blood count, chemistry profile, creatinine phosphokinase, clotting, serum CEA, serum CA 19-9, HLA typing, storage of PBMCs, comet assay, urinalysis and chest X-ray. Disease assessment was performed by CT. Patients were offered the possibility of additional disease assessment using FDG-PET imaging.

2.1.6 Drug administration

MFECP1 was administered intravenously at a fixed dose of 3000U/m^2 over 2 hours in a total volume of 300ml of 0.9% sodium chloride. Each vial of BIP prodrug was reconstituted with 10ml sodium bicarbonate solution and filtered through a 0.22μm filter. The three BIP prodrug bolus administrations were each separated by 1 hour. Each bolus was given into a fast running drip of 5% w/v dextrose through a Hickman Line

(central venous catheter). The dose of BIP prodrug for the first cohort of patients was 200mg/m^2 per bolus i.e. 600mg/m^2 per treatment.

2.1.7 Measurement of serum CPG2 activity

Serum CPG2 was measured by Dr Surinder Sharma, Dr Hassan Shahbakhti and Natalie Griffin.

Serum CPG2 activity was measured by high performance liquid chromatography 5 minutes after completion of MFECP1 infusion and then up to hourly until serum CPG2 activity was < 0.002U/ml.

2.1.8 Pharmacokinetics of BIP prodrug

Pharmacokinetic measurement and analysis was performed by Professor Caroline Springer's laboratory (Institute of Cancer Research, Sutton, UK).

Plasma samples for analysis of BIP prodrug concentrations were taken at 2, 5, 10, 15, 30 and 60 minutes following the 3rd prodrug bolus. Plasma BIP prodrug concentrations were quantified by HPLC. Concentration of BIP prodrug extrapolated back to time zero (C$_0$), volume of distribution at steady state (V$_{ss}$), area under the curve extrapolated to infinity (AUC$_{INF}$), rate of clearance and elimination half-life were determined using a non-compartmental model and WinNonlin software.

Pharmacokinetic profile of the activated prodrug was not possible because the activated prodrug has a half life in the order of seconds.

2.1.9 Prodrug activation

Single cell comet assays were performed by Dr Janet Hartley (Department of Oncology, UCL, UK). Comet assays were used to indirectly measure DNA interstrand crosslinks, which was used as a surrogate marker for prodrug activation. Blood for comet assay was taken approximately 60 minutes after the last prodrug administration. Comet assays were performed on peripheral blood lymphocytes and tumour tissue (where available).

2.1.10 Post treatment evaluation

Patients were followed up until Day 58 of treatment. History, physical examination, performance status assessment, HAMA and HACPA were performed weekly. Toxicity was assessed according to the National Cancer Institute CTC. Full blood count and chemistry profile were examined twice weekly in the fist 4 weeks and weekly thereafter. Serum creatinine kinase was measured 4-6 hours after the first prodrug bolus of each treatment and was also measured the day after prodrug administration. Serum CEA and serum CA 19-9 were measured every 2 weeks from Day 16. Blood clotting was measured on days 30 and 58. ECG was recorded on Day 23. A blood sample on day 37 was used to extract PBMCs in order to assess T_h lymphocyte response to MFECP1.

Patients were evaluated for response by CT on Days 29 and 57. Response was assessed according to RECIST criteria. Optional disease assessment with FDG-PET imaging was performed on Days 23 and 54. Response was assessed by semi-automatic volume of interest drawing (Green *et al.*, 2008). A significant reduction in tumour activity was defined as >15% reduction in total counts in the volume(s) of interest.

2.1.11 Measurement of HACPA

HACPA was measured by Dr Surinder Sharma, Dr Hassan Shahbakhti and Natalie Griffin.

Blood was assessed for HACPA formation before treatment and weekly during follow up period. HACPA were measured by ELISA. 96 well ELISA plates were coated with CPG2. (96 well ELISA plates were supplied by Nunc-Immuno™. CPG2 was supplied by Centre for Applied Microbiology Research (Porton Down, Sutton, UK). Serum samples were added to the plates at dilutions of 1:100 and 1:1000. Peroxidase-labelled goat anti-human IgG F(ab')$_2$ was used as a secondary antibody. (Peroxidase-labelled goat anti-human IgG F(ab')$_2$ was supplied by Sigma-Aldrich: Ref A2290). After adding substrate results were quantified using a plate reader. Plates were read using a Thermo Labsystems Opsys MR Dynex

Technologies plate reader. The cut-off value for a positive result was an absorbance of 0.2 at 490nm wavelength. The cut-off value was obtained by assaying 50 pre-treatment serum samples at 1:100 dilutions and taking the cut-off as 3x the standard deviation above the mean absorbance. A serum sample from a previous patient that had been found to be HACPA positive was used as the positive control.

2.2. Materials and methods for immunogenicity experiments

Experiments were performed at:

1. The Department of Infectious Diseases and Immunity, Hammersmith Hospital, Du Cane Road, London, United Kingdom in collaboration with Professor Daniel Altmann (Professor of Immunology).
2. The Department of Oncology, 1st floor, Royal Free Hospital, Pond Street, London, United, Kingdom.
3. Biovation Ltd / Antitope Ltd, Babraham Research Campus, Babraham, Cambridge, United Kingdom.

All experiments were done in accordance with COSHH (control of substances hazardous to health). Radioactive work was done in accordance with Radiation Protection guidelines. All animal work was carried out in accordance with the guidelines of the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) and the Animal (Scientific Procedures) Act 1986. Blood samples were taken from members of staff and students in the Commonwealth Building, Imperial College (Hammersmith campus) with specific ethics committee approval. Blood samples were taken from ADEPT patients for analysis of T-cell immunogenicity on patients 35 to 43 inclusive. Ethics committee approval was granted as part of the ADEPT protocol amendment of 4th April 2006.

2.2.1. Extraction of PBMCs from peripheral blood

A blood sample (12ml from healthy volunteer and 50ml from an ADEPT patient) was taken from a peripheral vein into sodium heparin vacutainer tubes (BD Diagnostics, USA). Blood samples were taken by an

appropriately trained member of staff. The blood sample was transferred to a microbiological safety cabinet. The blood was mixed with an equal volume of sterile phosphate buffered saline (PBS) pH 7.4 (Invitrogen, USA). 20ml of the blood/PBS/heparin mixture was very slowly layered onto 15ml of Histopaque (Sigma-Aldrich, USA) in a 50ml centrifuge tube. This was repeated so that all the blood/PBS/heparin mixture was layered onto Histopaque. Density gradient centrifugation was performed by centrifuging samples at 1500rpm for 25 minutes at room temperature with no brake. The PBMCs were seen as a buffy layer and this layer was removed using a sterile plastic disposable pipette. The PBMCs were placed into a new 50ml centrifuge tube and the tube filled to a total volume of 50ml with AIM-V media (Invitrogen, USA). The sample was centrifuged at 1500rpm for 10 minutes at room temperature with brake on. The supernatant was discarded, the pellet resuspended in the small residual volume and the centrifuge tube filled to 50ml with AIM-V media. The sample was centrifuged again (1500rpm, 10 minutes, room temperature, brake on). The supernatant was discarded, the pellet resuspended in the small residual volume and 10mL AIM-V media added.

2.2.2 Calculating the concentration of lymphocytes in a cell suspension

The sample was mixed to prevent settling of cells. A $10\mu l$ sample of cell suspension was removed and mixed with $90\mu l$ of 0.2% trypan blue solution (Sigma-Aldrich, USA). A $10\mu l$ sample of the dye/cell suspension mixture was placed onto a plastic microscope slide with quantitative grid (Hycor Biomedical, USA). The sample was inspected at 20x magnification using a microscope. The number of viable lymphocytes in 4 large squares was counted (viable cells appear as large, rounded, white cells) and this value divided by 4 to obtain the mean number of viable cells per large square. The cell concentration was calculated as follows:

$$\text{Cell concentration in cell suspension mixture} = \text{mean number of viable cells per large square} \quad \text{X} \quad 10^5$$

The cell concentration was adjusted as follows whenever the calculated cell concentration was different to the required cell concentration. When the concentration was too high additional media was added to bring the cell concentration to the desired figure. When the concentration was too low the sample was centrifuged at 1500 revolutions per minute (rpm) for 10 minutes at room temperature with brake on. The supernatant was discarded and the cell pellet resuspended in the small residual volume. The appropriate volume of media was then added to create the required cell concentration.

2.2.3 Mice: immunization and lymphocyte extraction

All DRB1*0101 transgenic mice were kindly supplied by Professor Daniel Altmann. Immunization material consisted of MFECP1 immunization (i.e. MFECP1 solution and adjuvant) or control immunization (i.e. sterile PBS and adjuvant). The adjuvant was TiterMax®Gold (CytRx Corporation, USA). MFECP1 immunization material was prepared by thoroughly mixing equal volumes of MFECP1 solution and adjuvant. The concentration of MFECP1 stock solution varied according to the batch number and therefore the quantity of MFECP1 injected per mouse is described in the results. (The volume injected per mouse was constant i.e. 50µl). A single batch of MFECP1 was used for each immunization experiment. Control immunization material was prepared by thoroughly mixing equal volumes of sterile PBS and adjuvant. Immunization material was mixed using a 1ml syringe and an orange needle. Mice were each immunized with 50µl of immunization material into the foot pad of the left hind foot. The day of immunization was described as Day 0.

Mice were culled between days 10 and 13. The mice were killed by cervical dislocation. The popliteal lymph node from the left hind leg of each mouse was identified and removed. Each lymph node was placed into a 50ml Falcon tube containing 5ml of murine media. Murine media was HL1 media (Lonza, Switzerland), plus 1% L-glutamine (Invitrogen, USA), 1% penicillin/streptomycin and 0.7% 2-mercaptoethanol (VWR

International, USA). Each lymph node was homogenized using the barrel of a 1ml syringe and filtered through a 70μm nylon cell strainer (BD Biosciences, USA). Each cell suspension was placed into a separate 15ml centrifuge tube and centrifuged at 1500rpm for 15 minutes at room temperature with brake on. For each tube the supernatant was discarded, the pellet resuspended in the residual volume and 1ml of murine media added to each tube. The cell concentration was then calculated as per section 2.2.2.

2.2.4 T-cell proliferation assays

T-cell proliferation assays were performed in 96 well clear flat-bottom cell culture plates (Corning, USA). Plates were prepared by placing 100μl of MFECP1 solution (50μg/ml), negative control solution (media only) or positive control solution into the appropriate wells.

Media for human T_h lymphocytes was AIM-V$^®$ media and media for murine T_h lymphocytes was HL1 media plus 1% L-glutamine, 1% penicillin/streptomycin and 0.7% 2-mercaptoethanol. MFECP1 was diluted with the appropriate media to produce a solution of 50μg/ml. Positive controls were Staphylococcal enterotoxin B (SEB) 20ng/ml (Sigma-Aldrich, USA) and Tuberculin Purified Protein Derivative (PPD) 1000U/ml (Chiron Vaccines Evans, USA). SEB and PPD were diluted in the appropriate media according to whether the T_h lymphocytes were human or murine.

An equal volume (100μl) of cell solution was added to each well. Hence the final "in-well" concentration of MFECP1 was 25μg/ml and the final "in-well" concentration of SEB and PPD was 10ng/ml and 500U/ml respectively.

Each experimental condition was performed in triplicate i.e. performed in 3 wells. 100μL of cell suspension at a concentration of 3.5 x10^6 cells/ml

was added to each of the wells. The plate was then incubated at 37^oC with 5% CO_2 for 5 days.

After 5 days of incubation a 37MBq/ml / 1mCi/ml (methyl-^{3}H) thymidine solution (GE Healthcare, USA) was diluted 1 in 100 with the appropriate media (depending on whether cells were human or murine). Ten micro litres of the (methyl-^{3}H) thymidine/media mixture was placed in each well so that each well was pulsed with $1\mu Ci$. The cells were incubated for 8 hours at 37^0C with 5% CO2. Plates were then either harvested immediately or frozen at -20^0C and harvested within 5 days (half life of (methyl-^{3}H) thymidine is 12.3 years).

Plates that had been frozen were defrosted. The plates were harvested onto glass fibre filtermats (PerkinElmer Life Sciences, USA) using a cell harvester (Mach IIIm, Tomtec). The filtermats were dried in a standard electric oven at 125^0C until completely dry (approximately 10 minutes). Each filtermat was placed into a plastic sample bag (PerkinElmer Life Sciences, USA) and enough scintillation liquid (PerkinElmer Life Sciences, USA) added to soak the filtermat (approximately 10ml). Each sample bag was sealed and the filtermats read on a beta scintillation counter (PerkinElmer Life Sciences, USA).

The raw results of each well were described as counts per minute (cpm). The mean cpm for each experimental condition was calculated. Stimulation indices (S.I.) for antigens were calculated as:

$$S.I. = \frac{\text{mean cpm with antigen}}{\text{mean cpm with negative control}}$$

2.2.5 Peptide experiments

T-cell epitope mapping of CPG2 was performed by Biovation Ltd. (now Antitope Ltd) by Dr Matthew Baker.

Individual peptides spanning the amino acid sequence of CPG2 were incubated *in vivo* with PBMCs from 20 healthy volunteers. T-cell proliferation assays were used to measure T-cell stimulation.

2.2.5.1 Peptides

Peptides (Mimotopes, Australia) spanning the amino acid sequence of CPG2 were used. The peptides were 15 amino acids long (15mers) and each overlapped the adjacent peptide by 12 amino acids (Figure 2.1). The amino acid sequences of the 128 peptides are shown in Appendix 2. The peptides, which were supplied as a freeze-dried product, were dissolved in DMSO to produce a stock-solution of 5mM.

| Peptide 87 | N | A | D | V | R | Y | A | R | N | E | D | F | D | A | A | | | |
| Peptide 88 | | | | V | R | Y | A | R | N | E | D | F | D | A | A | M | K | T |

Figure 2.1

Example of overlapping 15 mer peptides.

2.2.5.2 Preparation of PBMCs from volunteer blood samples

(Method from Biovation Ltd.)

PBMCs were isolated from whole blood. Blood samples from healthy volunteers were obtained from the National Blood Service, Long Road, Cambridge. Extraction of PBMCs was performed in a class II microbiological safety cabinet. Blood was centrifuged in falcon tubes at 1500rpm for 10 minutes at room temperature. The upper layer (plasma) was removed using a sterile disposable Pasteur pipette and disposed. Each falcon tube was filled to 50ml with sterile PBS and the sample

mixed. 25ml of blood/PBS mixture was carefully layered onto 15ml of Ficoll-Paque PLUS (GE Healthcare, USA). Each falcon tube was centrifuged at 400 x g for 30 minutes at room temperature with no brake. The buffy layer (PBMCs) was removed from each sample using a sterile plastic disposable pipette and placed in a new falcon tubes. Each 20ml of PBMCs was washed with 30ml of PBS. Samples were centrifuged at 1500rpm for 10 minutes at room temperature. The supernatant was carefully poured off and disposed. Cells were resuspened in PBS and carefully layered onto 10ml of Ficoll-Pacque PLUS. Each sample was centrifuged at 2000rpm for 30minutes at room temperature with no brake. The buffy layer was removed from each sample using a sterile plastic disposable pipette and placed in a single new falcon tube. The PBMCs were washed with at least 1x volume of PBS. The sample was centrifuged at 1500rpm for 10 minutes at room temperature. The supernatant was poured off and disposed. Cells were resuspened in 50ml PBS. The sample was centrifuged at 1500rpm for 10 minutes at room temperature. The supernatant was poured off and disposed. Cells were resuspened in 30ml AIM-V media.

A 10µL sample was taken and the cells counted (see section 2.2.2) so that the total number of cells in the 30ml sample could be calculated. The 30ml sample was centrifuged at 1500rpm for 10 minutes at room temperature. The supernatant was poured off and disposed. Cells were resuspended in AIM-V media to a concentration of 2×10^7 cells per ml.

2.2.5.3 T-cell proliferation assays in peptide experiments

PBMCs (5×10^8 cells) were incubated in a flat bottom flask for 1 hour at $37^{o}C$. Non-adherent (responder) cells were removed and the flask rinsed with 10ml AIM V media. Responder cells were pelleted and frozen at –$80^{o}C$. 15ul GM-CSF (800units/ml) and 15ul of IL-4 (500 units/well) were added to adherent (stimulator cells) cells and incubated at $37^{o}C$ with 5% CO_2. After 3 days of culture 0.5ml AIM V containing 15ul GM-CSF (800U/ml) and 15ul IL-4 (500U/ml) were added and cells were cultured for

another 2-3 days. 5ul IL-1α (50U/ml) was added for the last 24 hours. Cells were collected and washed twice in 10-20 ml AIM V media.

Stimulator cells were thawed and washed in AIM V and resuspended in 1ml AIM V, Mitomycin C was added at a final concentration of 50ug/ml. Cells were incubated at $37^{o}C$, washed 3x in AIM V and resuspended ($4x10^{5}$ cells/ml).

T-cell proliferation assays were performed in 96 well plates. 100ul of peptide solution (5μM) was added to the appropriate wells. Each peptide was tested in triplicate. In negative control wells 100ul of AIM V was added. Positive control was Keyhole limpet hemocyanin (KLH) supplied by Thermo Fisher Scientific (USA). KLH was dissolved in AIM-V to produce a solution of 200μg/ml and 100ul was added to positive control wells. 50ul of stimulator cells was added to each well and plates were incubated at $37\ ^{o}C$ for 1 hour. 50ul of responder cells (4×10^{6}cells/ml) was added to each well, plates were incubated for 48 hours and 10U IL 2 added. Cells were incubated for a further 6 days, then pulsed with ^{3}H-thymidine for 18 hours, plates harvested and read on a beta scintillation counter. Stimulation indices were calculated (see section 2.2.4) for each peptide and control.

2.2.6 Cytokine secretion assay and fluorescence-activated cell sorting (FACS)

A 15ml blood sample was taken from each healthy volunteer into sodium heparin tubes. This 15ml sample was referred to as the "experimental sample". In one volunteer an additional 5ml of blood was taken to calibrate the flow cytometry machine. This 5ml sample was referred to as the "calibration sample". The 15ml experimental sample was divided equally into 3 centrifuge tubes. Each 5ml blood sample was incubated with MFECP1, SEB or media. The concentration of MFECP1 in the blood/MFECP1 mixture was 10μg/ml. The concentration of SEB in the blood/SEB mixture was 1μg/ml. In each 5ml sample 76μl of blood was first removed. 76μl of MFECP1 solution (0.66mg/ml), 76μl of SEB solution

(66μg/ml) or 76μl of media (AIM-V) were added to the appropriate blood samples. (The cells were human and therefore the media used to dilute SEB stock to 66μg/ml was AIM-V). The 5ml calibration blood sample was incubated with media (AIM-V) i.e. 76μl of blood was first removed and then 76μl of media was added. Each sample was incubated for 13 hours at 37^OC with 5% CO_2.

After the 13 hour incubation period, 45ml of 1x erythrocyte lysing solution (ELS) was added to each 5ml blood sample. ELS (10x) was prepared with 41.4g NH_4Cl (Sigma-Aldrich, USA), 5g KHCO3 (Sigma-Aldrich, USA), 1ml 0.5M EDTA (VWR International, USA) and made up to 500ml with distilled water. The pH of the ELS (10x) solution was adjusted to 7.3 using concentrated NaOH or concentrated HCl as appropriate. The pH of the solution was measured with an electronic pH meter (Spectronic analytical instruments, UK). ELS (1x) was prepared by mixing 50ml ELS (10x) with 450ml distilled water.

The mixtures of blood, ELS and antigen (or media negative control) were incubated at room temperature for 10 minutes and each sample mixed several times during the 10 minutes. The samples were then centrifuged at 1200rpm for 10 minutes with brake on. The supernatant was removed with a sterile disposable plastic pipette. Each cell pellet was resuspended in 15ml of cold buffer (4^OC). Buffer was prepared with 495.5ml PBS and 2.5ml of 0.5% foetal bovine serum (Autogen Bioclear, UK).

