# HPMA copolymer - aminoellipticine conjugates: Mechanism of action

by

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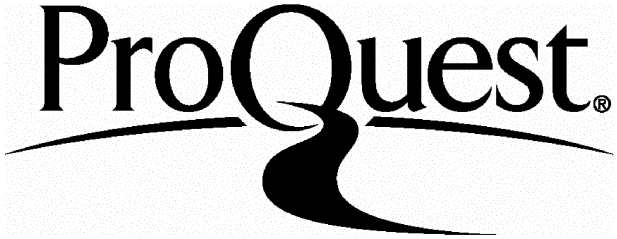

**This work is dedicated to my family and to Yuk.**

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## Abstract

Over the past two decades cancer chemotherapy has resulted in a small number of previously fatal cancers becoming curable. Many cancers, particularly the so-called solid tumours, do not respond well to conventional chemotherapy. To maximise tumour targeting and minimise host tissue toxicity a large number of drug delivery systems have been proposed. Polymer-anticancer conjugates based on N-(2-hydroxypropyl) methacrylamide (HPMA) have recently entered early clinical trial. It has been shown that HPMA copolymer conjugates preferentially extravasate into solid tumours and are retained there by a process known as the 'enhanced permeability and retention' (EPR) effect.

A natural anticancer agent, derived from ellipticine, namely 6-(3-aminopropyl) ellipticine (APE) was selected for conjugation to HPMA copolymers. In this study a series of HPMA copolymer-APE conjugates were synthesised, containing a variety of drug loadings (1.07-6.10%w/w) conjugated via the tetrapeptide linker (Gly-Phe-Leu-Gly). These conjugates were designed to be localised in tumours following injection and to be taken up by tumour cells via the process of endocytosis before liberating APE, mediated by cathepsin B present in the lysosome. These conjugates were shown to form complex intramolecular micelles in solution which resulted in the conjugates of a high drug loading showing reduced APE release *in vitro* (20%/5h) compared to the medium and low drug loading conjugates (55%/5h), suggesting hindered enzyme access. All conjugates showed a marked reduction in haemolysis, a common problem with ellipticines, compared to APE alone. Anti-tumour activity was observed in the s.c. B16F10 murine melanoma model and also in the CORL-23 human non small cell lung carcinoma xenograft in mice, particularly for the conjugate of medium APE loading.

This thesis also examined the extravasation and intratumoural distribution of HPMA copolymer-anticancer conjugates using HPMA copolymer doxorubicin (PK1) as a model conjugate in the rat dorsal window chamber model.

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## Abbreviations

| | |
|---|---|
| AML | Acute myeloid leukaemia |
| APE | 6-(3-Aminopropyl)-ellipticine |
| ATRP | Atom transfer radical polymerisation |
| DIVEMA | Divinylether maleic acid |
| DMF | Dimethyl formamide |
| DMSO | Dimethyl sulphoxide |
| EDTA | Ethylenediaminetetraacetic acid |
| EPR | Enhanced permeability and retention effect |
| FCS | Foetal calf serum |
| FTIR | Fourrier-transform infrared spectroscopy |
| GFLG | Glycine-Phenylalanine-Leucine-Glycine |
| GPC | Gel permeation chromatography |
| GSH | Reduced glutathione |
| HAMA | Human anti-mouse antibody formation |
| HPLC | High performance liquid chromatography |
| HPMA | *N*-(2-Hydroxypropyl)methacrylamide |
| $IC_{50}$ | Inhibitory concentration for 50% of cells |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| LDH | Lactate dehydrogenase |
| MPEG | Monomethoxy poly(ethylene glycol) |
| MTD | Maximum tolerated dose |
| MTT | 1-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide |
| $M_w$ | Weight average molar mass |
| $M_n$ | Number average molar mass |
| NAp | *p*-Nitroanilide |
| NCI | National Cancer Institute |
| ONp | *p*-Nitrophenol |
| PBS | Phosphate buffered saline |
| PEG | poly (Ethylene glycol) |
| PK1 | HPMA copolymer Gly-Phe-Leu-Gly-doxorubicin |
| PK2 | HPMA copolymer Gly-Phe-Leu-Gly-doxorubicin/galactosamine |
| RES | Reticulo-endothelial system |
| Rf | Reference factor for TLC |
| RI | Refractive index |
| s.c. | Subcutaneous |
| SD | Standard deviation |
| SE | Standard error |
| SMANCS | Poly(styrene-co-maleic acid)-neocarzinostatin |
| TLC | Thin layer chromatography |
| TNF | Tumour necrosis factor |
| UV | Ultraviolet |
| VEGF | Vascular endothelial growth factor |

# Chapter One

## *General Introduction*

## 1.1 General Introduction

Globally approximately 10 million people are diagnosed with cancer each year and this number is estimated to double by the year 2020 because of the world's ageing population. Cancer is the cause of 6 million deaths every year worldwide (Sikora, 1999). In the United Kingdom a half of all cancer deaths are caused by just three cancer types – lung, large bowel and prostate (men) or breast (female) (CRC CancerStats, 2000). These are so-called solid tumours and the main modalities of their treatment are surgery and radiotherapy.