The samples were centrifuged at 1200rpm for 10 minutes at 4^OC with brake on. The supernatant was removed with a sterile disposable plastic pipette. Each cell pellet was resuspended in 160µl of cold medium (4^OC). Medium was prepared with 64ml RPMI (Invitrogen, USA) and 16ml human serum albumin type AB (Cambrex Corporation,USA).

Interferon gamma (IFN-γ) secretion assay kit was supplied by Miltenyi-Biotec, Germany. 20µl of Cytokine Catch Reagent was added to all

samples except the calibration sample. 20 µl of cold medium (4 ^{O}C) was added to the calibration sample. The samples were incubated on ice for 5 minutes. 7ml of warm medium (37°C) was added to each sample. All samples were incubated at 37 ^{O}C for 45 minutes and the sample was turned every 5 minutes. The tubes were removed from the incubator and placed on ice. 8ml of cold buffer (4°C) was added to each sample. The samples were centrifuged at 1200rpm for 10 minutes at 4°C with brake on. The supernatant was removed with a sterile disposable plastic pipette. The cell pellet of each experimental sample was resuspended in 160µl of cold buffer (4°C). The cell pellet of the calibration sample was resuspended in 400µl of cold buffer (4°C). 80 µl of each experimental sample (i.e. not the calibration sample) was placed into FACS tubes. 10µl of cytokine detection antibody on PE, 10µl of anti-CD3 on FITC (Immunotech, France) and 10µl anti-CD4 on PE-CY5 (BD Biosciences, USA) were added to each FACS tube.

The calibration sample was divided into 4 and each 100µL added to a FACS tube labeled "Calibration 1", "Calibration 2", "Calibration 3" and "Calibration 4". Nothing was added to Calibration 1. 5µl of anti-CD3 on FITC was added to Calibration 2. 5µl of anti-CD3 on PE (BD Biosciences, USA) was added to Calibration 3. 5µl of anti-CD3 on PE-CY5 (BD Biosciences, USA) was added to Calibration 4.

All the FACS tubes were then incubated in the dark and on ice for 10 minutes. 3ml of cold buffer (4°C) was added to each FACS tube. Each sample was centrifuged at 1200rpm for 10 minutes at 4°C with brake on. The supernatant was removed with a sterile disposable plastic pipette. Each cell pellet was resuspended in 100µl of cell fix (1x) (BD Biosciences, USA). The samples were kept in the dark at 4°C until they were ready to be analysed (later the same day).

Flow cytometry was kindly performed by Dr Rebecca Ingram using a FACSCalibur machine and Flow Jo software.

Chapter 3

Treatment, pharmacokinetics and toxicity of ADEPT with MFECP1 and BIP prodrug (Phase I/II trial).

3.1 Introduction

Successful ADEPT therapy has a number of requirements. These include a suitable antigen target, a suitable antibody-enzyme product (safe, minimally immunogenic, can be uniformly manufactured), a high concentration of enzyme on tumour, a very low concentration of enzyme on non-tumour, a prodrug that is safe and catalysed efficiently by the targeted enzyme and an active cytotoxic drug that causes minimal systemic side-effects. These design criteria provide for a safe system that can be given more than once, is clinically effective and practically feasible.

ADEPT is complex and a number of clinical trials have been conducted to develop the system (Table 1.2). The most recent clinical trial used a single treatment of ADEPT using MFECP1 and BIP prodrug (Mayer *et al.*, 2006). Thirty one patients were treated with a single treatment of ADEPT. The MTD was MFECP1 $3000U/m^2$ followed at a median of 13 hours (from the end of the MFECP1 infusion) by a total prodrug dose of $806.4mg/m^2$ when serum CPG2 enzyme activity was <0.002U/ml. MFECP1 localization was demonstrated on tumour biopsies up to 15 hours after administration of MFECP1 $3000U/m^2$. Three patients were treated at the MTD (patients 26, 27, 28) in whom no grade 3 or 4 treatment related adverse events occurred. All adverse events at the MTD for a single ADEPT treatment are shown in Table 3.1.

Table 3.1

Treatment related adverse events at the maximum tolerated dose of a single ADEPT treatment with MFECP1 and BIP prodrug

Adverse event	Number of patients (%)	
	CTC Grade 1	CTC Grade 2
Nausea	1 (33%)	1 (33%)
Vomiting	1 (33%)	1 (33%)
Fatigue	1 (33%)	2 (66%)
Constitutional – other*	1 (33%)	0 (0%)
Platelets	1 (33%)	0 (0%)
Leucocytes (total WBC)	0 (0%)	1 (33%)
Neutrophils/Granulocytes	0 (0%)	1 (33%)
Haemoglobin	1 (33%)	1 (33%)
AST	1 (33%)	0 (0%)

Data shown is from 3 patients (patients 26, 27 and 28) who were all treated at the maximum tolerated dose (MTD) of a single ADEPT treatment using MFECP1. MTD was MFECP1 3000U/m^2 followed by a total prodrug dose of 806.4mg/m^2. Adverse events shown were possibly, probably or almost certainly related to ADEPT. All data from Cancer Research UK database. Adverse events described according to common toxicity criteria (CTC) version 2. ADEPT: antibody directed enzyme prodrug therapy, WBC: white blood cells, AST: aspartate aminotransferase *pyrexial (maximum 37.9^{O}C) onset 3 hours after prodrug, completely resolved after 15 hours.

In mice bearing human carcinoma xenografts repeated treatments of ADEPT produced increased efficacy compared with a single ADEPT treatment (Sharma *et al.*, 2005). It is hypothesized that a similar effect occurs in patients i.e. that repeat treatment will produce more anti-tumour efficacy than a single treatment. Since repeat treatments of ADEPT with

MFECP1 have never previously been given to patients, the first priority was to assess the safety of repeat treatments. A principal objective of this thesis therefore is to conduct and analyse a phase I/II clinical trial of repeat ADEPT using MFECP1. In particular the aims of the trial are to investigate toxicity and to identify the maximum number of tolerated treatments and the maximum tolerated TPD. Toxicity results are presented in this chapter and anti-tumour activity is discussed in Chapter 4.

3.2 Patient characteristics

Between April 2005 and November 2007 twelve patients were registered onto the study and all patients received at least two ADEPT treatments. Patient characteristics are listed in Table 3.2. Eleven patients completed the follow-up period. One patient (patient 41) died as a consequence of disease progression on Day 25 (serum CA19-9 increased 84% from Day 1 to Day 19). The study was completed in January 2008.

3.3 Treatment received

Details of treatment received are shown in Table 3.3. CPG2 enzyme levels were <0.002U/ml at the time of prodrug administration for all treatments.

Patients in the first cohort (patients 32, 33 and 34) each received 2 scheduled treatments. Each treatment consisted of MFECP1 ($3000U/m^2$) followed by prodrug at $200mg/m^2$ x3. (Patient 32 remained HACPA negative after the first 2 ADEPT treatments. This patient was therefore eligible for further treatment which was given on days 43/44 and 45/46.) No DLTs occurred in the first cohort (2 treatments) and therefore the next patient (patient 35) received 3 treatments at a prodrug dose of $200mg/m^2$ x3 per treatment.

Table 3.2
Patient characteristics in repeat-treatment ADEPT phase I/II trial

Characteristic	Number of patients (n=12)	%
Sex		
Male	10	83
Female	2	17
Age (years)		
Median	66	
Range	36 – 77	
Type of cancer		
Adenocarcinoma colon	7	58
Adenocarcinoma oesophagus	2	17
Adenocarcinoma pancreas	1	8
Adenocarcinoma gallbladder	1	8
Carcinoma of unknown primary	1	8
Number of previous chemotherapy regimes (including adjuvant)		
Median	2	
Range	1 – 3	
WHO Performance status		
0	4	33
1	8	67
Sites of Metastases		
Lung	9	
Distant lymph node	8	
Liver	5	
Local nodal disease	2	
Soft tissue	3	
Bone	1	
Peritoneum	1	
Adrenal gland	1	
Spleen	1	

		Day 1	Day 2	Day 3	Day 4	Day 8	Day 9
Patient	Total prodrug dose (mg/m^2)	MFECP1	Prodrug (mg/m^2)	MFECP1	Prodrug (mg/m^2)	MFECP1	Prodrug (mg/m^2)
32	1200	✓	600	✓	600	NA	NA
33	1200	✓	600	✓	600	NA	NA
34	1200	✓	600	✓	600	NA	NA
35	1800	✓	600	✓	600	✓	600
36	900	✓	450	✓	450	✓	ND*
37	1350	✓	450	✓	450	✓	450
38	900	✓	300	✓	300	✓	300
39	300	✓	150	✓	150	✓	ND**
40	450	✓	150	✓	150	✓	150
41	450	✓	150	✓	150	✓	150
42	900	✓	300	✓	300	✓	300
43	700	✓	300	✓	300	✓	100***

Table 3.3

ADEPT treatment received in repeat-treatment ADEPT phase I/II trial

✓ = MFECP1 3000U/m^2 per treatment administered. NA: not applicable, ND: not done.

Patients 32, 33, 34, 36 and 39 completed 2 scheduled treatments. Patient 32 received 2 additional treatments (MFECP1 3000U/m^2 per treatment and prodrug 600mg/m^2 per treatment) on days 43/44 and 45/46 (details not shown on table).

Patients 35, 37, 38, 40, 41, 42 and 43 completed 3 scheduled treatments.

* Patient 36 did not complete the 3rd treatment; MFECP1 was administered but prodrug was not given because the patient developed a symptomatic pulmonary embolus.

** Patient 39 did not complete the 3rd treatment; MFECP1 was administered but prodrug was not given due to very slow MFECP1 clearance.

** In patient 43 only 1 bolus of prodrug was given following the 3rd MFECP1 infusion because the patient was found to be thrombocytopaenic (after the 1st bolus of prodrug was given) and therefore no further prodrug was given.

Patient 35 experienced grade 4 thrombocytopaenia and was given a pool of platelets as prophylaxis against haemorrhage. Patient 35 also experienced grade 4 neutropaenia and grade 3 febrile neutropaenia. Both the grade 4 thrombocytopaenia requiring platelet transfusion and the febrile neutropaenia constituted a DLT. Following the significant myelosuppression in patient 35 the PI decided that expanding the cohort and treating further patients at the same dose should not be performed as this could harm patient safety.

In order to attempt to reduce the risk of myelosuppression a substantial protocol amendment was approved by the MHRA in April 2006 and contained 3 features. Firstly the next cohort of patients would receive 3 treatments at a reduced dose of 150mg/m^2 x3. Secondly if further DLT occurred the cohort could be expanded or a new cohort opened with a reduced prodrug dose. Following a dose reduction the new cohort would continue with the same number of treatments as the previous cohort. In the event that toxicity was less than grade 2 a new cohort would open and the number of treatments would be increased by one. Thirdly the definition of DLT relating to thrombocytopaenia was changed from grade 4 thrombocytopaenia of ≥ 5 days or requiring platelet transfusion to any grade 3 thrombocytopaenia (this was changed at the request of CRUK in order to harmonize the definition with that used in other CRUK sponsored phase I trials).

Patient 36 was scheduled to receive 3 treatments, each with a prodrug dose of 150 mg/m^2 x3. However, only 2 treatments were completed because the patient developed a pulmonary embolus on day 9 before the prodrug (of the 3rd treatment) had been given. The PI decided not to proceed with prodrug. Patient 36 experienced a grade 2 thrombocytopaenia and therefore the cohort was expanded. Patient 37 received 3 treatments at 150 mg/m^2 x3. This patient experienced DLT (febrile neutropaenia and grade 4 thrombocytopaenia.) The PI and CRUK decided to open a new cohort with a reduced prodrug dose of 100 mg/m^2 x3.

Patient 38 received 3 treatments at 100 mg/m^2 x3 and experienced grade 3 thrombocytopaenia. Under the new definition of DLT (substantial protocol amendment 2006) the grade 3 thrombocytopaenia constituted a DLT. To further lower the risk of myelosuppression the PI and CRUK decided to open a new cohort with a reduced prodrug dose of 50 mg/m^2 x3. Patient 39 was scheduled to receive 3 treatments at a prodrug dose of 50 mg/m^2 x3. However following the 3rd MFECP1 infusion, serum CPG2 enzyme activity did not fall <0.005U/ml until 59.5 hours after the end of the infusion. Prodrug was not given following this 3rd MFECP1 infusion because MFECP1 retention on tumour was unlikely to be adequate after this length of time (Mayer *et al.*, 2006). Hence patient 39 only completed 2 ADEPT treatments. In patient 39 the MFECP1 had cleared successfully on previous infusions (on the 1st and 2nd MFECP1 infusions the time interval between the end of the MFECP1 infusion and prodrug was 18.4 hours and 14.1 hours respectively). Patient 39 developed grade 2 neutropaenia and therefore the cohort was expanded. Patient 40 and 41 both received 3 treatments of 50 mg/m^2 x3 without DLT.

An unplanned interim analysis of efficacy for patients 32 to 41 identified that efficacy was only seen at a TPD ≥ 900mg/m^2. A substantial protocol amendment therefore increased the TPD to 900mg/m^2 (3 treatments at 100 mg/m^2 x3). In addition the definition of DLT relating to thrombocytopaenia was redefined as < 20 x10^9/l. Patient 42 received 3 treatments at a prodrug dose of 100 mg/m^2 x3 (TPD 900mg/m^2) and developed a DLT (platelets were 19 x10^9/l). Patient 43 only received the first of three planned prodrug bolus injections on day 9 (3rd treatment) because of thrombocytopaenia. The patient subsequently developed a DLT on Day 19 (platelets were 18 x10^9/l).

3.4 Time interval between MFECP1 and prodrug

A total of 33 ADEPT treatments (MFECP1 followed by prodrug) were given, including 2 additional treatments given to patient 32. The median time interval from the end of the MFECP1 infusion to the start of prodrug

administration was 14.6 hours with a range of 13.7 to 22.3 hours (Table 3.4).

Table 3.4

Time interval (hours) from end of MFECP1 infusion to start of BIP prodrug administration in repeat-treatment ADEPT phase I/II trial

Patient	Treatment 1	Treatment 2	Treatment 3
32 (cycle 1)	15.2	14.3	NA
32 (cycle 2)	14.2	14.3	NA
33	15.8	13.7	NA
34	13.9	14.8	NA
35	14.3	14.1	14.5
36	22.3	18.3	BIP not given *
37	14.5	14.7	14.8
38	14.5	14.7	14.5
39	18.4	14.1	BIP not given **
40	14.4	14.5	14.6
41	14.8	14.4	14.1
42	14.8	14.7	17
43	18.6	17.4	16.8

NA: not applicable. ** Patient 36 did not complete the 3rd treatment; MFECP1 was administered but prodrug was not given because the patient developed a symptomatic pulmonary embolus. **serum CPG2 enzyme activity did not fall <0.005U/ml until 59.5 hours after the end of the infusion, therefore BIP prodrug not given.

3.5 Pharmacokinetics of BIP prodrug

Pharmacokinetic data was recorded after the 3rd prodrug bolus i.e. when patients had already received 2 bolus administrations of BIP prodrug. Despite the possibility that BIP prodrug could accumulate following each bolus, previous clinical data using the same prodrug found no evidence of significant prodrug accumulation (Francis *et al*, 2002). Measurement after the 3rd prodrug bolus allowed collection of data at 60 minutes that would not have been meaningful after the 1st or 2nd boli because of the administration of more prodrug i.e. the 2nd or 3rd bolus.

Initially pharmacokinetic data was recorded after the 3rd prodrug bolus of every treatment. However data from patient 32, 33, 34 and 35 suggested that repeat treatments did not significantly affect BIP prodrug pharmacokinetics. Consequently the protocol was modified so that from patient 36 onwards pharmacokinetic data was only recorded after the 3rd prodrug bolus of the first and last treatments.

Pharmacokinetic data was recorded on all patients and included 24 treatments (Table 3.5). The median half life of the BIP prodrug was 13.6 minutes (range 9.8 minutes to 18.2 minutes). This is similar to the median half life of 10.0 minutes reported in the single treatment ADEPT trial (Mayer *et al.*, 2006). Median half life varied little during the 1st, 2nd or 3rd treatments (13.6, 11.8 and 13.9 minutes respectively).

3.6 Safety

Toxicities that were deemed at least possibly related to ADEPT are shown in Table 3.6. Thrombocytopaenia and neutropaenia were the principal toxicities. A higher TPD was associated with increased grades of thrombocytopaenia and neutropaenia (Table 3.7).

The median time of platelet nadir was day 25 (range 17 – 30). Patient 35 experienced grade 4 thrombocytopaenia and was given a platelet transfusion as prophylaxis against haemorrhage. A patient with lung metastases (patient 37) developed a clinically small haemoptysis during a

period of grade 3 thrombocytopaenia and was therefore given a platelet transfusion. The median time taken for platelet recovery following platelet nadir was 13 days (range 7 to 41).

In patients who developed grade 3 or 4 neutropaenia the median time of the nadir was Day 32 (range day 27 to 37). One patient with grade 3 neutropaenia (patient 37) and one patient with grade 4 neutropaenia (patient 35) developed neutropaenic fever. The median time taken for neutrophil recovery in patients with grade 3 or 4 neutropaenia was 14 days (range 7 to 46). The most frequent non-laboratory adverse events were nausea and fatigue.

Patient 39 was diagnosed with acute myeloid leukaemia (AML) 8 months after commencing ADEPT. The patient had received 2 ADEPT treatments with a TPD of 300mg/m^2. In addition to ADEPT the patient had received 2 other cytotoxic regimes i.e. ECX (epirubicin, cisplatin, capecitabine) and gemcitabine with carboplatin. The patient's first cancer was an anaplastic large cell carcinoma of unknown primary. The time interval between the patient's first exposure to a cytotoxic drug and the development of AML was 15 months.

Adverse events that were considered to be caused by MFECP1 are discussed in detail in Chapter 5.

Patient	Treatment number	TPD per treatment (mg/m^2)	Elimination half-life (min)	C_0 (µg/ml)	AUC$_{INF}$ (µg.ml.min)	Clearance (ml/min)	Vss (ml)
					Table 3.5		

Table 3.5

BIP prodrug pharmacokinetics in repeat treatment ADEPT phase I/II trial

Patient	Treatment number	TPD per treatment (mg/m^2)	Elimination half-life (min)	C_0 (µg/ml)	AUC$_{INF}$ (µg.ml.min)	Clearance (ml/min)	Vss (ml)
32	1	600	10.0	44.2	432	778	9493
	2	600	10.8	53.0	539	624	8038
	3	600	12.8	60.0	502	645	8647
	4	600	9.3	49.9	438	740	8488
33	1	600	10.3	55.4	557	707	8894
	2	600	10.4	62.6	617	638	7926
34	1	600	11.7	53.9	562	544	7587
	2	600	12.8	59.9	551	555	8165
35	1	600	13.5	257	1100	351	4070
	2	600	14.6	54.1	805	480	8277
	3	600	13.9	48.0	963	401	7741
36	1	450	14.8	46.8	530	509	8582
37	1	450	18.2	63.5	1389.5	180.6	4348.4
	3	450	18.1	71.6	1615.4	155.4	3803.8
38	1	300	9.8	24.9	333.0	615.6	8264.8
	3	300	19.5	41.3	853.2	240.3	5963.5
39	1	150	16.7	18.0	358.1	279.3	5999.0
40	1	150	13.7	27.5	260.5	383.8	5706.6
	3	150	12.9	17.2	267.9	373.3	6285.0
41	1	150	13.6	15.3	190.8	445.6	6827.0
	3	150	10.8	32.6	316.4	268.7	3388.4
42	1	300	17.4	32.0	628.4	350.1	8135.4
	3	300	14.8	29.4	520.1	423.0	7506.6
43	1	300	13.0	32.6	446.2	403.4	6218.0

Two minute post-dose value in treatment 1 of patient 35 was extremely high. This was probably an artefact due to inadequate flushing of the central venous cannula resulting in contamination of the sample with prodrug. TPD: total prodrug dose.

Adverse event	Grade 1 – 4	Grade 1	Grade 2	Grade 3	Grade 4
Leucocytes (total WBC)	92	8	42	25	17
Neutrophils/Granulocytes	75	8	25	8	33
Lymphopaenia	67	0	0	67	0
Platelets	92	8	25	50	8
Haemoglobin	50	25	25	0	0
Bilirubin	25	17	8	0	0
ALT	33	17	8	8	0
AST	33	25	8	0	0
GGT	8	0	8	0	0
Febrile neutropaenia	17	NA	NA	17	0
Nausea	75	42	33	0	NA
Vomiting	50	42	8	0	0
Diarrhoea	8	8	0	0	0
Fatigue	50	42	0	8	0
Fever (no neutropaenia)	17	17	0	0	0
Rigors/chills	17	17	0	0	NA
Taste disturbance	17	17	0	NA	NA
Haemoptysis	8	0	NA	8	0
Secondary malignancy	8	NA	NA	NA	8
Supraventricular arrhythmia	8	0	0	8	0

Table 3.6

The percentage of patients experiencing adverse events in repeat treatment ADEPT phase I/II trial

Adverse events (AEs) shown were possibly, probably or almost certainly related to ADEPT. AEs defined according to Common Toxicity Criteria (CTC); version 2. NA: not applicable i.e. no such category exists in CTC version 2. Data from CRUK database. All data on patient 32 relates to cycle 1 only i.e. 2 scheduled treatments.

WBC: white blood cells, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma glutamyl transpeptidase

Table 3.7

Thrombocytopaenia and neutropaenia in relation to number of treatments and total prodrug dose in repeat treatment ADEPT phase I/II trial

Patient	Number of treatments	Total prodrug dose (mg/m^2)	Thrombocytopaenia (CTC grade)	Neutropaenia (CTC grade)
39	2	300	1	2
36		900	2	0
32		1200	1	1
33		1200	3	2
34		1200	3	1
40	3	450	2	2
41		450	2	0
43		700	3	4
38		900	3	3
42		900	3	4
37		1350	3	4
35		1800	4	4

CTC: common toxicity criteria.

All data on patient 32 relates to cycle 1 only i.e. 2 scheduled treatments.

3.7 Maximum tolerated TPD for repeat ADEPT treatments

The definition of DLT in relation to thrombocytopaenia was changed during the study by protocol amendment. Definition 1 (patients 32 to 35 inclusive) was grade 4 thrombocytopaenia with 1 or more of the following: duration ≥ 5 days, bleeding or requiring platelet transfusion. In April 2006 the definition was changed: Definition 2 (patients 36 to 41 inclusive) was ≥ grade 3 thrombocytopaenia. The final protocol amendment (July 2007) increased the minimum TPD to 900mg/m^2. Since it was anticipated that grade 3 thrombocytopaenia could occur at this dose, the definition of DLT was changed: Definition 3 (patients 42 and 43) was platelet count < 20x10^9/l.

On study completion a single definition of DLT was applied to all patients to determine the MTD. The definition regarding thrombocytopaenia was Definition 3 (platelet count < 20x10^9/l), all other criteria were as defined in Materials and Methods (Chapter 2). Details of DLTs are shown in Table 3.8.

The maximum tolerated TPD with 2 treatments was 1200mg/m^2. Three patients (patients 32, 33, 34) received this dose. Two patients experienced grade 3 thrombocytopaenia (platelet nadir was 33 x10^9/l in patient 33 and 48 x10^9/l in patient 34). There were no grade 4 adverse events

The maximum tolerated TPD with 3 treatments was 450mg/m^2. This schedule was received by patients 40 and 41. Complete follow up data is only available on patient 40 because patient 41 died on day 25 due to disease progression. Patient 40 experienced no grade 3 or 4 adverse events.

	Table 3.8		
	Dose limiting Toxicity (DLT) in repeat treatment ADEPT phase I/II trial		
Number of completed ADEPT treatments	**Total prodrug dose (mg/m^2)**	**DLT***	**Patient**
2	300	No	39
	900	No	36
	1200	No	32**
	1200	No	33
	1200	No	34
3	450	No	40
	450	No	41
	700	Yes: Platelets < 20 x10^9/l	43
	900	No	38
	900	Yes: Platelets < 20 x10^9/l	42
	1350	Yes: 1. Febrile neutropaenia 2. Platelets < 20 x10^9/l	37
	1800	Yes: 1. Febrile neutropaenia 2. Platelets < 20 x10^9/l	35

* The definition of DLT with regard to thrombocytopaenia was a platelet count < 20 x10^9/l.

** All data on patient 32 relates to cycle 1 only i.e. 2 scheduled treatments.

3.8 Combined analysis of toxicity in single and repeat treatment patients

A combined analysis of the results from all 43 patients who have received ADEPT with MFECP1 was performed. Thirty one patients received 1 treatment, 5 patients received 2 treatments and 7 patients received 3 treatments. The toxicity results of the single ADEPT treatment phase I trial have previously been published (Mayer *et al.*, 2006). The combined analysis in this MD (Res) thesis represents a novel analysis of all available data.

3.8.1 Combined analysis of myelosuppression

Myelosuppression was seen in single and repeat treatment patients. Severity of myelosuppression increased as the TPD increased (Table 3.9).

In addition, as the *number* of treatments increased the dose of prodrug necessary to cause myelosuppression decreased. For example in patients who received MFECP1 $3000U/m^2$ the lowest TPD that was associated with $\geq$ grade 3 thrombocytopaenia after 1, 2 or 3 treatments was $1613mg/m^2$, $1200mg/m^2$ and $700mg/m^2$ respectively (Table 3.9).

There is some evidence that myelosuppression is increased when prodrug is given at higher serum enzyme levels. Forty three patients received a total of 64 ADEPT treatments (including 2 additional treatments given to patient 32). Prodrug was given when serum enzyme activity was $\leq 0.005U/ml$ in 63 of 64 treatments. However in 1 treatment (patient 3) prodrug was administered when serum enzyme activity was $0.017U/ml$. Patient 3 developed grade 3 neutropaenia and grade 3 thrombocytopaenia despite being in the lowest prodrug dose cohort (Table 3.10).

Table 3.9
Thrombocytopaenia and neutropaenia in relation to number of treatments and total prodrug dose in patients that received MFECP1 3000U/m^2

Patient	Number of treatments	Total prodrug dose (mg/m^2)	Thrombocytopaenia (CTC grade)	Neutropaenia (CTC grade)
26	1	806.4	0	0
27		806.4	1	2
28		806.4	0	0
24		1612.8	3	4
25		1612.8	1	3
39	2	300	1	2
36		900	2	0
32		1200	1	1
33		1200	3	2
34		1200	3	1
40	3	450	2	2
41		450	2	0
43		700	3	4
38		900	3	3
42		900	3	4
37		1350	3	4
35		1800	4	4

CTC: common toxicity criteria. ** All data on patient 32 relates to cycle 1 only i.e. 2 scheduled treatments.

At a fixed TPD of 1613mg/m^2 a reduction in MFECP1 dose permitted earlier prodrug administration but was associated with greater myelosuppression (Table 3.11). The increase in myelosuppression, despite serum CPG2 activity being < 0.002U/ml, was probably caused by CPG2 activity in non-tumour tissue other than plasma e.g. liver, lung.

Table 3.10

Serum enzyme activity at time of prodrug administration and thrombocytopaenia / neutropaenia. Data on 6 patients who each received a total prodrug dose of 37.3mg/m^2 with MFECP1 5000U/m^2.

Patient number	Serum CPG2 activity (U/ml)	Thrombocytopaenia CTC grade	Neutropaenia CTC grade
1	≤ 0.005	2	0
2	≤ 0.005	1	0
3	**0.0167**	**3**	**3**
5	≤ 0.005	0	0
6	≤ 0.005	0	0
7	≤ 0.005	0	0
CTC: common toxicity criteria			

Table 3.11

MFECP1 to prodrug time interval and myelosuppression

Patient	MFECP1 dose (U/m^2)	Serum CPG2 activity* (U/ml)	MFECP1-prodrug interval (hours)		Neutropaenia CTC	Thrombocytopaenia CTC
21	5000	<0.002	17.0	mean = 16.5	0	0
22	5000	<0.002	16.0		0	1
24	3000	<0.002	9.7	mean = 11.8	4	3
25	3000	<0.002	13.8		3	1

*serum CPG2 activity at time of BIP prodrug administration.

MFECP1-prodrug interval was time from end of MFECP1 infusion to BIP prodrug administration.

CTC: Common Toxicity Criteria (version 2)

3.8.2 Combined analysis of hepatic toxicity

Two patients (patient 23 and 25) experienced grade 4 elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). A liver biopsy in patient 23 revealed hepatic necrosis. The liver dysfunction resolved completely. Grade 4 hepatic impairment was only seen in patients who received a TPD *per treatment* of $\geq 1613mg/m^2$. Grade 4 hepatic impairment did not occur in patients who received repeat ADEPT; the highest TPD per treatment in repeat ADEPT patients was $600mg/m^2$.

3.8.3 Combined analysis of renal toxicity

Grade 4 renal dysfunction occurred in one patient (patient 23). This patient experienced grade 4 elevation of serum creatinine and uric acid. A renal biopsy revealed acute tubular necrosis. Temporary haemofiltration was required and the renal toxicity subsequently resolved. This patient also experienced grade 4 elevations of serum ALT and AST. The patient had received a single ADEPT treatment of MFECP1 $5000U/m^2$ followed by a TPD of $3225.6mg/m^2$. This was the largest TPD per treatment given to any patient. No other patient had any elevation of serum creatinine or uric acid.

3.9 Conclusions

This chapter had two aims. Firstly to investigate treatment delivery and safety of repeat ADEPT treatments with MFECP1 and BIP prodrug. Secondly to identify risk factors for toxicity by examining all available clinical data i.e. single and repeat treatment patients.

Twelve patients were treated in a phase I/II clinical trial of repeat ADEPT treatment. The median time interval between the end of the MFECP1 infusion and prodrug administration was 14.6 hours. Dose limiting toxicity (thrombocytopaenia and febrile neutropaenia) prevented the number of treatments being increased above 3. The maximum tolerated TPD was $1200mg/m^2$ for 2 treatments and $450mg/m^2$ for 3 treatments. The most

frequent adverse events (any grade) were leucopaenia, thrombocytopaenia, neutropaenia and nausea. The most frequent grade 3 or 4 adverse events were lymphopaenia, thrombocytopaenia and leucopaenia.

Combined analysis of all data identified risk factors for myelosuppression and hepatic toxicity. Grade 4 hepatic toxicity only occurred in patients who received a TPD *per treatment* ≥ 1613mg/m^2. Increased TPD or number of treatments was positively associated with an increase in myelosuppression. Myelosuppression may also be associated with increased serum CPG2 activity at time of prodrug and reduced MFECP1-prodrug interval.

These results indicate that repeat ADEPT can be given safely and have identified clinically relevant risk factors for toxicity. In the following chapter the efficacy of repeat ADEPT treatment is investigated.

Chapter 4

Efficacy of repeat ADEPT treatment with MFECP1 and BIP prodrug (Phase I/II trial).

4.1 Introduction

The primary objective of the phase I/II clinical trial was to determine the maximum number of tolerated treatments and the maximum tolerated TPD. Whilst formal assessment of efficacy is performed at a later stage in drug development (phase II and phase III), a phase I trial offers an opportunity to obtain early data on tumour response. Evidence of tumour response in a phase I trial would favour a drug's continued development. Furthermore if responses do occur then these responses may provide early evidence of which drug doses are associated with response. This information may help in the selection of the recommended phase II dose.

This chapter aims to examine evidence of efficacy in repeat treatment ADEPT patients. In addition a combined analysis of all available efficacy data is performed i.e. single and repeat treatment patients. This combined analysis includes FDG-PET results from single-treatment patients, data that was not included in the published data on the single-treatment trial (Mayer *et al.*, 2006).

4.2 Tumour response by CT imaging

Eleven of 12 patients (92%) were evaluated for response with a Day 29 and Day 57 CT scan. No complete or partial responses were seen. The results of

disease assessment by CT are shown in Table 4.1. Patients are listed according to TPD received. Patient 35 had a 10% reduction in tumour size (RECIST criteria) on Day 29 imaging (Figure 4.1) and a 4% reduction on Day 57 imaging.
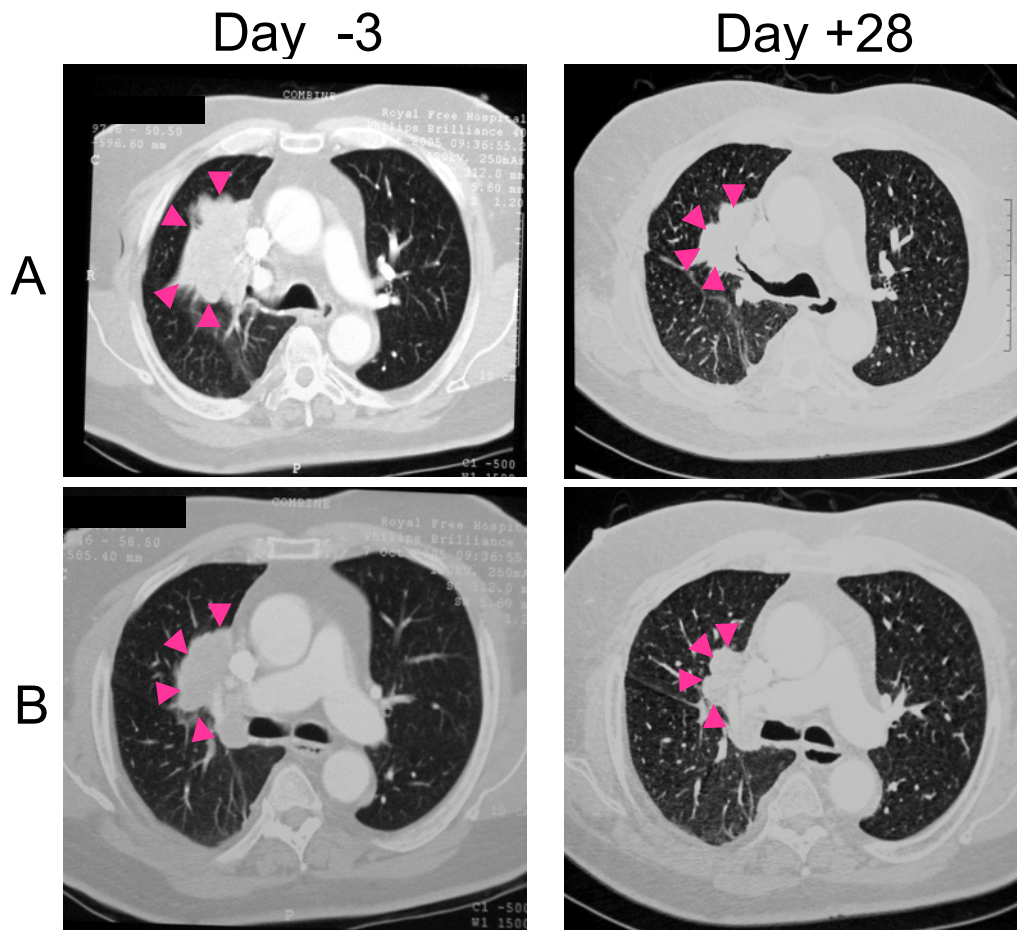
Figure 4.1

Reduction in the size of a metastasis following ADEPT. The images were taken before (Day -3) and after ADEPT (Day 28). The images in row A are taken at a different anatomical level to the images in row B. The location of a metastasis in the right lung is indicated with pink arrowheads. Images are from patient 35 who received 3 ADEPT treatments with a total prodrug dose of 1800mg.m^2. By RECIST criteria reduction in longest diameter was 10% i.e. stable disease.

Patient	Total prodrug dose (mg/m^2)	CT Day 29 RECIST criteria (% change or new lesion)	CT Day 57 RECIST criteria (% change or new lesion)
		Table 4.1 **Disease assessment by CT imaging in repeat treatment ADEPT phase I/II trial**	
39	300	SD (13% increase)	PD (28% increase)
40	450	SD (4% increase)	SD (7% increase)
41	450	Not examined	Not examined
43	700	SD (18% increase)	SD (18% increase)
36	900	SD (3% increase)	SD (12% increase)
38	900	SD (3% increase)	SD (7% increase)
42	900	PD (New enlarged lymph node)	PD (New enlarged lymph node)
32	1200	SD (5% increase)	SD (16% increase)
33	1200	SD (10% increase)	PD (29% increase)
34	1200	PD (New bone lesion)	PD (New bone lesion)
37	1350	SD (1% decrease)	SD (2% decrease)
35	1800	SD (10% decrease)	SD (4% decrease)

Percentage change in measured target lesions relative to the pre-ADEPT CT scan. CT: computerized tomography. RECIST: response evaluation criteria in solid tumours. PD (progressive disease) is an increase in the sum of longest diameters of target lesions of ≥ 20% or the appearance of one or more new lesions. SD (stable disease) is defined as neither sufficient shrinkage to qualify for PR (≥30% decrease in the sum of the longest diameters of target lesions) nor a sufficient increase to qualify for PD.

4.3 FDG-PET imaging

Eleven patients chose to undergo an optional FDG-PET scan prior to treatment. Post-treatment imaging was performed on Day 23 and Day 54 in 10 and 8 patients respectively. Three patients were unable to have post treatment imaging due to symptomatic deterioration. The results of disease assessment by FDG-PET coincidence imaging are shown in Table 4.2. A significant reduction in tumour activity (>15%) was seen in 3 patients at Day 23.

4.4 Serum CA19-9 measurement

The serum tumour marker CA19-9 was elevated at baseline in 8 patients. Following ADEPT the serum CA19-9 fell in 3 patients, all of whom received a TPD ≥ 900 mg/m^2. Reductions in serum CA19-9 of 64%, 63% and 27% occurred in patients 32, 35 and 38 respectively (Figure 4.2). These 3 patients are the same patients that responded on FDG-PET.
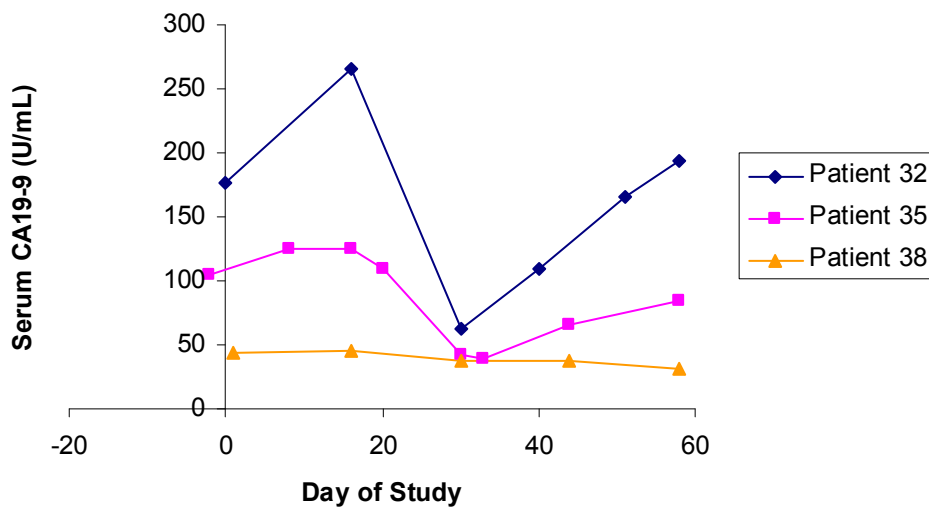
Figure 4.2

Reduction in serum CA19-9 in patients 32, 35 and 38 following ADEPT. All patients commenced treatment on Day 1. Data shown on patient 32 represents the response to the first 2 treatments that patient 32 received.