Drug chemotherapy has resulted in a number of cancers becoming largely curable such as Hodgkin's disease and childhood leukaemia and it is hoped that this success in the treatment of the so-called soft tissue tumours can be emulated with improved treatments for solid tumours. Effective treatment of soft tissue tumours results from the fact that they have characteristics of a disseminated disease. Solid tumours, however, form a more localised tumour mass and therefore it is often difficult to achieve the high local drug concentration essential to achieve effective treatment and simultaneously minimise side-effects (reviewed in De Vita et al, 1993). The main difficulties associated with systemic chemotherapy of solid tumours are: achieving a sufficient local drug concentration (reviewed by Jain, 1989); achieving drug penetration throughout the tumour mass (Tunggal et al, 1999) and not least the development of resistance to therapy (reviewed by Krishna & Mayer, 2000). A number of strategies have been employed as means of overcoming these difficulties (Table 1.1). They include use of combination chemotherapy of different agents acting via different mechanisms of action, development of more effective analogues of existing agents, synthesis or identification of new compounds and not least the use of drug delivery systems to optimise tumour targeting and minimise drug access to sites of toxicity. Drug delivery systems have the potential advantage that the technology used may be applied to a broad spectrum of agents. One promising new approach being developed for improved tumour targeting is the use of polymer-drug conjugates and a number of these systems have recently entered Phase I/II clinical trials (reviewed by Duncan, 2000).

Those polymer-drug conjugates in clinical testing utilise established anticancer agents but this approach may potentially benefit anticancer agents which have failed

**Table 1.1:** Methods for overcoming the current limitations of cancer chemotherapy. Key: MOPP (mechlorethamine, vincristine, procarbazine, prednisone) CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone).

| Type of therapy | Examples | Condition treated or stage of development | References |
|---|---|---|---|
| Combination chemotherapy | MOPP | Hodgkin's disease | reviewed by McCaffrey & Bajorin, 1998 |
| | CHOP | Non-Hodgkin's lymphoma | |
| Analogues of existing agents | Temozolamide (analogue of dacarbazine) | Malignant glioma | Martindale, 1993 |
| | Epirubicin (an anthracycline) | Breast cancer | |
| Novel cytotoxic compounds (e.g. natural products) | Ecteinascidin 743 | Phase I | Ryan et al, 2001 |
| | Combretastatin A4 | Phase I | Tozer et al, 1999 |
| Biologics | Interferon α-2a | Kaposi's sarcoma | Martindale, 1993 |
| | Interleukin-2 | Metastatic renal cell carcinoma | |
| Drug delivery systems | Liposomes | Various tumour types | Gregoriadis, 1995 |
| | Antibodies | | Buske et al, 1999 |
| | Polymer-drug conjugates | | Duncan, 2000 |

clinical development due to toxicity difficulties. This thesis will focus on the synthesis and biological testing of a novel polymer-drug conjugate of such an agent, *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer 6-(3-aminopropyl)-ellipticine (APE), with a view to developing a clinical candidate. In addition, this study has examined the process of polymer-drug conjugate extravasation into tumour tissue using the rat dorsal window chamber technique (Hill et al, 1999). Here the drug delivery systems in research and use in cancer chemotherapy will be introduced and the emerging field of polymer therapeutics will be reviewed with emphasis on the design of polymer-drug conjugates, in particular conjugates of HPMA copolymers. The biological rationale for the development of these conjugates will also be described. As this study focuses on a natural product conjugate the current use of natural product anticancer agents is reviewed focusing on the ellipticine family of compounds.

## 1.2 Drug delivery systems in cancer therapy

Drug delivery systems have been developed either to improve tumour targeting or to allow prolonged controlled release of drug and sometimes they achieve both. Tumour selective localisation of chemotherapy would achieve higher tumour drug concentrations and could thus improve efficacy and decrease the incidence of side effects (reviewed by Connors, 1996) (i.e. improve the therapeutic index). Improved targeting also opens the possibility of using highly toxic agents like some of the novel natural product drugs proposed as chemotherapy and discussed later (Section 1.5).

### *1.2.1* Polymeric implants for controlled release and drug targeting

This study focuses on the use of water soluble polymers for targeting and controlled release but it is important to state that polymers, in the form of polymeric implants (particles, discs and rods), have already been widely developed for controlled release and targeting of anti-tumour agents (reviewed by Fung & Saltzman, 1997).