Table 4.2

Disease assessment by FDG-PET imaging in repeat treatment ADEPT phase I/II trial

Patient	Total prodrug dose (mg/m^2)	Response by FDG-PET coincidence imaging on Day 23 (% change)	Response by FDG-PET coincidence imaging on Day 54 (% change)
39	300	SD (7.0% increase)	N.Q.
40	450	SD (8.5% increase)	PD (45.2% increase)
41	450	NE	NE
43	700	NQ	NQ
36	900	NQ	NQ
38	900	PR (15.2% decrease)	SD (5.4% increase)
42	900	PD (60.6% increase)	PD (68.6% increase)
32	1200	PR (20.7% decrease)	SD (6.1% increase)
33	1200	PD (36.6% increase)	NE
34	1200	PD (692% increase)	NE
37	1350	NE	NE
35	1800	PR (27.2% decrease)	PD (48.6% increase)

Response defined according to the method described by Green (Green *et al.*, 2008). Partial response (PR) is a reduction >15%, progression of disease (PD) is an increase >10%, stable disease (SD) relates to values between PR and PD. PR, SD and PD shaded in green, blue and pink respectively. Percentage change is relative to the pre-ADEPT baseline FDG-PET coincidence scan. Patient 37 declined FDG coincidence imaging. Patient 41 was unable to undergo follow up imaging due to symptomatic deterioration. Patients 33 and 34 were unable to undergo the Day 53 scan due to symptomatic deterioration. NQ: not quantifiable. NE: not examined.

4.5 Combined analysis of efficacy in single and repeat treatment patients

A combined analysis of the results from all 43 patients who have received ADEPT (with MFECP1 and BIP prodrug) was performed. The median number of previous chemotherapy regimes received prior to ADEPT (including adjuvant therapy) was 2 (range 1 to 5).

No objective response (complete response or partial response on RECIST) was seen on CT imaging. Two patients had minor CT responses i.e. 9% reduction in patient 24 and 10% reduction in patient 35.

Quantification of FDG-PET tumour response was performed in 22 patients. Tumour activity on FDG-PET imaging reduced significantly (> 15%) in 4 patients. The primary site of tumour in these patients was peritoneal serous carcinoma (patient 24), adenocarcinoma of the pancreas (patient 32) and adenocarcinoma of the colon (patients 35 and 38).

In 3 of the 4 patients who had a significant response on FDG-PET imaging, data from a subsequent FDG-PET was available. In all 3 of these patients the significant reduction in tumour activity was seen on the Day 23 scan. However in each of these patients tumour activity had increased above the pre-ADEPT imaging on the Day 54 scan. The median interval in days between the 2 scans was 35 days (range 28 to 38).

Responses on FDG-PET were only seen in patients that received a TPD ≥ 900 mg/m^2. There was a significant correlation between percentage change of tumour activity on FDG-PET and TPD (p=0.031, Spearman Rank correlation test).

Serum CA19-9 was elevated at baseline in 30 patients. Following ADEPT serum CA19-9 fell in 9 patients. The 3 largest falls in serum CA19-9 (64% in

patient 32, 63% in patient 35 and 27% in patient 38) all occurred in patients that received a TPD ≥ 900 mg/m^2. A summary of adverse events and efficacy in patients who received a TPD ≥ 900mg/m^2 is shown in Table 4.3.

4.6 Efficacy with 2 ADEPT treatments given at maximum tolerated TPD

Using an MFECP1 dose of 3000U/m^2 the maximum tolerated TPD was 450mg/m^2 with 3 treatments, 1200mg/m^2 with 2 treatments and 806.4mg/m^2 with 1 treatment. (In the single ADEPT treatment trial MFECP1 3000U/m^2 was only examined at 2 TPD levels: 806.4mg/m^2 and 1612.8mg/m^2). Combined analysis of efficacy indicated that efficacy was only seen when TPD ≥ 900mg/m^2.

There was evidence of efficacy with 2 ADEPT treatments given at maximum tolerated TPD (1200mg/m^2). Three patients were treated with this regime (patients 32, 33 and 34). Efficacy was seen in one of these patients (patient 32) on FDG-PET (21% reduction in activity) and serum CA19-9 (64% reduction). The FDG-PET response appeared short lived and tumour activity had increased above baseline 35 days later i.e. on the Day 54 scan. With this regime both grade 3 lymphopaenia and grade 3 thrombocytopaenia occurred in 2 patients (66%). One patient (33%) had grade 2 neutropaenia. Non-laboratory adverse events were grade 1 only. The most frequently occurring non-laboratory adverse events were nausea (66%), vomiting (66%) and fatigue (66%).

Table 4.3

Adverse events and efficacy (CT and FDG-PET) with TPD ≥ 900mg/m^2

Number of treatments	Patient Number	TPD (mg/m^2)	Grade of Toxicity (CTC)					CT	FDG-PET
			Creatinine	Platelets	Neutrophils	ALT	AST		
1	21	1613	0	0	0	1	0	SD	NQ
	22	1613	0	1	0	1	1	SD	Disease progression
	24	1613	0	3	4	2	2	SD	**Response**
	25	1613	0	1	3	4	4	PD	NE
	23	3226	4	3	2	4	4	PD	Disease progression
2	32	1200	0	1	1	2	1	SD	**Response**
	33	1200	0	3	2	0	0	SD	Disease progression
	34	1200	0	3	1	0	0	PD	Disease progression
3	42	900	0	3	4	3	1	PD	Disease progression
	38	900	0	3	3	1	1	SD	**Response**
	36	900	0	2	0	0	0	SD	NQ
	37	1350	0	3	4	2	1	SD	NE
	35	1800	0	4	4	0	0	SD	**Response**

No grade 4 toxicity occurred following 2 treatments with a TPD of 1200mg/m2 (shaded green). CT is Day 29 result. FDG-PET is Day 23 result. Response on FDG-PET defined according to the method described by Green (Green *et al.*, 2008). ALT: alanine transaminase, AST: aspartate transaminase, SD: stable disease, PD: progression of disease, NQ: not quantifiable, NE: not examined: TPD: total prodrug dose, CTC: common toxicity criteria.

4.7 Conclusions

The aim of this chapter was to investigate evidence of efficacy with repeat ADEPT treatments. This was achieved by assessment of CT, FDG-PET and serum CA19-9. No complete responses or partial responses were seen on CT imaging. One patient had a 10% reduction of tumour size on CT by RECIST criteria. Clinically significant responses on FDG-PET imaging occurred in 3 patients.

Combined analysis of data from single and repeat ADEPT treatments indicated that FDG-PET responses only occurred in patients who received a TPD $\geq$ 900mg/m^2. Efficacy was seen in 1 of 3 patients that received 2 ADEPT treatments given at the maximum tolerated TPD.

Chapter 5

The immune response to MFECP1

5.1 Introduction

MFECP1 is a non-human, recombinant fusion protein. Hence administration of MFECP1 to patients could cause activation of elements of the immune system e.g. T_h lymphocytes and B-lymphocytes. The aim of the work presented in this chapter was to increase understanding of the immune response to MFECP1. The immune response to MFECP1 is important because antibodies to MFECP1 could reduce patient safety and adversely affect the function of MFECP1.

The first question asked was whether increasing the number of MFECP1 infusions would increase the incidence of immunological adverse events. This is possible because data from the single-treatment ADEPT trial indicated that patients could become HACPA positive as early as day 7. Since a patient receiving 3 ADEPT treatments would receive the 3rd MFECP1 infusion on day 8, HACPA could be present at the time of MFECP1 administration.

The second question was whether patients receiving repeat MFECP1 infusions were more likely to become HACPA positive than those who received a single treatment.

Finally the nature of the T_h lymphocyte response to MFECP1 was investigated. Since T_h lymphocyte activation is required for the development of high affinity, long-lived antibody responses the possibility of identifying and mutating clinically relevant T-cell epitopes was investigated. The focus

was placed on addressing the CPG2 component of MFECP1 since this was likely to be the most immunogenic sequence within MFECP1. T_h lymphocytes were isolated from healthy volunteers and a patient receiving ADEPT, as well as from mice transgenic for human MHC class II, in order to investigate the role of T_h lymphocytes in the development of immune responses against MFECP1.

5.2 Safety of MFECP1

During the single-treatment and repeat-treatment ADEPT trials a total of 66 MFECP1 infusions were administered to 43 patients (including 2 additional treatments given to patient 32). Adverse events related to MFECP1 occurred in 8 of 43 patients (19%) and were grade 1 only (Table 5.1).

MFECP1 infusion reactions, defined as MFECP1-related adverse event(s) that started after MFECP1 administration but before BIP prodrug, occurred in 11% (7/66) of infusions and were grade 1 only. Infusion reactions occurred with 12% of 1st-dose infusions and 9% of non-1st dose infusions. Hence the results indicated that prior administration of MFECP1 did not increase the risk of an infusion reaction (Table 5.2).

Table 5.1

Adverse events related to MFECP1 in single and repeat treatment ADEPT patients

Patient	Adverse event	CTC grade	Day	Comments	MFECP1 infusion reaction ‡
1	rigors/chills	1	1	50 minutes after starting MFECP1	Yes
	fatigue	1			
	allergy/immunology – other*	1			
2	headache	1	1	Day 1	Yes
11	hypertension	1	1	2 hours after starting MFECP1	Yes
12	fever (no neutropaenia)	1	2	5 hours after BIP prodrug	No
	fever (no neutropaenia)	1	3	Day 3 i.e. after MFECP1 and BIP prodrug	No
14	nausea	1	1	4 hours after starting MFECP1	Yes
	vomiting	1			
28	constitutional – other**	1	2	3 hours after BIP prodrug	No
35	fever (no neutropaenia)	1	1	1 hour after starting MFECP1	Yes
	rigors/chills	1	8	After 3rd MFECP1 infusion	Yes
36	nausea	1	3	After 2nd MFECP1 infusion	Yes

Adverse events listed were considered possibly, probably or almost certainly related to MFECP1. CTC: Common Toxicity Criteria (CTC), version 2.
* Patient felt cold / shivery and felt "heat" in throat. Looked pale and had cool peripheries. ** Pyrexia. ‡ MFECP1 infusion reaction defined as MFECP1-related adverse event(s) that started after MFECP1 administration but before BIP prodrug administration.

Table 5.2

MFECP1 infusion reactions and number of MFECP1 infusions received; results from single and repeat treatment ADEPT patients

	1st MFECP1 administration (n=43)	2nd MFECP1 administration (n=12)	3rd MFECP1 administration (n=10)	4th MFECP1 administration (n=1)
Number (%) of infusions complicated by infusion reaction	5 (12%)	1 (8%)	1 (10%)	0 (0%)

5.3 HACPA production

HACPA formation was measured by ELISA before treatment and weekly during follow up period. The cut-off value for a positive result was obtained by assaying 50 pre-treatment serum samples at 1:100 dilutions and taking the cut-off as 3x the standard deviation above the mean absorbance.

HACPA data was obtained from all repeat-treatment patients. These results were combined with those from single-treatment patients to provide a combined data set from 42 patients. No follow up HACPA data was available on patient 31 due to clinical deterioration. The combined HACPA results (Table 5.3) showed that all patients became HACPA positive during the 58 day follow-up period. In patients who received repeat MFECP1 infusions the median day of becoming HACPA positive was day 16 (range 15 to 58).

Table 5.3

HACPA production in relation to number of MFECP1 infusions; results from single and repeat treatment ADEPT patients

Number of MFECP1 infusions	Number of HACPA positive patients (%)	Day of HACPA onset or median day of HACPA onset (range), in HACPA positive patients
1*	12/30 (40%)	day 14 (7 to 42)
2	2/2 (100%)	day 16 and day 19
3	9/9 (100%)	day 16 (15 to 37)
4	1/1 (100%)	day 58

* Data updated from single ADEPT treatment study (Mayer et al, 2006)

One patient received a total of 4 MFECP1 infusions (2 scheduled infusions plus 2 additional infusions) and became HACPA positive on day 58. This patient was able to receive the 2 additional infusions (on day 43 and 45) as per protocol.

Four patients received ciclosporin immunosupression in an attempt to reduce HACPA formation. Ciclosporin was given to patients 36, 37, 38 and 39 who became HACPA positive on day 18, 15, 23 and 15 respectively. Patient 36 had a grade 4 allergic reaction (anaphylaxis) to intravenous ciclosporin shortly after beginning the infusion. This allergic reaction resolved completely the same day after supportive treatment. The patient was switched from ciclosporin to alternative immunosupression (oral tacrolimus).

Ciclosporin was also considered responsible for grade 2 nausea in patient 37 and grade 1 elevation of creatinine in patient 39. Since ciclosporin appeared to increase toxicity without producing any delay in HACPA formation, it was not given after patient 39.

Patients who received more than 1 MFECP1 infusion were significantly more likely to become HACPA positive (Log-rank p = 0.001) than patients who received a single infusion (Figure 5.1). A significant positive relationship was also found between total MFECP1 dose and HACPA formation (p=0.013, Cox Regression analysis).
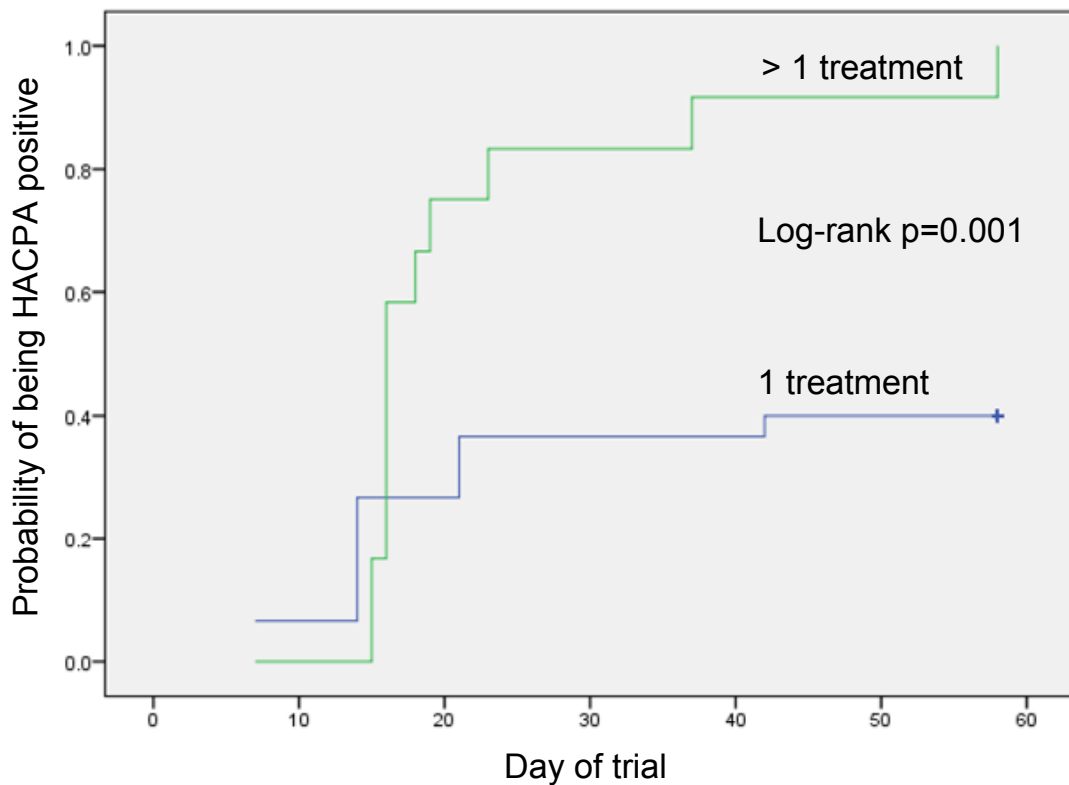
Figure 5.1

Kaplan Meier curves for probability of being HACPA positive following a single or repeat MFECP1 infusion. Single infusion = blue line, repeat infusion = green line. Data based on results of 42 patients. (+ = data censored)

5.4 Human T_h lymphocyte response to MFECP1: healthy volunteers

The *in vitro* response to MFECP1 was studied using T_h lymphocytes from 6 healthy volunteers none of whom had received MFECP1. PBMCs were incubated for 5 days with MFECP1 in T-cell proliferation assays. SEB and PPD were used as positive controls. The results of these experiments are illustrated in Figure 5.2 and showed that significant lymphocyte stimulation, a stimulation index (SI) of ≥ 2, occurred in 83% i.e. 5 of the 6 volunteers.

T-cell proliferation assays from healthy volunteers.

Figure 5.2

In vitro response of peripheral blood mononuclear cells (PBMCs) from healthy volunteers to MFECP1. T-cell proliferation assays using PBMCs from 6 healthy volunteers. Cells were incubated with MFECP1, SEB, PPD or negative control (medium only). Results are shown as stimulation indices (S.I.). Significant proliferation (S.I. ≥ 2) occurred in 5 of 6 donors when incubated with MFECP1.

5.5 Human T$_h$ lymphocyte response to MFECP1: ADEPT patient

PBMCs from an ADEPT patient were incubated with MFECP1 in a T-cell proliferation assay. The PBMCs were prepared from a blood sample taken from patient 43 on day 80. Due to a low yield of PBMCs the concentration of cells added to wells was 1.5 x10^6 cells/ml rather than the usual concentration of 3.5 x10^6 cells/ml. The patient received 3 MFECP1 infusions and became HACPA positive on day 16. The patient did not receive ciclosporin and had not experienced any adverse events related to MFECP1. Results from the T-cell proliferation assay showed that incubation of PBMCs with MFECP1 produced a SI of 2.5, consistent with significant stimulation (Figure 5.3).

T-cell proliferation assay from ADEPT patient.
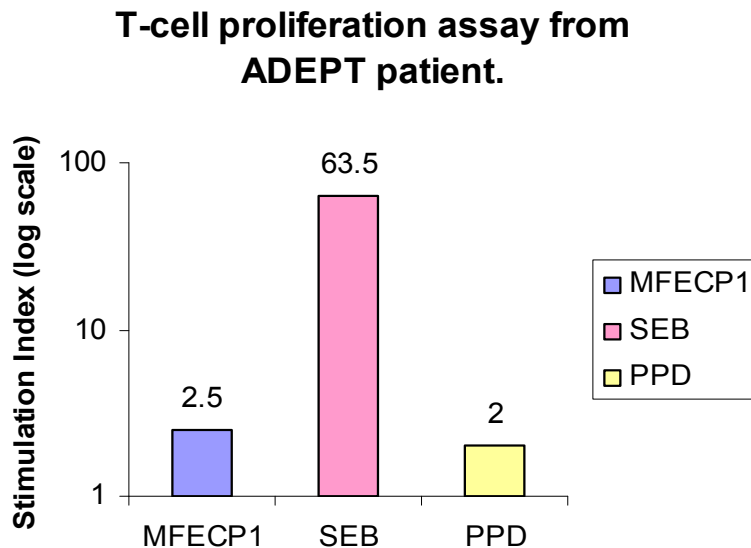
Figure 5.3

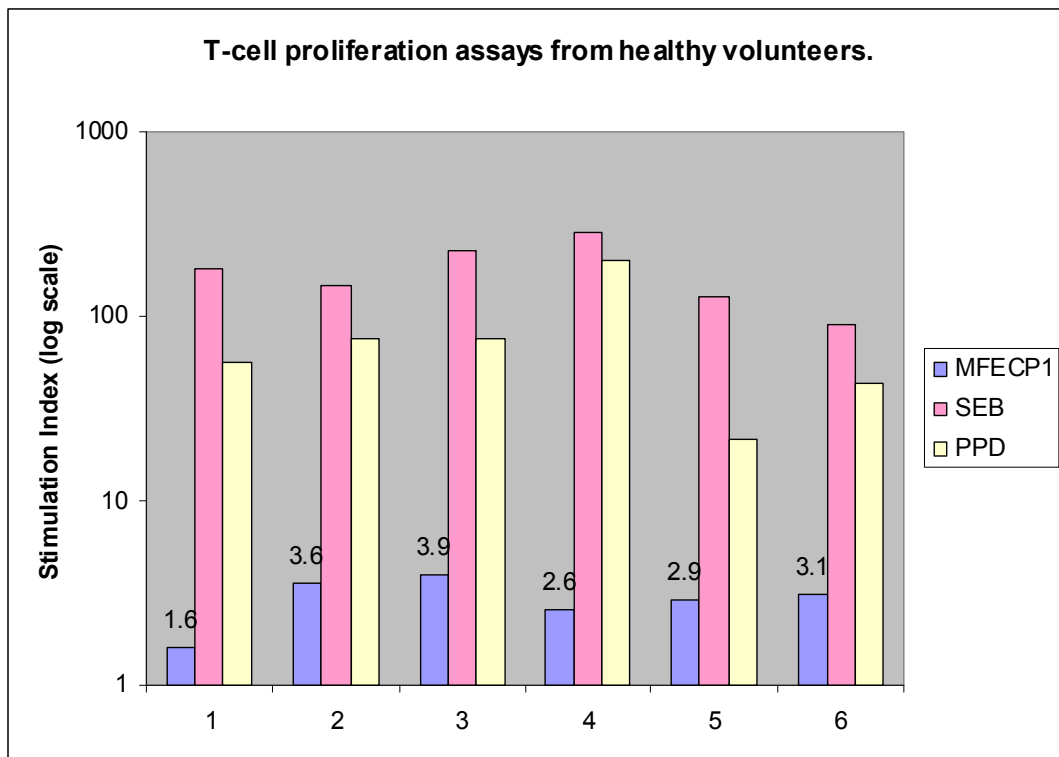*In vitro* response of peripheral blood mononuclear cells (PBMCs) from an ADEPT patient to MFECP1. A T-cell proliferation assay using PBMCs from patient 43. Cells were incubated with MFECP1, SEB, PPD or negative control (medium only). Results are shown as stimulation indices (S.I.). Significant proliferation (S.I. ≥ 2) occurred following incubation of PBMCs with MFECP1.

5.6 Human T_h lymphocyte response: IFN-γ secretion assay

Secondary immune responses are characterized by rapid, large responses to an antigen that an organism has previously been exposed to. In section 5.4 it was demonstrated that T_h lymphocytes from 5 of 6 healthy volunteers responded after a 5 day *in vitro* incubation with MFECP1. T_h lymphocyte proliferation at 5 days suggested that the reaction was a secondary immune response. Further experiments were therefore conducted to investigate whether there was evidence of lymphocyte activation earlier than 5 days.

Blood was taken from 3 healthy volunteers. All 3 volunteers (volunteer 2, 3 and 6) had given a previous blood sample for the T-cell proliferation experiments (see Figure 5.4) and had responded to MFECP1. Whole blood was incubated for 13 hours at 37°C with MFECP1, SEB (positive control) or media only (negative control). The red blood cells were then lysed and T-cell stimulation was measured by using an IFN-γ secretion assay. T-cells were first labelled with a catch reagent specific for IFN-γ (this is a proprietary mAb-mAb complex that binds to the cell surface at one end and binds to IFN-γ at the other). Cells were incubated at 37°C to allow IFN-γ secretion from stimulated cells. IFN-γ that bound to the catch reagent was then labelled using a second IFN-γ specific mAb that was conjugated to R-phycoerythrin (PE) for detection by flow cytometry. In addition cells were labelled for CD3 and CD4. Cells were then fixed and flow cytometry performed.

Results showed that incubation of PBMCs with MFECP1 increased IFN-γ release compared to incubation with medium alone in CD3 positive cells (Figure 5.4).

Figure 5.4

Response of CD3+ cells (measured by IFN-γ release) from 3 healthy volunteers after
incubation with MFECP1, negative control (media only) or positive control (SEB). A.
Percentage of CD3 positive cells releasing IFN-γ after incubation for 13 hours with MFECP1,
negative control or positive control. B. IFN-γ release in CD3 positive cells from volunteer 3
after incubation with MFECP1 (blue line), negative control (red line) or positive control (green
line).

To identify which CD3+ cells were responding to MFECP1 subset analysis
was performed on CD4+ and CD4- cells.

Results from the CD4+ subset suggested that IFN-γ secretion is increased
by incubation with MFECP1 compared with medium. The apparent increase
in IFN-γ secretion from CD4+ cells following incubation with MFECP1 is
shown in Figure 5.5. Incubation with MFECP1 did not appear to increase
IFN-γ secretion in CD4- cells (Figure 5.6 and Figure 5.7).
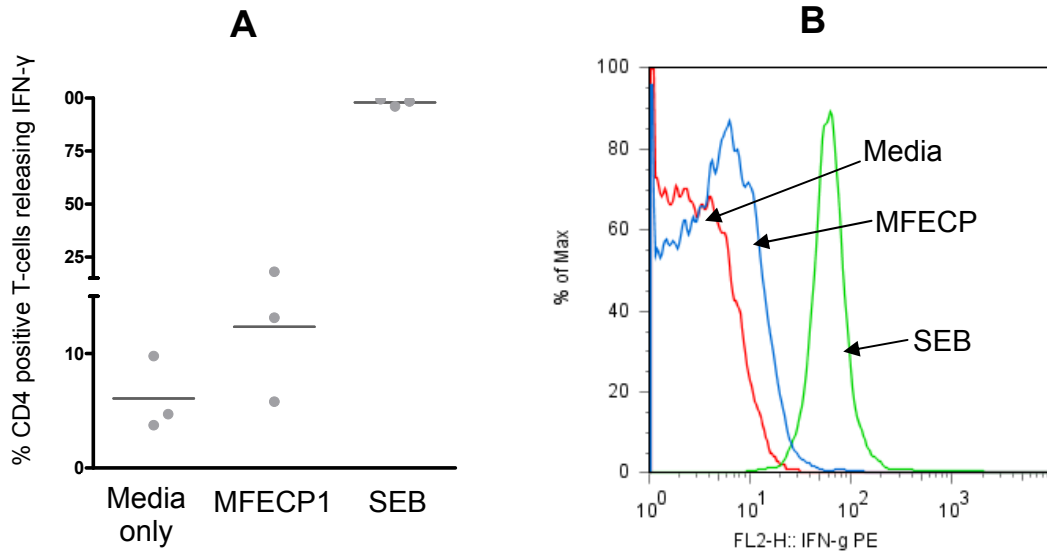
Figure 5.5

Response of CD4+ cells (measured by IFN-γ release) from 3 healthy volunteers after incubation with MFECP1, negative control (medium only) or positive control (SEB). **A.** Percentage of CD4 positive cells releasing IFN-γ after incubation for 13 hours with MFECP1, negative control or positive control. **B.** IFN-γ release in CD4 positive cells from volunteer 3 after incubation with MFECP1 (blue line), negative control (red line) or positive control (green line).

The stimulation of CD4+ cells from healthy volunteers by MFECP1 is particularly significant because it occurred after a short incubation i.e. 13 hours. Stimulation of CD4+ cells after such a short incubation would strongly suggest that healthy donors were pre-primed against components of MFECP1 and generate T cell memory which can produce rapid T-lymphocyte responses upon challenge with MFECP1 *in vitro*.
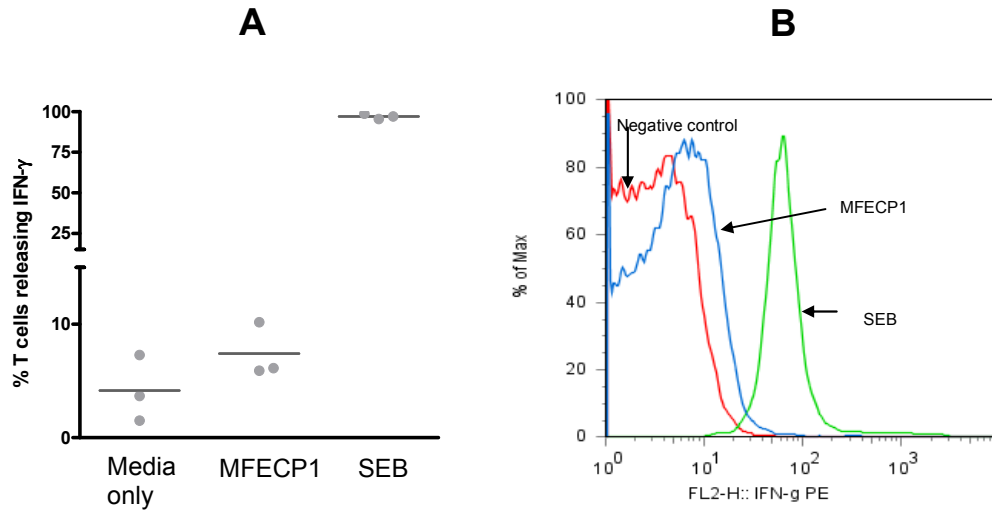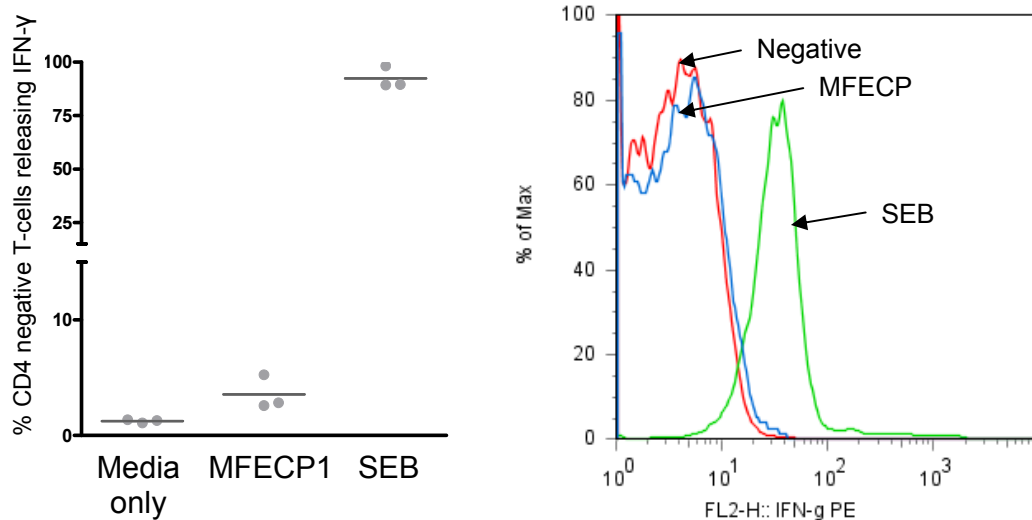
Figure 5.6

Response of CD4- cells (measured by IFN-γ release) from 3 healthy volunteers after incubation with MFECP1, negative control (medium only) or positive control (SEB). A. Percentage of CD4 negative cells releasing IFN-γ after incubation for 13 hours with MFECP1, negative control or positive control. B. IFN-γ release in CD4 negative cells from volunteer 3 after incubation with MFECP1 (blue line), negative control (red line) or positive control (green line)
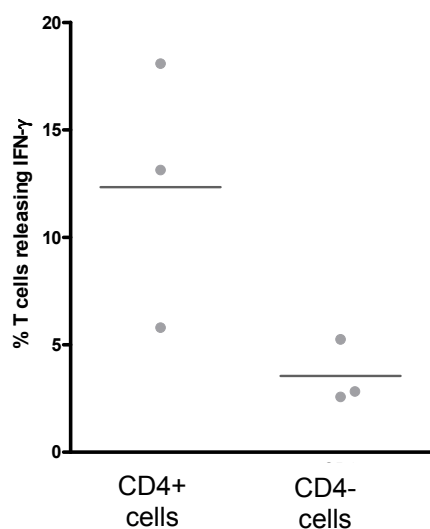
Figure 5.7

Percentage of CD4+ and CD4- cells releasing IFN-γ after incubation with MFECP1 for 13 hours; results from 3 healthy volunteers.

5.7 Mapping T-cell epitopes on CPG2: healthy volunteers

Experiments shown in section 5.7 were performed by Biovation Ltd. as part of a collaboration between the ADEPT development team at UCL and Biovation Ltd. (a biotechnology company with expertise in de-immunization of proteins and now known as Antitope Ltd.).

In order to identify amino acid sequences on CPG2 that induce T_h lymphocyte proliferation, PBMCs from 20 healthy volunteers were incubated with 128 different overlapping 15mer peptides that spanned the entire sequence of CPG2. T_h lymphocyte activation was assessed using T-cell proliferation assays. The results of these T-cell proliferation assays are summarized in Figure 5.8. Full results are shown in Appendix 2. Whilst 81/128 peptides did not cause significant proliferation of PBMCs from any of the 20 donors, 47/128 peptides caused significant proliferation in at least one donor.

31 peptides reacted with 1 volunteer

12 peptides reacted with 2 volunteers

3 peptides reacted with 3 volunteers

1 peptide reacted with 4 volunteers
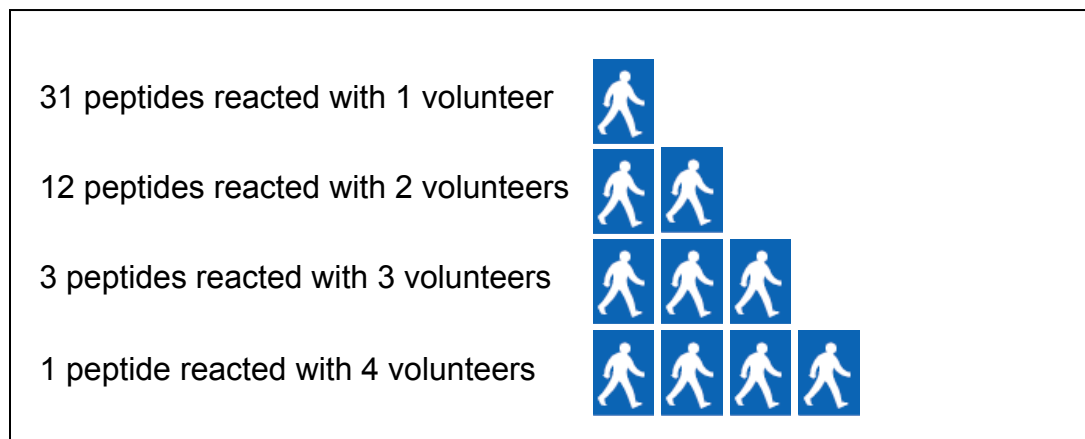
Figure 5.8

Cartoon to illustrate the results of T-cell proliferation assays using peripheral blood mononuclear cells from 20 healthy volunteers incubated with 15 mer peptides spanning sequence of carboxypeptidase G2.

The data from these T-cell proliferation assays was analysed by Biovation in conjunction with the known structure of CPG2. Eight T_h lymphocyte epitopes were identified. The predicted binding of these 8 epitopes with HLA was analysed *in silico* and 23 amino acid substitutions were proposed to disrupt peptide/HLA binding. The proposed mutations are shown in Table 5.4. A model indicating the position of individual mutations for a single epitope is shown in Figure 5.9.

Table 5.4

Identification of 8 T_h lymphocyte epitopes on CPG2 and proposed amino acid substitutions

Epitope	Mutation identity	Amino acid and location	Substitution	Secondary structure information
A	1	I 74	T	B-sheet exposed
	2	L 83	A / G	B-sheet exposed
	3	M 85	K	B-sheet exposed
B	4	Y 110	T	B-sheet exposed
	5	I 114	A / G	Loop, cleft, partly exposed
	6	V 123	A / G	A-helix interior
	7	L 125	A / G	A-helix interior
C	8	L 145	G or T	B-sheet interior
D	9	V 171	A / G	B-sheet interior
	10	L 172	T	B-sheet interior
	11	F 174	A / G	B-sheet interior
	12	L 184	A / G	B-sheet interior
E	13	L 211	A / G	Loop exterior
	14	V 213	A	Loop exterior
	15	L 216	A	A-helix exterior
F	16	L 236	A / G	Loop partly exposed
	17	W 240	A (H)	B-sheet interior
	18	I 242	A, T	B-sheet exterior
	19	V 248	T	Loop exterior
G	20	V 291	A / G	B-sheet
	21	V 293	A / G	B-sheet
H	22	L 311	A / G	a-helix
	23	V 312	A / G	a-helix

Figure 5.9

Model of CPG2 monomer with amino acid substitutions at 3 locations designed to disrupt T-cell epitope A. Amino acid 74 (isoleucine) shown in red has been changed to threonine. Amino acid 83 (leucine) shown in green has been changed to alanine. Amino acid 85 (methionine) shown in yellow has been changed to lysine.

5.8 MFECP1 response of T_h lymphocytes from transgenic mice

Human and particularly patient blood is a resource of limited availability. It was hypothesized that mice transgenic for human MHC could offer a viable alternative; particularly as the mice could be immunized with MFECP1. The *in vitro* response of T_h lymphocytes from a transgenic mouse strain (HLA-DRB1*0101 mice) to MFECP1 was investigated. The experiment was performed using 20 male DRB1*0101 mice; 10 were immunized with MFECP1 plus adjuvant, 10 were immunized with PBS plus adjuvant. Mice that were injected with MFECP1 received 16.5μg of MFECP1 each. On days 12 to 13 the mice were culled and lymphocytes extracted from the popliteal lymph node adjacent to the immunization site. The cells were incubated for 5 days in T-cell proliferation assays with MFECP1, SEB or medium.

The results from these experiments indicated that T_h lymphocytes from control immunized DRB1*0101 mice did not respond to MFECP1 (SI < 2) whilst T_h lymphocytes from 9 of the 10 MFECP1-immunized DRB1*0101 mice responded. These results are shown in Figure 5.10.
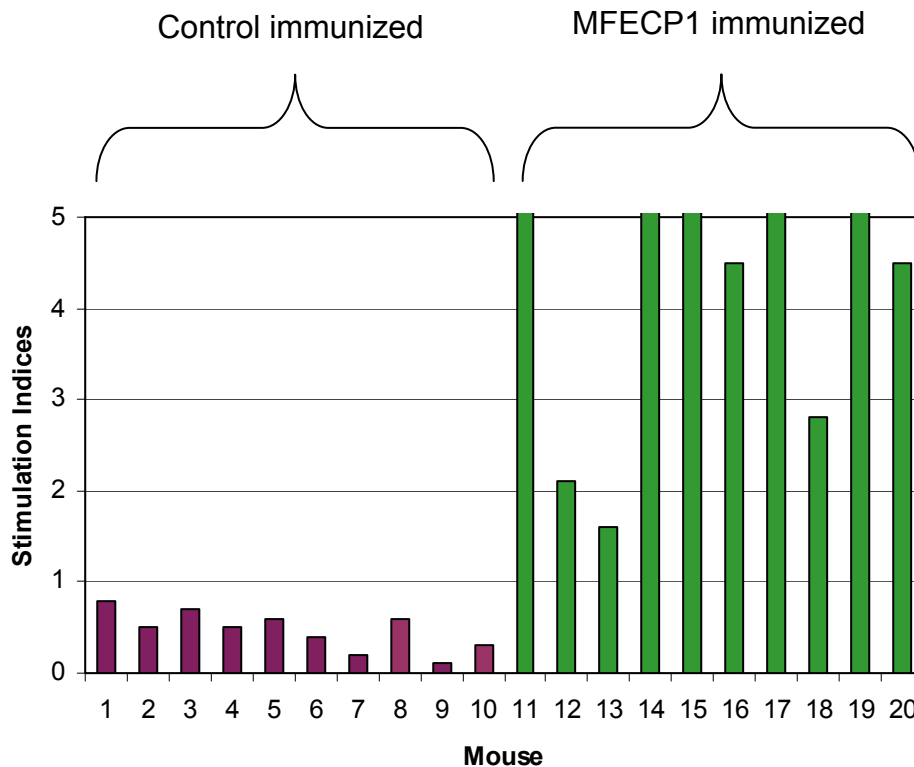
Figure 5.10

Stimulation indices of T_h lymphocytes from DRB1*0101 transgenic mice incubated with MFECP1 in T-cell proliferation assays. Mice 1-10 had been immunized with control immunization (PBS and TiterMax adjuvant). Mice 11-20 had been immunized with MFECP1 immunization (MFECP1 and TiterMax adjuvant). Twelve to 13 days after immunization the mice were culled and lymphocytes extracted from the popliteal lymph node adjacent to the immunization site. Cells were incubated for 5 days with medium, MFECP1 or SEB. T_h lymphocytes from control immunized mice did not proliferate *in vitro* when incubated with MFECP1 i.e. stimulation indices <2. T_h lymphocytes from 9 of 10 MFECP1 immunized mice did proliferate *in vitro* when incubated with MFECP1 i.e. stimulation indices ≥ 2. Off scale data for mice 11, 14, 15, 17 and 19 were 9.6, 23.4, 57.1, 36.4 and 11.3 respectively.

5.9 Conclusions

The aim of this chapter was to increase understanding of the immune response to MFECP1.

Data from 66 MFECP1 infusions indicated that infusion reactions were mild (grade 1 only) and occurred in 11% of infusions. Repeat administration of MFECP1 did not increase the risk of an infusion reaction but significantly increased the risk of a patient becoming HACPA positive.

In vitro MFECP1 induced proliferation of T_h lymphocytes in 83% of healthy volunteers. The induction of IFN-γ release from T_h lymphocytes by MFECP1 after an incubation of only 13 hours strongly suggested that the response was a secondary immune response. Healthy volunteers may have been previously exposed to T-cell epitopes on MFECP1 through natural exposure to CPG2 from *Pseudomonas* bacteria.

In collaboration with Biovation, 8 possible T-cell epitopes on CPG2 were identified by incubating PBMCs from 20 healthy donors with overlapping 15 mer peptides spanning the sequence of CPG2. Suggested mutations to remove the T-cell epitopes were proposed.

In view of the limited availability of donor and patient blood investigations were made into whether transgenic mice may be suitable candidates for epitope mapping. Although T_h lymphocytes from control immunized mice did not respond to MFECP1 *in vitro*, T_h lymphocytes from 90% of MFECP1-immunized mice did respond. The results indicate that MFECP1-immunized mice may provide an alternative source of T_h lymphocytes for T-cell epitope mapping experiments.

Chapter 6

Discussion

6.1 Introduction

The aim of ADEPT is to produce significant clinical efficacy with low toxicity and low immunogenicity. The results presented in this MD (Res) thesis provide new data that increases understanding of the safety of ADEPT, risk factors for adverse events, the immune response to ADEPT and efficacy. In the light of this new data two important questions can be discussed. What are the conditions required for successful ADEPT? Were these conditions achieved?

6.2 The conditions required for successful ADEPT

An ideal ADEPT system would:

- Use a safe antibody-enzyme

- Deliver sufficient enzyme to tumour

- Deliver sufficient prodrug to tumour

- Have very low toxicity (in particular antibody-enzyme would not be present in non-tumour tissue at time of prodrug administration)

- Be non-immunogenic

- Be suitable for repeat treatment

- Have high clinical efficacy

Pre-clinical and clinical ADEPT trials have aimed to achieve these conditions. Pre-clinical investigations of ADEPT with MFECP1 and BIP prodrug have produced positive results (Sharma *et al.*, 2005). A single ADEPT treatment caused significant tumour growth delay in LS174T and SW1222 xenografts. Furthermore efficacy was significantly increased when repeat treatments were given. In LS174T tumours there was prolonged growth delay and in SW1222 tumours there was complete regression of tumours.

Were the conditions that were achieved in these pre-clinical investigations achieved in patients? How do the characteristics of ADEPT with MFECP1 and BIP prodrug compare to an ideal ADEPT system?

6.2.1 Safety of MFECP1

The clinical results demonstrated that repeat administration of MFECP1 was well tolerated and prior administration of MFECP1 did not increase the risk of an infusion reaction. This compares favourably with streptokinase (a licensed non-human enzyme) in which 50% of non-1st dose infusions were associated with allergic reactions (White *et al.*, 1990). In total 66 MFECP1 infusions were administered and all infusion reactions were mild (grade 1). It appears based on the current evidence that repeat administration of MFECP1 is safe. It is possible that more serious infusion reactions may have occurred if MFECP1 had been given to a larger number of patients. However since the rate of grade 3/4 infusion reactions is currently 0% (0/66) it is unlikely that such reactions are common. It should be noted that MFECP1 was given under very specific safety conditions i.e. only administered to HACPA negative patients. MFECP1 has never been given to a HACPA positive patient and therefore we do not know the safety of MFECP1 in such circumstances. It is probable that administration of MFECP1 to a HACPA positive patient would increase the risk of an infusion reaction. For example a significantly increased risk of infusion reactions was reported when the

chimeric mAb infliximab (used to treat inflammatory bowel disease) was given to patients with anti-infliximab antibodies (Miele *et al.*, 2004). The administration of MFECP1 to HACPA positive patient is therefore not advised.

6.2.2 Targeting and delivery of CPG2 activity to tumour

In mice bearing LS174T xenografts co-localization of CPG2 with CEA in tumour tissue was demonstrated by immunohistochemistry (Sharma *et al.*, 2005). This co-localization was also demonstrated in tumour biopsies from single-treatment patients following MFECP1 infusion (Mayer *et al.*, 2006).

In mice the mean tumour CPG2 activity at time of prodrug administration (6 hours after MFECP1) was 1.55U/g in LS174T xenografts and 1.29U/g in SW1222 xenografts. Was this level of tumour CPG2 activity achieved in patients at the time of prodrug administration? In the single-treatment ADEPT trial CPG2 activity within tumour was estimated by the administration of radiolabelled MFECP1 followed by single photon emission computed tomography (SPECT) imaging. In two patients, the SPECT scan was performed within 1 hour of prodrug administration; this is the best available data with which to assess tumour CPG2 activity at time of prodrug. At times within an hour of prodrug administration, estimated median tumour CPG2 activities in patients (0.007U/g and 0.006U/g in patients 18 and 21 respectively) were significantly less than the tumour CPG2 activities achieved in mice (1.55U/g and 1.29U/g in LS174T and SW1222 xenografts respectively). Details are shown in Table 6.1.

Table 6.1

Tumour CPG2 activity at time of prodrug in mice and patients

	Mice	Mice	Patient 18$^\diamond$	Patient 21$^\diamond$
Tumour type	LS174T*	SW1222*	adenocarcinoma of colon	adenocarcinoma of colon
MFECP1 dose	1000U/kg	1000U/kg	5000U/m^2	5000U/m^2
Time of prodrug**	6, 7 and 8 hours	6, 7 and 8 hours	20.6, 21.6 and 22.6 hours	18.4, 19.4 and 20.4 hours
Serum CPG2 activity at time of prodrug (U/ml)	0.0011 (SD=0.0004)	0.0038 (SD=0.0005)	<0.002	<0.002
Tumour CPG2 activity at time of prodrug (U/g)	1.55[***] (SD = 0.20)	1.29[***] (SD = 0.22)	0.07[‡] (0.06 to 0.10)	0.06[‡] (0.03 to 0.08)

* human colonic adenocarcinoma xenograft, ** time after start of MFECP1, *** mean value, ‡ median and range of CPG2 activity values from >1 tumour in each patient, $^\diamond$ in patients 18 and 21 time interval between prodrug and single photon emission computed tomography (SPECT) imaging was ≤ 1hour.. CPG2 activity in mice measured on tumour tissue using high-performance liquid chromatography assay. CPG2 activity in patient tumour estimated using radiolabelled MFECP1 and SPECT. Patient 17 received [123]Iodine radiolabel; patient 18 received [131]Iodine radiolabel. Mice data from Sharma et al, 2005. Patient data from CRUK database. Both patient 18 and 21 received a single treatment of ADEPT. CPG2 = carboxypeptidase G2. SD = standard deviation.

Mice CPG2 activity was measured on tumour tissue by an indirect high-performance liquid chromatography assay (HPLC) whereas in patients CPG2 activity was estimated using a non-invasive technique i.e. SPECT. However tumour CPG2 activity results obtained by SPECT analysis have previously been shown to correlate well with direct measurement of CPG2 activity in tumour tissue measured by HPLC (Napier *et al.*, 2000). Hence the available evidence suggests that tumour CPG2 activity at time of prodrug administration was significantly less in patients compared to mice.

Furthermore even at much earlier time points following MFECP1 administration, the tumour CPG2 activity in patients was still significantly less than the values associated with efficacy in mice (Table 6.2).

Table 6.2

Estimated CPG2 activity in patient tumour 3 - 4 hours after start of MFECP1 infusion

Patient	MFECP1 dose (U/m^2)	Time interval from radiolabel to SPECT (hours)	Estimated CPG2 activity in tumour (U/g).
17	5000	3.2	0.13
18	5000	4.4	0.13* (0.11 - 0.15)

Estimation of CPG2 activity was performed by single photon emission computed tomography analysis. Patient 17 received MFECP1 radiolabelled with [123]Iodine and patient 18 received MFECP1 radiolabelled with [131]Iodine. *Median value (and range) from 4 separate tumours assessed in patient 18. Data from CRUK database.

Why was tumour CPG2 activity less in patients compared to mice? The answer does not appear to be due to differences in plasma half life since the alpha half life of MFECP1 (approximately 30 minutes) is very similar in mice and patients (Sharma *et al.*, 2005; Mayer *et al.*, 2006). It is possible that delivery of MFECP1 into tumour was greater in mice than patients. Since CPG2 localization following MFECP1 administration occurs in viable tumour (as opposed to necrotic tumour) it is possible that the difference observed was due to a greater proportion of viable tumour tissue in mice compared to patients.

6.2.3 Prodrug and activated prodrug in tumour

Activated prodrug is effective *in vitro* against proliferating LoVo colorectal tumour cells following a 1 hour (IC_{50} 0.34µM) or 1 minute incubation (IC_{50} 1.57µM) (Blakey *et al.*, 1996). Activated prodrug is also effective *in vitro* against quiescent LoVo cells (IC_{50} 1.9µM after one hour incubation). Exposure of tumour cells to activated prodrug causes DNA interstrand crosslinks (Webley *et al.*, 2001) and apoptosis (Monks *et al.*, 2001).

Ideally the concentration of BIP prodrug and activated prodrug would be measured in tumour and non-tumour tissue. In mice the half life of BIP prodrug following a TPD of 45mg/kg given intraperitoneally was 9 minutes (Astra Zeneca data, 1998). This is similar to the half life measured in patients after intravenous administration i.e. 14.5 minutes after a TPD of 47mg/m^2 (Francis *et al.*, 2002) and 10.3 minutes (mean value) after TPDs ranging from 37mg/m^2 to 3225mg/m^2 (Mayer *et al.*, 2006).

It is also desirable to know what quantity of activated prodrug was being produced in tumour and in non-tumour tissue. However due to the very short half life of activated prodrug it was not possible for this to be measured in clinical trials. However it *was* possible to measure the biological effects of activated prodrug i.e. DNA interstrand crosslinks.

Single cell gel electrophoresis (comet) assay can be used to assess DNA interstrand crosslinks. The method is based on the observation that when a cell is irradiated (to provide a fixed quantity of random DNA strand breakage), the cell membrane lysed and the cell placed in an electric field, the smaller fragments of DNA produced by DNA strand breakage move towards the anode. These smaller DNA fragments form a structure that resembles the tail of a comet. DNA interstrand crosslinks can be detected because (in contrast to DNA strand breakage) they *reduce* the size of the tail moment.

Comet assays have been used in pre-clinical and clinical ADEPT studies (Webley *et al.*, 2001; Francis, 2002, Mayer, 2006). In pre-clinical studies tumour tissue from treated mice was compared with tumour tissue from untreated mice. In clinical studies comet assays were performed on circulating lymphocytes before and after prodrug and on tumour tissue which was biopsied after prodrug. Tumour biopsies taken from patients after prodrug were compared with pre-prodrug circulating lymphocytes, since no pre-prodrug biopsy was available as a control. The absence of a pre-prodrug tumour control means that some caution needs to be applied when interpreting comet assays performed on tumour from patients.

What TPD was required to produce DNA interstrand crosslinks or clinical efficacy? The doses of BIP prodrug associated with pharmacodynamic effect on tumour in pre-clinical and clinical ADEPT studies are shown in Table 6.3. ADEPT using BIP prodrug has been investigated in nude mice bearing human xenografts (Blakey *et al.*, 1996, Webley *et al.*, 2001, Sharma *et al.*, 2005). In these pre-clinical investigations BIP prodrug was given intraperitoneally and the TPD was expressed in mg/kg. It is possible to calculate the human equivalent TPD (for details see www.fda.gov/cder/cancer/animalframe.htm). First the mouse mg/kg dose is converted into the mouse mg/m^2 dose by multiplying the mouse mg/kg dose

by 3. Second, doses in mg/m^2 are assumed to scale 1:1 between mice and humans when normalized to body surface area i.e. mouse mg/m^2 dose assumed to equal human mg/m^2 dose. The bioavailability of intraperitoneal BIP prodrug was assumed to be 100%.

In pre-clinical investigations human equivalent TPDs of 630mg/m^2 and 675mg/m^2 were associated with tumour regression and DNA interstrand crosslinks respectively. In clinical trials using MFECP1 a TPD of 806mg/m^2 was associated with DNA interstrand crosslinks and a TPD ≥ 900mg/m^2 produced efficacy on FDG-PET.

It has been reported that a TPD of 37mg/m^2 produced DNA interstrand crosslinks in tumour tissue in a clinical trial with a F(ab')$_2$ - CPG2 chemical conjugate and BIP prodrug (Francis *et al.*, 2002). This TPD is significantly less than the other pre-clinical and clinical results. This low result does not appear to be secondary to a high tumour CPG2 activity because a tumour biopsy on day of prodrug in the same patient showed a CPG2 activity of 0.010U/g (PhD thesis of Francis RJ, 2005, UCL). The DNA interstrand crosslinks may have been secondary to extensive prodrug activation in non-tumour since the serum CPG2 activity at time of prodrug was 0.025U/ml. This was approximately 10 times higher than the serum CPG2 at time of prodrug in the clinical trials using MFECP1.

Hence it appears that in the phase I trials of ADEPT with BIP prodrug and MFECP1, effective prodrug dose conditions were achieved in patients provided that the TPD was 806mg/m^2 (to produce DNA interstrand crosslinks) or ≥ 900mg/m^2 (to produce efficacy on FDG-PET).

				Table 6.3				
				Doses of BIP prodrug associated with pharmacodynamic effect on tumour in pre-clinical and clinical ADEPT studies				
Study setting	**Data Source**	**TPD**	**Route**	**Human equivalent TPD* (mg/m^2)**	**Number of treatments**	**Antibody-enzyme**	**Tumour**	**Pharmacodynamic effect on tumour**
Pre-clinical mice studies	Blakey *et al.*, 1996	210mg/kg	ip	630mg/m^2	Single	F(ab')$_2$ conjugated to CPG2	LoVo xenograft	Tumour regression
	Webley *et al.*, 2001	225mg/kg	ip	675mg/m^2	Single	""	LS174T xenograft	Tumour DNA interstrand crosslinks
	Sharma *et al.*, 2005	210mg/kg	ip	630mg/m^2	Repeat	MFECP1	SW1222 xenograft	Tumour regression
Clinical	Francis *et al.*, 2002	37mg/m^2	iv	37mg/m^2	Single	F(ab')$_2$ conjugated to CPG2	Carcinoma colon**	Tumour DNA interstrand crosslinks
	Mayer *et al.*, 2006	806mg/m^2 (patient 26)	iv	806mg/m^2	Single	MFECP1	Carcinoma colon	Tumour DNA interstrand crosslinks
	Wilkins 2010 MD(Res) thesis	≥ 900mg/m^2	iv	≥ 900mg/m^2	Single and repeat	MFECP1	carcinoma colon (#35, #38), pancreas (# 32), peritoneal (#24).	Responses on FDG-PET

* Conversion from mg/kg to mg/m^2 in mice by multiplying mg/kg x3. Doses assumed to scale 1:1 between mice and humans when normalized to body surface area (www.fda.gov/cder/cancer/animalframe.htm). BIP prodrug assumed to have 100% bioavailability when given ip. ** Data from PhD thesis of Francis RJ, 2005, UCL. TPD = total prodrug dose, ip = intraperitoneal, iv = intravenous, CPG2 = carboxypeptidase G2.

6.2.4 Toxicity

Myelosuppression was the principal toxicity of repeat ADEPT treatments and was the cause of all DLTs. It is not possible to attribute the frequency or severity of ADEPT induced thrombocytopaenia to poor patient characteristics e.g. low performance status or prior chemotherapy. For example the patient with the most severe thrombocytopaenia after 2 ADEPT treatments given at the maximum tolerated TPD (patient 33, platelet nadir 33×10^9/l) had a good overall health status (WHO performance status of 1) and had only received one previous line of chemotherapy.

In principle the myelosuppression could have been caused by any of the 3 drugs administered or generated during ADEPT i.e. MFECP1, BIP prodrug or activated prodrug

MFECP1 is a recombinant fusion protein of a scFv plus CPG2 and is unlikely to have caused myelosuppression. In contrast there is evidence that the severity of myelosuppression was positively associated with TPD. However this data does not clarify whether it is BIP prodrug or activated prodrug that caused the myelosuppression (or possibly a combination of the two).

The only data on the effects of BIP prodrug given alone come from an earlier ADEPT trial (Francis *et al.*, 2002; PhD thesis of Francis RJ, 2005, UCL). Three patients received BIP prodrug alone at TPDs of 15mg/m^2, 47mg/m^2 and 56mg/m^2. One patient (15mg/m^2) had grade 1 nausea, one patient (47mg/m^2) had grade 1 leucopaenia plus grade 1 neutropaenia and one patient (56mg/m^2) had no drug-related toxicities. In the current trial the TPD per treatment was significantly higher (150mg/m^2 to 600mg/m^2) and the TPD over all treatments ranged from 300mg/m^2 to 1800mg/m^2. Hence it is possible that BIP prodrug caused some myelosuppression.

However the most important cause of myelosuppression was probably activated prodrug. Two lines of evidence support this hypothesis. Firstly activated prodrug is an alkylating agent, a class of drugs well known to cause myelosuppression. Secondly at a fixed dose of MFECP1 and BIP prodrug, administration of prodrug at a higher serum CPG2 activity was associated with greater myelosuppression (Table 3.10). The most likely explanation for these findings is that when prodrug was given at higher serum CPG2 activity, greater amounts of activated prodrug was generated and this caused increased myelosuppression.

However if activated prodrug was the principal cause of myelosuppression and if it is assumed that activated prodrug would need to be present in the bone marrow to cause myelosuppression, how does activated prodrug come to be in the bone marrow? One possibility is that some activated prodrug was generated in bone marrow due to the presence of MFECP1 in the bone marrow at the time of prodrug administration. Although pre-clinical testing of the glycosylated fusion protein did measure CPG2 activity in plasma, liver, kidney, lung and spleen, CPG2 activity was not measured in bone marrow. CPG2 activity in bone marrow was also not measured in the single or repeat-treatment ADEPT trials. Hence it is not known whether activation of BIP prodrug within bone marrow is clinically significant.

An alternative cause of myelosuppression is the possibility that BIP prodrug is activated away from the bone marrow but then travels to it in the systemic circulation. This mechanism would require the activated prodrug to be stable long enough to travel from the site of generation to the bone marrow. The exact half life of the activated prodrug is not known although it is considered to be very short. During development of BIP prodrug 16 novel potential prodrugs (and their corresponding activated prodrugs) were assessed (Springer *et al.*, 1995). The shortest measured half-life of any of the activated prodrugs was 0.6 minutes (36 seconds). Several activated

prodrugs (including the activated prodrug of BIP prodrug) had hydrolysis rates that were too fast to measure accurately. Hence the half-life of the activated cytotoxic drug generated during the current ADEPT trial is considered < 36 seconds. Therefore although the half life of activated prodrug is short it may be long enough to allow some drug to reach the bone marrow.

Hence activated cytotoxic drug probably travels to the bone marrow after being produced in tumour and in non-tumour tissue e.g. plasma. Although CPG2 activity (U/g) is relatively low in non-tumour tissue the total mass of non-tumour tissue is high. There is clinical evidence to support the hypothesis that generation of activated prodrug in non-tumour tissues is a significant cause of myelosuppression. For example administration of prodrug when serum CPG2 activity > 0.005U/ml was associated with increased severity of myelosuppression. The increased myelosuppression was probably caused by increased production of activated prodrug in the plasma.

It is also possible that CPG2 activity in viscera (non-tumour tissue other than plasma) was a significant cause of myelosuppression. In mice CPG2 activity was detected in liver, lung, kidneys and spleen following administration of MFECP1 (Sharma *et al.*, 2005). Furthermore at 6 hours, when MFECP1 had cleared from plasma (CPG2 activity 0.001U/ml), enzyme activity in lung was 4 times higher (0.004U/g).

CPG2 activity in viscera may be a significant cause of myelosuppression in patients. When the time interval between MFECP1 and prodrug was reduced (as a result of a lower MFECP1 dose) myelosuppression increased even though prodrug was administered when serum CPG2 activity < 0.002U/ml (Table 3.11). The most likely explanation for this is that at earlier time points there was increased total CPG2 activity in organs e.g. liver, lung.

Other sites of prodrug activation were also considered but are unlikely to be clinically significant. The first was the possibility that some BIP prodrug was activated in the lumen of the central venous catheter (used to obtain large-bore venous access in patients). All patients had a central venous catheter inserted since BIP prodrug can cause venous irritation. The central venous catheter was used to administer the MFECP1 and the BIP prodrug. The line was thoroughly flushed with normal saline at the end of the MFECP1 infusion. Furthermore the BIP prodrug was typically given around 14 hours after the end of the MFECP1 infusion. However if some MFECP1 adhered to the plastic lumen of the central venous catheter and retained enzymatic activity, it is possible that some activated prodrug could have been generated in the lumen of the central venous catheter when BIP prodrug was administered.

The second possibility was that some BIP prodrug was activated by CPG2 in *Pseudomonas* bacteria which are occasionally present in gut flora. For this to occur there would need to be: i) movement of BIP prodrug into the lumen of the colon ii) activation of BIP prodrug by CPG2 iii) movement of activated prodrug across the colonic epithelium and into the portal circulation iv) movement of activated prodrug from the portal circulation to the bone marrow. Two pieces of evidence make this hypothesis unlikely. Firstly the half life of the activated prodrug is < 36 seconds and therefore there is unlikely to be time for activated prodrug to travel from the lumen of the colon to the bone marrow. Secondly the toxicity profile of ADEPT does not support the hypothesis. Contact between activated prodrug and the wall of the colon would potentially cause diarrhoea. However diarrhoea only occurred in 2 of the 43 ADEPT (grade 1 in patient 35 and grade 2 in patient 30).

The combined data from all 43 patients has identified clinical factors that modify the risk of myelosuppression (Table 6.4).

Table 6.4
Clinical factors and myelosuppression
Myelosuppression is positively associated with: ● Total prodrug dose ● Serum CPG2 activity at time of prodrug administration ● Number of ADEPT treatments **Myelosuppression can be negatively associated with:** ● Time interval between MFECP1 and prodrug

The combined analysis of all patients identified that grade 4 hepatic toxicity only occurred in patients who received a TPD *per treatment* ≥ 1613mg/m^2. This toxicity is probably caused by a high peak concentration of BIP prodrug.

One patient was diagnosed with AML 8 months after commencing ADEPT treatment. This event could be i) an unrelated chance event ii) caused by the patient having an independent risk factor (genetic or environmental) that increased the risk of both cancers iii) caused by treatment (or investigational procedures) undertaken during the management of the first cancer.

There is clear evidence that some chemotherapy regimes increase the risk of AML (Kirova *et al.*, 2008; Schaapveld *et al.*, 2008). However since many chemotherapy regimes contain more than one drug (and patients may receive several different chemotherapy regimes) it can be difficult to know the carcinogenic potential of individual drugs.

The patient who developed AML had received 3 lines of cytotoxic chemotherapy before being diagnosed with AML: i) ECX (epirubicin, cisplatin, capecitabine) ii) ADEPT iii) gemcitabine with carboplatin.

An increased risk of AML has also been reported with epirubicin and cisplatin (Pedersen-Bjergaard *et al.*, 1992) and carboplatin (Travis *et al.*, 1999). Gemcitabine and capecitabine are not known to increase the risk of AML.

The activated form of BIP prodrug is an alkylating agent, a class of drugs known to increase the risk of AML (Curtis *et al.*, 1992). It is possible that activated prodrug produced during ADEPT was associated with the subsequent development of AML. In total 70 patients have received ADEPT using BIP prodrug; 27 in the trial by Francis and coworkers (Francis *et al.*, 2002), 31 in the trial by Mayer and colleagues (Mayer *et al.*, 2006) and 12 in the current trial of repeat ADEPT treatments. The patient reported here is the only one of these patients to have been reported to have developed acute leukaemia.

6.2.5 Immunogenicity

Following repeat MFECP1 infusions 100% of patients developed HACPA whereas only 40% of patients who received a single MFECP1 infusion developed HACPA. The significant increase in ADA formation with multiple MFECP1 infusions compared to a single infusion (Log-rank $p = 0.001$) is consistent with pre-clinical ADEPT data and data from other therapeutic proteins.

In pre-clinical experiments 8 Balb/c mice were immunized repeatedly with MFECP-his (Mayer *et al.*, 2004). None of the mice produced anti-CPG2 after 1 immunization (day 15) whereas 4 of the 8 mice produced anti-CPG2 after 4 immunizations (day 57).

Braun and colleagues demonstrated in Balb/C mice that when a fixed total dose of IFN-α was given once, twice or three times per week, an increased frequency of administration was associated with higher titres of ADA i.e. frequency of administration increased ADA production independent of the total dose (Braun *et al.*, 1997). Furthermore patients with multiple sclerosis who received IFNβ-1a three times per week were significantly more likely to develop ADAs compared to patients who were treated once per week (Ross *et al.*, 2000).

A significant positive relationship was also found between total MFECP1 dose and HACPA formation. This result is probably because patients that received a higher total MFECP1 dose did so by receiving multiple MFECP1 infusions.

Since activation of T-lymphocytes is likely to be involved in the immunological response to MFECP1, the *in vitro* response of T- lymphocytes to MFECP1 and CPG2 was investigated. PBMCs from 83% of healthy volunteers proliferated in response to MFECP1. The most likely cause of a T-lymphocyte response after 5 days of incubation is a memory response i.e. the healthy volunteers had previously been exposed to T-cell epitopes on MFECP1.

To investigate whether *in vitro* stimulation could occur after a much shorter incubation PBMCs from 3 healthy volunteers were incubated with MFECP1 for 13 hours. T_h lymphocyte stimulation was assessed using an IFN-γ secretion assay. This experiment demonstrated that T_h lymphocytes were stimulated by MFECP1 after only 13 hours of incubation. The result is consistent with the response to MFECP1 being a secondary immune response rather than a primary immune response. This suggests that the healthy volunteers had been previously exposed to T-cell epitopes on CPG2.

This may have occurred through natural exposure to CPG2 in *Pseudomonas* bacteria.

The *in vitro* response of PBMCs from a treated ADEPT patient to incubation with MFECP1 was investigated. After 5 days of incubation MFECP1 induced significant stimulation (SI = 2.5). The magnitude of proliferation was similar to the results from healthy volunteers (median SI = 3.0, range 1.6 to 3.9). The blood sample from the treated ADEPT patient was taken on day 80. It is possible that proliferation would have been greater if the blood had been taken nearer to ADEPT treatment. Evidence to support this comes from similar experiments conducted on the non-human therapeutic protein streptokinase. Squire and colleagues demonstrated that compared to pre-streptokinase blood samples, lymphocytes taken 6 days after treatment showed a statistically significant elevation in stimulation indices (Squire *et al.*, 1999). However by 6 weeks the stimulation indices had returned to baseline values.

The activation of T_h lymphocytes implies that certain sequences within MFECP1 were behaving as T-cell epitopes. Since CPG2 is bacterial in origin it was considered that the amino acid sequence of CPG2 was probably the most immunogenic portion of MFECP1. The aim of the next experiment was therefore to find possible T-cell epitopes within CPG2. In collaboration with Biovation overlapping 15 mer peptides spanning the sequence of CPG2 were incubated with PBMCs (containing T_h lymphocytes and antigen presenting cells) from healthy volunteers. This *in vitro* data was used to identify 8 possible T-cell epitopes.

Since blood from healthy volunteers, and particularly patients, was a very limited resource it was considered desirable to have a non-human model to investigate the immune response to MFECP1. Mice transgenic for human MHC provide a possible alternative source of pre-clinical data. First the *in*

vitro response of T_h lymphocytes from mice transgenic for human DRB1*0101 to MFECP1 were investigated. Half of the mice were pre-immunized with MFECP1 plus adjuvant, the other half were immunized with PBS plus adjuvant. T_h lymphocytes activation was measured in T-cell proliferation assays. Nine of the 10 mice that had been pre-immunized with MFECP1 responded *in vitro* to MFECP1 whereas none of the mice pre-immunized with control responded *in vitro* to MFECP1. Since mice that were not immunized with MFECP1 did not respond *in vitro* to MFECP1, it appears that the mice had not been in contact with epitopes on MFECP1 during the course of their lives. However by pre-immunizing the mice with MFECP1 the mice produced T_h lymphocytes that respond to MFECP1 *in vitro*. It may be possible therefore for pre-immunized DRB1*0101 transgenic mice to be used in epitope mapping experiments on CPG2.

The results demonstrate that MFECP1 is immunogenic although infusions reactions to date have been mild. Production of HACPA limits the treatment window for MFECP1 to approximately 2 weeks. Strategies to reduce HACPA formation are discussed in section 6.3.1.

6.2.6 Delivery of repeat ADEPT treatments

In the pre-clinical investigation of repeat ADEPT (with MFECP1 and BIP prodrug) mice with LS174T xenografts received treatment 5 times in week 1 and then twice per week in weeks 2 and 3. Mice bearing SW1222 xenografts were given a different regime; 4 times in week 1 and then twice per week in weeks 2, 3 and 4.

In the repeat treatment phase I/II trial the first cohort of patients received 2 treatments. The protocol permitted the number of treatments to be progressively increased so that in theory 8 treatments could have been scheduled over an 11 day period. This would have achieved a schedule similar to that performed in pre-clinical experiments. However in the phase

I/II clinical trial the maximum number of scheduled treatments could not be increased above 3 due to myelosuppression. Repeat ADEPT was significantly more toxic in patients than in mice. Hence the repeat treatment ADEPT phase I/II trial was unable to achieve the number and frequency of treatments performed in pre-clinical investigations.

6.2.7 Evidence of efficacy

In patients with metastatic cancer new treatments aim to improve patient survival. Consequently survival is the ideal end-point in a phase III trial. In contrast the primary objectives of a phase I trial are to investigate safety, pharmacokinetics and relevant pharmacodynamic end-points. As part of pharmacodynamic evaluation it is desirable to obtain information on whether there is any effect on key aspects of tumour behaviour e.g. survival and growth. This information may be useful when choosing which drugs should be taken into phase II trials. Furthermore responses may allow preliminary assessment of which tumour types are most sensitive. This information would be helpful when deciding which tumour types to treat in a phase II trial.

The most frequently used technique to assess tumour response is to measure the size of the tumour before and after treatment. The method is relatively simple and results can be presented in a standardized format using RECIST criteria. A revised edition of RECIST (version 1.1) has recently been produced (Eisenhauer *et al.*, 2009).

However the use of tumour size to measure efficacy has weaknesses. For example some effective drugs may be cytostatic rather than cytotoxic and hence deliver significant clinical benefit without reducing the size of the tumour. This principle is demonstrated by a phase III randomized trial that compared sorafenib (an oral multi-kinase inhibitor) with placebo in patients with advanced previously untreated hepatocellular carcinoma (Llovet *et al.*,

2008). Sorafenib was associated with a significant increase in median survival even though sorafenib only produced tumour shrinkage in 2% of patients (objective response by RECIST). However in a clinical trial investigating a cytotoxic drug e.g. ADEPT, measurement of tumour size appears appropriate since an effective cytotoxic drug would be expected to cause tumour shrinkage.

Changes in tumour metabolism following treatment can also provide clinically relevant information on efficacy. FDG-PET is the most frequently used technique to measure changes in tumour metabolism. For example in patients with gastrointestinal stromal tumours treated with imatinib, reductions in tumour metabolism on FDG-PET after 8 days of treatment were associated with a significantly increased progression free survival (Stroobants *et al.*, 2003). FDG-PET has also been useful in measuring the response of gastrointestinal carcinomas to cytotoxic chemotherapy (Ott *et al.*, 2006). A draft framework for PET Response Criteria in Solid Tumours (PERCIST) has recently been proposed (Wahl *et al.*, 2009).

In the single-treatment and repeat-treatment ADEPT trials efficacy was measured using CT and FDG-PET. Response on FDG-PET was measured using a semi-automatic technique to quantify total activity within a tumour volume of interest. Tumour response defined according to this technique has been positively associated with prolonged survival in patients receiving cytotoxic chemotherapy for solid tumours (Green *et al.*, 2008; Francis *et al.*, 2007).

Four of the 22 ADEPT patients who underwent FDG-PET had significant reductions in tumour activity on functional imaging. Three observations suggest that the changes measured by FDG-PET reflected significant biological change in the tumours. Firstly the 4 patients that responded on FDG-PET include the 2 patients that had small reductions on CT (10% and

9% size reductions). Secondly the 4 patients that responded on FDG-PET also include the 3 patients with the largest falls in serum CA19-9. Thirdly there was a significant correlation between percentage change of tumour activity on FDG-PET and TPD.

6.3 Challenges to ADEPT

It is apparent from an examination of the current ADEPT system that only some of the conditions required for successful ADEPT are being achieved. Positive features have been the development of a safe antibody-enzyme (MFECP1), adequate delivery of BIP prodrug to tumour and some evidence of clinical efficacy. However three important characteristics need to be improved:

1) Antibody-enzyme immunogenicity

2) Myelosuppression

3) Tumour CPG2 activity at time of prodrug

Since the 2nd and 3rd points are intimately entwined they are discussed together in section 6.3.2.

6.3.1 Reduction of antibody-enzyme immunogenicity

In patients who received repeat ADEPT treatments HACPA developed at a median of day 16 and the earliest time that any patient became HACPA positive was day 15. Hence the time period in which ADEPT can safely be given is approximately 2 weeks. During this time it would be possible in theory to give 8 treatments. In practice however the number of treatments was limited by myelosuppression not immunogenicity.

For short courses of treatment HACPA would not be relevant. For example in a patient that received 2 ADEPT treatments with a TPD of 1200mg/m^2, the final MFECP1 infusion would be given on day 3, i.e. before HACPA develops.

However taking a broad perspective, immunogenicity remains an important independent challenge to clinical ADEPT development. Future developments to reduce myelosuppression may re-expose immunogenicity as the rate-limiting factor in the delivery of repeat treatments.

There are a number of potential strategies to reduce immunogenicity in ADEPT. One option is to pre-treat patients with an immunosuppressant drug. Administration of ciclosporin (an inhibitor of activated T_h lymphocytes) to ADEPT patients delayed HACPA production but increased adverse events (Bagshawe and Sharma, 1996). Corticosteroids may provide an alternative less toxic means of transient immunosupression. In a randomized double-blind, placebo controlled trial of patients with Crohn's disease who were receiving the chimeric monoclonal antibody infliximab, pre-treatment with intravenous hydrocortisone significantly reduced ADA titres at 8 and 16 weeks (Farrell *et al.*, 2003). However the percentage of patients who were considered ADA positive was not significantly reduced.

In a randomized trial of patients receiving intravenous radiographic contrast medium, pre-treatment with oral corticosteroid 12 hours and 2 hours before the administration of contrast, significantly reduced the incidence of allergic reactions (Lasser *et al.*, 1987). The effectiveness and safety of corticosteroid pre-treatment in ADEPT has not been investigated.

Identification and removal of T-cell epitopes on a bacterial enzyme is technically challenging but remains the most elegant solution. One group has achieved limited success with efforts to eliminate T_h lymphocyte

epitopes on a bacterial protein. Warmerdam and colleagues used a combination of *in silico* techniques and *in vitro* assays to identify an immunodominant T-cell epitope on staphylokinase (Warmerdam *et al.*, 2002). PBMCs from 10 healthy volunteers (covering 95% of HLA-DR haplotypes in European and North American populations) were used to generate T-cell clones specific for staphylokinase. Incubation with individual 17 mer peptides (overlapping by 12 amino acids) revealed a peptide (C3) that stimulated T-cell clones derived from 9 of the 10 donors. The promiscuous binding of this peptide with multiple HLA types suggested that this peptide contained an immunodominant epitope. To identify precisely the epitope(s) within C3, the binding of 11 mer peptides (derived from C3) to HLA-DR1 was analysed *in silico*. This identified two overlapping 11 mer peptides within the C3 region that bound strongly to HLA-DR1. The effect of single alanine substitutions at each amino acid location on these 2 peptides was assessed by measuring proliferation of T cell clones in response to the variant peptides and by *in-silico* calculation of binding to HLA. This information was used to produce 3 variant staphylokinase proteins containing 2, 3 or 4 amino acid substitutions which retained biological activity. Although the immunogenicity of the C3 region was eliminated, proliferation assays on one of the staphylokinase variants demonstrated that the intact protein continued to stimulate T-cells primed with the staphylokinase variant. There was no evidence for the creation of a new epitope in the C3 region and therefore the persistent immunogenicity is almost certainly caused by other epitopes outside the C3 region.

Interestingly Harding and colleagues have identified four CD4 positive T-cell epitopes on the bacterial enzyme β-lactamase, an enzyme that can be used in ADEPT systems (Harding *et al.*, 2005). They used 117 15-mer peptides (overlapping by 12 amino acids) that spanned the entire sequence of β-lactamase. Individual peptides were incubated with PBMCs from 65 community donors in T-cell proliferation assays. The 4 peptides that caused

stimulation in the greatest percentage of donors were identified as 4 possible T-cell epitopes. For each of the 4 peptides variants with a single alanine replacement at each amino acid location were produced. Each of these variant peptides was incubated with PBMCs from 66 community donors. By selection of the peptides that produced the least *in vitro* response, four alanine substitutions were chosen for each epitope. Complete enzyme mutants were produced incorporating these mutations and 2 mutant epitopes were identified that did not significantly interfere with enzyme function or stability. Both of these modified epitopes were incorporated into a novel B-lactamase. The novel B-lactamase was expressed at high levels and retained enzyme function. Novel B-lactamase produced significantly less stimulation than wild-type B-lactamase when incubated with PBMCs from 30 community donors. This is the first report of successful identification and removal of T-cell epitopes from a bacterial enzyme used in ADEPT systems. This novel B-lactamase can be incorporated into a fusion protein but has not been used in an ADEPT clinical trial.

6.3.2 Reducing myelosuppression and increasing CPG2 activity in tumour

A successful ADEPT system requires that at the time of prodrug administration tumour CPG2 is high and non-tumour CPG2 activity is low / absent. Failure to achieve the former would be expected to reduce efficacy; failure of the latter would be expected to increase myelosuppression.

There is evidence that the current ADEPT system may be failing in both of these requirements.

In pre-clinical ADEPT studies MFECP1 achieved the required design characteristics. Tumour CPG2 activity at time of prodrug (1.29U/g in SW1222 xenografts) was sufficient to produce significant tumour regression

when given as repeated treatments with BIP prodrug. Furthermore repeat ADEPT therapy caused less than 2% weight loss in mice.

However in patients, when MFECP1 had cleared from the circulation (serum CPG2 activity <0.002U/ml) tumour CPG2 activity was 0.006U/ml to 0.007U/ml i.e. significantly less than in mice. It is possible that the rapid clearance of MFECP1 from the systemic circulation may be an explanation for the apparent sub-optimal retention of MFECP1 on tumour. MFECP1 was designed to clear rapidly from the circulation so that a clearing antibody would not be required. Rapid clearance of MFECP1 was achieved by post-translational mannose glycosylation (a consequence of production in *P.pastoris*). MFECP1 binds to mannose receptors on liver sinusoidal endothelial cells and is taken into the cells (Kogelberg *et al.*, 2007).

Clinical data also suggests that CPG2 on non-tumour may be a significant cause of myelosuppression. In summary, at the time of prodrug administration the available evidence suggests that tumour CPG2 activity in patients was probably too low and total non-tumour CPG2 activity was too high.

It is important at this point to comment on the concept of tumour to non-tumour ratios. This concept is often included in ADEPT publications e.g. ratio of CPG2 activity in tumour compared to plasma. Despite its frequent use this concept could be misleading and should probably be avoided. It is likely that *absolute* values are more important than ratios. For example a CPG2 activity ratio of 500:1 (tumour versus plasma) may appear desirable but if the absolute value of plasma CPG2 activity is too high then there will probably be unacceptable myelosuppression. Similarly a CPG2 activity ratio of 500:1 may not produce efficacy if the absolute CPG2 activity in tumour is inadequate.

Can the dose / schedule of MFECP1 be modified to improve the selectivity of CPG2 distribution at the time of prodrug administration? The effect of MFECP1 dose on tumour CPG2 was examined in pre-clinical investigations in mice bearing LS174T xenografts. When the MFECP1 dose was increased from 600U/kg to 1200U/kg tumour CPG2 activity at 6 hours increased from 0.31U/g to 1.6U/g (Sharma *et al.*, 2005). Similarly plasma CPG2 activity at 6 hours increased from 0.0009U/ml to 0.0017U/ml. At this dose schedule tumour CPG2 activity increased approximately 5 times while serum CPG2 increased 2 times (and remained < 0.002U/ml).

The effect of MFECP1 dose on tumour CPG2 activity in patients is unknown. Six single-treatment ADEPT patients had quantification of tumour CPG2 by SPECT analysis (1 received MFECP1 3000U/m^2 and 5 received MFECP1 5000U/m^2). Although the scans were taken at different times, making comparison difficult, no obvious effect on tumour CPG2 was seen (Table 6.5).

Table 6.5

Estimated tumour CPG2 activity and MFECP1 dose

Patient	MFECP1 dose (U/m^2)	Time of scan after radiolabel (hours)	Median estimated CPG2 activity on SPECT (range)
17	5000	3.2	0.13*
18	5000	4.4	0.13 (0.11 – 0.15)
26	**3000**	**11.1**	**0.07 (0.05 – 0.007)**
21	5000	17.4	0.06 (0.03 – 0.08)
19	5000	19.6	0.04 (0.03 – 0.06)
20	5000	20.6	0.12 (0.12 – 0.16)
18	5000	21.3	0.07 (0.06 – 0.10)

* single tumour assessed. Tumour CPG2 activity estimated using radiolabelled MFECP1 and single photon emission computed tomography. Patient 17 received [123]Iodine radiolabel; patient 18, 19, 20, 21, 26 received [131]Iodine radiolabel. Data from CRUK database.

It is possible that a MFECP1 dose significantly > 5000U/m^2 may increase tumour CPG2. However such a dose may also increase plasma CPG2 activity and could therefore increase myelosuppression

Similarly there may also be no simple method to reduce total non-tumour CPG2 activity without compromising other ADEPT characteristics. For example increasing the time interval from the end of the MFECP1 infusion to the administration of prodrug would be expected to reduce total non-tumour

CPG2 activity. However it would also be expected to reduce tumour CPG2 activity.

Hence there is unlikely to be a new dose/schedule of MFECP1 that would significantly increase tumour CPG2 activity and significantly reduce total non-tumour CPG2 activity.

Should repeat ADEPT with MFECP1 and BIP prodrug be recommended to enter phase II trials? This decision requires a balanced view of the current evidence, weighing the positive and negative aspects. The most positive finding was a response on FDG-PET in 1 of the 3 patients that received 2 ADEPT treatments at the maximum tolerated TPD.

However there were also a number of significant negative considerations:

1) FDG-PET responses were short-lived.

2) None of the 13 patients who received a TPD ≥ 900mg/m^2 had a PR on CT imaging by RECIST criteria.

3) At the only schedule in which the maximum tolerated TPD was ≥ 900mg/m^2 (2 ADEPT treatments with a TPD of 1200mg/m^2) 66% of patients developed grade 3 thrombocytopaenia. This frequency of grade 3 thrombocytopaenia appeared higher than in conventional out-patient cytotoxic drug regimes. For example FOLFOX 6 (oxaliplatin, fluorouracil, folinic acid) produced G3/4 thrombocytopaenia in 1% of patients when used as 2nd line treatment of metastatic colorectal cancer (Tournigand *et al.*, 2004).

4) Tumour CPG2 activity associated with efficacy in mice may not have been achieved in patients.

5) Due to instability of reconstituted BIP prodrug the time interval from the start of reconstitution to expiry of the reconstituted BIP prodrug product was 20 minutes. Reconstitution and labeling required approximately 5 minutes and drug administration to the patient was over 5 minutes. Hence there was a maximum of 10 minutes to transport the reconstituted BIP prodrug from pharmacy to the patient. This would be logistically difficult in routine medical practice.

After consideration of all available evidence concerning ADEPT with MFECP1 and BIP, the most appropriate conclusion was that the current system should not be recommended to enter a phase II clinical trial.

6.4 ADEPT in perspective

Currently the most frequently adopted strategy in oncology drug development is to attempt to disrupt signaling pathways using small molecule enzyme inhibitors or mAbs. These non-cytotoxic drugs have added to the previous gains made by cytotoxic drugs (e.g. trastuzumab in breast cancer) and transformed the management of some tumours in which cytotoxic drugs were ineffective (e.g. sunitinib in renal cell carcinoma). However in many tumour types cytotoxic drugs are likely to remain an important part of systemic drug therapy. Therefore efforts to optimize cytotoxic delivery remain very relevant. In this context ADEPT can be seen as an incorporation of recombinant technology into a cytotoxic-based strategy. Despite the difficulties associated with the current ADEPT system (MFECP1 and BIP prodrug) ADEPT as a generic system continues to offer appealing characteristics and pre-clinical development of ADEPT is likely to continue.

The challenges faced in ADEPT and the techniques used to address them are issues that affect many areas of drug development in oncology. For example the issues of immunogenicity and mechanistic trial design are relevant to the development of many new compounds.

For example immunogenicity is potentially important in the development of any new protein therapeutic. Both wholly/partially foreign proteins and therapeutic proteins that are designed to replace an absent or deficient endogenous protein can be significantly affected by immunogenicity. The immunological reactions to these two types of therapeutic proteins however can be quite different. In the case of non-human proteins/sequences the consequences of ADA formation are primarily hypersensitivity, altered clearance and neutralization of the therapeutic protein. In contrast administration of recombinant endogenous proteins e.g. erythropoietin, can lead to breaking of tolerance and ADAs that neutralize both the therapeutic protein and any endogenous protein. This situation is rare but can have very serious clinical effects (Casadevall *et al.*, 2002). Hence the consequences of ADA formation can vary widely in clinical type and severity. Consequently a risk-based approach to the evaluation of immunogenicity in clinical trials has been proposed (Shankar *et al.*, 2007). For example the administration of a human recombinant protein to replace an endogenous protein with a unique and essential function, is regarded as higher risk because of the possibility of ADAs leading to neutralization of the endogenous counterpart. In contrast the administration of a non-human protein (e.g. CPG2) that is not designed to interfere with human protein function is regarded as lower risk. It should be noted however that anaphylaxis can occur even with lower risk therapeutic proteins.

Just as immunogenicity is a widespread issue in oncological drug development, so too are the changes in phase I trial design that have taken place since the first ADEPT clinical trial. The adoption of a mechanistic trial

design in ADEPT phase I trials (i.e. the use of biomarkers) mirrors widespread changes in the design of phase I trials in oncology. Recent ADEPT phase I trials have incorporated multiple biomarkers including biomarkers of MFECP1 delivery (e.g. SPECT), biological effect biomarkers (e.g. tumour COMET assays) and efficacy biomarkers (e.g. FDG-PET). Biomarker data is essential to understand a complex drug system. Furthermore biomarker data can be combined with pharmacokinetic data to build a computer model of ADEPT (Fang and Sun, 2008).

The use of multiple biomarkers in ADEPT clinical trials reflects a sea-change of phase I trial design in oncology. A review of over 2000 American Society of Clinical Oncology phase I trial abstracts reported that the proportion of studies incorporating one or more biomarkers increased significantly over time; 14% in 1991 compared with 26% in 2002 (Goulart *et al.*, 2007). In 39% of journal articles reporting on phase I trials, a biomarker supported the proposed drug mechanism of action. However only 25% of phase I trials reported in journal articles used multiple (4 or 5) biomarkers.

Biomarkers require close collaboration between the laboratory and clinic, add expense and may require patients to undergo additional investigations. However the data they provide can assist in both trial design and pre-clinical investigations. The data can allow trial investigators to know whether a drug achieved the desired effects. When the system deviates from the desired path it may be possible to rapidly adjust the trial design to correct deviations. For example during the repeat-treatment ADEPT phase I/II trial, detection by FDG-PET (an imaging biomarker) that efficacy only occurred when TPD ≥ 900mg/m^2 led to modification of the trial protocol to adjust the dose of prodrug. Hence mechanistic trial design has been integral to the development of ADEPT.

6.5 Conclusions

The aim of this MD (Res) thesis was to investigate the conditions required for successful repeat ADEPT treatments. In particular the thesis aimed to increase understanding of toxicity, efficacy and immunogenicty. ADEPT consisted of a recombinant antibody-enzyme (MFECP1) that targeted CEA followed by BIP prodrug.

A phase I/II clinical trial of repeat ADEPT treatments was conducted to obtain clinical and mechanistic data. This data was combined with the results of a single-treatment ADEPT phase I trial (also using MFECP1 and BIP prodrug) to provide a dataset on 43 patients.

The toxicity profile was defined and risk factors for adverse events identified. Myelosuppression was the principal toxicity and was probably caused by activated prodrug travelling to the bone marrow after production in tumour and non-tumour tissue. The maximum number of tolerated treatments was three. MFECP1 appeared to be safe and repeat MFECP1 administration did not increase the risk of infusion reactions. Repeat MFECP1 infusions significantly increased the incidence of HACPA. *In vitro* investigations indicated that MFECP1 causes activation and proliferation of T-lymphocytes.

Four patients had significant reductions in tumour activity on FDG-PET following ADEPT treatment. Response on FDG-PET only occurred in patients who received a TPD ≥ 900mg/m^2. One of three patients treated with 2 ADEPT treatments at the maximum tolerated TPD responded on FDG-PET.

This MD (Res) thesis has significantly increased understanding of the conditions required for successful ADEPT treatment.

7. Publications in support of the thesis

7.1 Abstracts

Wilkins D.K., Mayer A., Sharma S.K., Tolner B., Francis R.J., Springer C.J, Hartley JA., Boxer G.M., Bell J., Green A.J., Parker S., Griffin N., Shahbakhti H., Grudus E., Chester K.A. and Begent RHJ. Evidence of Efficacy of Antibody Directed Enzyme Prodrug Therapy (ADEPT) in a Phase I Trial in Patients with Advanced Carcinoma. Late-breaking abstract # LB200. American Association of Cancer Research 2008 annual meeting, April 2008, San Diego, USA. (oral presentation at plenary session given by Professor Richard Begent)

Wilkins D.K., Mayer A., Sharma S.K., Tolner B., Francis R.J., Springer C.J, Hartley JA., Boxer G.M., Bell J., Green A.J., Griffin N., Shahbakhti H., Grudus E., Chester K.A. and Begent RHJ. Efficacy in a Phase I/II Trial of Antibody Directed Enzyme Prodrug Therapy (ADEPT) in Patients with Gastrointestinal Carcinoma. Antibody Engineering, December 2007, San Diego, USA. (poster presentation)

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DK Wilkins, B Tolner, S Sharma, L Robson, M Baker, T Jones, RJ Ingram, D Altmann, RHJ Begent, A Mayer and K Chester. Identifying and mutating T-cell epitopes to reduce the Immunogenicity of a therapeutic enzyme in

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DK Wilkins, KA Chester, RHJ Begent, B Tolner, L Robson and A Mayer. Reducing the Immunogenicity of a therapeutic enzyme in Antibody Directed Enzyme Prodrug Therapy (ADEPT) by identifying and mutating T-cell epitopes. 23rd International Conference on Advances in application of Monoclonal Antibodies in Clinical Oncology, June 2006, Mykonos, Greece. (poster presentation)

7.2 Publications

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9. Appendices

Appendix 1

	Table 9.1					
	Planned treatment schedule for the administration of repeat ADEPT.					
	Number of treatments					
	2	**3**	**4**	**5**	**6**	**7**
Day	**Cohort 1**	**Cohort 2**	**Cohort 3**	**Cohort 4**	**Cohort 5**	**Cohort 6**
1	MFECP1	MFECP1	MFECP1	MFECP1	MFECP1	MFECP1
2	Prodrug x3	Prodrug x3	Prodrug x3	Prodrug x3 MFECP1	Prodrug x3 MFECP1	Prodrug x3 MFECP1
3	MFECP1	MFECP1	MFECP1	Prodrug x3 MFECP1	Prodrug x3 MFECP1	Prodrug x3 MFECP1
4	Prodrug x3	Prodrug x3	Prodrug x3	Prodrug x3	Prodrug x3	Prodrug x3 MFECP1
5						Prodrug x3
6						
7		MFECP1	MFECP1	MFECP1	MFECP1	MFECP1
8		Prodrug x3	Prodrug x3	Prodrug x3	Prodrug x3 MFECP1	Prodrug x3 MFECP1
9			MFECP1	MFECP1	Prodrug x3 MFECP1	Prodrug x3 MFECP1
10			Prodrug x3	Prodrug x3	Prodrug x3	Prodrug x3
On days where prodrug and MFECP1 are shown on the same day it was planned that prodrug would be given in the morning and MFECP1 in the evening.						

Appendix 2

Table 9.2
Results of T-cell proliferation assays using PBMCs from 20 healthy donors incubated with overlapping 15mer peptides from CPG2.

Peptide	Identity of responding donor (volunteer number)	Epitope	Peptide sequence
1	5		QKRDNVLFQAATDEQ
2			DNVLFQAATDEQPAV
3			LFQAATDEQPAVIKT
4			AATDEQPAVIKTLEK
5			DEQPAVIKTLEKLVN
6			PAVIKTLEKLVNIET
7			IKTLEKLVNIETGTG
8	5		LEKLVNIETGTGDAE
9			LVNIETGTGDAEGIA
10			IETGTGDAEGIAAAG
11			GTGDAEGIAAAGNFL
12			DAEGIAAAGNFLEAE
13			GIAAAGNFLEAELKN
14			AAGNFLEAELKNLGF
15			NFLEAELKNLGFTVT
16			EAELKNLGFTVTRSK
17	18		LKNLGFTVTRSKSAG
18			LGFTVTRSKSAGLVV
19			TVTRSKSAGLVVGDN
20			RSKSAGLVVGDNIVG
21			SAGLVVGDNIVGKIK
22			LVVGDNIVGKIKGRG
23	5		GDNIVGKIKGRGGKN
24			IVGKIKGRGGKNLLL
25	1, 6, 16, 18	A	KIKGRGGKNLLLMSH
26	1, 9		GRGGKNLLLMSHMDT
27			GKNLLLMSHMDTVYL
28	9		LLLMSHMDTVYLKGI
29	1, 18, 19		MSHMDTVYLKGILAK
30			MDTVYLKGILAKAPF

Peptide	Identity of responding donor (volunteer number)	Epitope	Peptide sequence
31			VYLKGILAKAPFRVE
32	1		KGILAKAPFRVEGDK
33			LAKAPFRVEGDKAYG
34			APFRVEGDKAYGPGI
35			RVEGDKAYGPGIADD
36			GDKAYGPGIADDKGG
37	5, 10	B	AYGPGIADDKGGNAV
38	10		PGIADDKGGNAVILH
39	1		ADDKGGNAVILHTLK
40	1, 18		KGGNAVILHTLKLLK
41	1, 18		NAVILHTLKLLKEYG
42	1, 18		ILHTLKLLKEYGVRD
43	18		TLKLLKEYGVRDYGT
44			LLKEYGVRDYGTITV
45			EYGVRDYGTITVLFN
46			VRDYGTITVLFNTDE
47			YGTITVLFNTDEEKG
48	10	C	ITVLFNTDEEKGSFG
49	10, 18		LFNTDEEKGSFGSRD
50	10		TDEEKGSFGSRDLIQ
51			EKGSFGSRDLIQEEA
52	10		SFGSRDLIQEEAKLA
53			SRDLIQEEAKLADYV
54			LIQEEAKLADYVLSF
55	1		EEAKLADYVLSFEPT
56	5, 7		KLADYVLSFEPTSAG
57			DYVLSFEPTSAGDEK
58	7	D	LSFEPTSAGDEKLSL
59	5, 7		EPTSAGDEKLSLGTS
60	7, 15		SAGDEKLSLGTSGIA
61			DEKLSLGTSGIAYVQ
62			LSLGTSGIAYVQVNI
63	4		GTSGIAYVQVNITGK
64			GIAYVQVNITGKASH
65			YVQVNITGKASHAGA
66	9, 16		VNITGKASHAGAAPE
67			TGKASHAGAAPELGV
68	18	E	ASHAGAAPELGVNAL
69	16		AGAAPELGVNALVEA
70	9, 16, 20		APELGVNALVEASDL

Peptide	Identity of responding donor (volunteer number)	Epitope	Peptide sequence
71			LGVNALVEASDLVLR
72			NALVEASDLVLRTMN
73			VEASDLVLRTMNIDD
74			SDLVLRTMNIDDKAK
75			VLRTMNIDDKAKNLR
76			TMNIDDKAKNLRFNW
77	18	F	IDDKAKNLRFNWTIA
78	1, 18		KAKNLRFNWTIAKAG
79	10, 18		NLRFNWTIAKAGNVS
80			FNWTIAKAGNVSNII
81	18		TIAKAGNVSNIIPAS
82	1		KAGNVSNIIPASATL
83	18		NVSNIIPASATLNAD
84			NIIPASATLNADVRY
85			PASATLNADVRYARN
86			ATLNADVRYARNEDF
87			NADVRYARNEDFDAA
88			VRYARNEDFDAAMKT
89			ARNEDFDAAMKTLEE
90			EDFDAAMKTLEERAQ
91			DAAMKTLEERAQQKK
92			MKTLEERAQQKKLPE
93			LEERAQQKKLPEADV
94			RAQQKKLPEADVKVI
95			QKKLPEADVKVIVTR
96			LPEADVKVIVTRGRP
97	1, 3, 18	G	ADVKVIVTRGRPAFN
98			KVIVTRGRPAFNAGE
99			VTRGRPAFNAGEGGK
100	3		GRPAFNAGEGGKKLV
101	16		AFNAGEGGKKLVDKA
102	9		AGEGGKKLVDKAVAY
103	18		GGKKLVDKAVAYYKE
104	16		KLVDKAVAYYKEAGG
105			DKAVAYYKEAGGTLG
106			VAYYKEAGGTLGVEE
107			YKEAGGTLGVEERTG
108	5		AGGTLGVEERTGGGT
109			TLGVEERTGGGTDAA
110			VEERTGGGTDAAYAA

Peptide	Identity of responding donor (volunteer number)	Epitope	Peptide sequence
111			RTGGGTDAAYAALSG
112	3		GGTDAAYAALSGKPV
113			DAAYAALSGKPVIES
114			YAALSGKPVIESLGL
115			LSGKPVIESLGLPGF
116	3		KPVIESLGLPGFGYH
117			IESLGLPGFGYHSDK
118			LGLPGFGYHSDKAEY
119			PGFGYHSDKAEYVDI
120			GYHSDKAEYVDISAI
121			SDKAEYVDISAIPRR
122			AEYVDISAIPRRLYM
123			VDISAIPRRLYMAAR
124	18		SAIPRRLYMAARLIM
125			PRRLYMAARLIMDLG
126			LYMAARLIMDLGAGK
127	11		AARLIMDLGAGKHHH
128			LIMDLGAGKHHHHHH

A responding donor (to a particular peptide) was a donor whose PBMCs proliferated significantly more to peptide than negative control i.e. stimulation index ≥ 2. Peptides that reacted with 1 donor are shown in blue. Peptides that reacted with 2 donors are shown in yellow. Peptides that reacted with 3 donors are shown in orange. Peptides that reacted with 4 donors are shown in pink. Experiments performed at Biovation Ltd.

10. Acknowledgments