For example Zoladex® is a biodegradable rod of poly(lactide-co-glycolide) implanted subcutaneously (s.c.) into the abdomen as a means of delivering the luteinizing hormone analogue, goserelin acetate, for the treatment of prostate cancer (Debruyne et al, 1988). There are a number of formulations available for one month or three month sustained therapy and the administered dose is 3.6mg per 28 days therapy (Martindale, 1993). Zoladex® was one of the first controlled release products to find

use in cancer chemotherapy. Gliadel is a polymeric implant (wafer) used to achieve "local" targeting in the treatment of malignant glioma. The biodegradable wafer Gliadel® contains carmustine (3.85%w/w) incorporated into a copolymer matrix composed of 1,3-bis(p-carboxyphenoxy)propane and sebacic acid (20:80 molar ratio) (Dang et al, 1996). After surgical removal of glioma Gliadel® wafers are placed in the surgical site. This treatment has been shown to prolong patient survival by 50% following malignant glioma removal (Brem et al, 1995). This is a method of restricting delivery to a tumour site as carmustine is rapidly metabolised and penetrates just 2mm into the surrounding brain tissue and therefore toxicity to healthy tissue is abrogated (reviewed by Wang et al, 1999).

Localised delivery of chemotherapy is only useful for treatment of local disease but would not be able to treat metastatic disease. For effective treatment of metastatic disease it is necessary to target tumours after intravenous (i.v.) administration. Several systems have been developed using this principle, including antibodies, liposomes and polymer therapeutics. These systems are described in more detail in the following sections.

### *1.2.2* Antibodies

Antigen-antibody reactions are potentially highly specific so the use of monoclonal antibodies as an active targeting system was seen as a wonderful opportunity to achieve tumour-specific targeting (Kohler & Milstein, 1975). It was a natural extension of this concept to attach drugs via a linker to the antibodies to achieve active tumour targeting (reviewed by Trail & Bianchi, 1999). Since then many anticancer drugs, immunotoxins and radioactive agents have been conjugated to antibodies for use as cancer chemotherapeutic agents.

These include conjugates of immunotoxins such as a Fab' fragment of a monoclonal anti-CD22 antibody conjugated to deglycosylated ricin-A chain which gave partial responses in 38% of evaluable patients in a Phase I study against patients with refractory B-cell lymphoma (Vitetta et al, 1991). Antibody conjugates containing radionuclides have also been widely studied such as [$^{131}$I]iodide which has been conjugated to a number of antibodies (e.g. anti-CD20 which was studied in a Phase I trial against B-cell non-Hodgkin's lymphoma and showed 50% complete remission and a further 29% partial responses in patients (Kaminski et al, 1996)). Antibodies have

also been conjugated to classical cytotoxic agents such as doxorubicin. An example is doxorubicin conjugated to the BR96 antibody via a hydrazone linkage (Firestone et al, 1996) which gave a maximum tolerated dose (MTD) of 25mg/m$^2$ (doxorubicin equivalent), when administered by i.v. infusion every three weeks, in a recent Phase I study against metastatic colon and breast cancer (Saleh et al, 2000). The first antibody-drug conjugate to enter clinical use in 2000 was the anti-CD33 calicheamicin immunoconjugate (Mylotarg™) which is used for the treatment of acute myeloid leukaemia (AML). CD33 is an antigen present on maturing normal haematopoietic and AML cells but not on normal haematopoietic cells. Calicheamicin, an anti-tumour antibiotic (Lee et al, 1987), is released upon internalisation of the construct into CD33 positive cells killing these cells whilst sparing normal stem cells. In this case the target antigen is expressed on a high percentage of desired target cells (about 90%) (Sievers et al, 1999).

In recent years interest has grown in the use of antibodies that can stimulate an immune response against the tumour. It has become apparent that the body is capable of removing a large tumour burden in this manner (Kranz et al, 1998). Herceptin® is an antibody to the human epidermal growth factor receptor (HER-2), often over-expressed in breast cancer, and has been developed both in single agent trials but also particularly in combination with established agents (doxorubicin and paclitaxel). It has shown responses in clinical trials itself but is especially useful in combination chemotherapy (reviewed by Baselga, 2001).

Originally antibodies were developed using murine systems but these mouse antibodies were immunogenic as they were recognised as foreign proteins. Administration gave rise to the so-called human anti-mouse antibody formation (HAMA) which resulted in rapid clearance of antibodies, particularly on repeated administration (reviewed by Buske et al, 1999). The development of 'humanised' or chimeric antibodies has significantly reduced this problem. The main difficulties remaining for antibodies as carriers are the general lack of specific identifiable targets within tumours, poor tumour penetration and the difficulty of maintaining antibody reactivity for antibody-drug conjugates. Drug loading capacity can also be limited (reviewed by Kranz et al, 1998).

Recent years have seen an increasing number of studies using antibodies in combination with liposomes (reviewed by Allen & Moase, 1996) and polymer conjugates (reviewed by Rihova, 1998). The aims of these studies being to improve tumour targeting and in the case of polymer conjugates reduce antibody immunogenicity.

*1.2.3* Liposomes

Liposomes were first proposed by Bangham (1965) and they were amongst the earliest systems developed for tumour targeting. Liposomes are self-assembling colloidal particles consisting of a lipid bilayer which encloses a fraction of the surrounding aqueous medium. This medium usually contains the drug of interest which is carried either in the bilayer or in the aqueous core (reviewed by Allen, 2000). The limitations of liposomes include: rapid clearance by the reticulo-endothelial system (RES) (Papahadjopoulos et al, 1991), extravasation into a tumour in an unpredictable manner (Uster et al, 1998 and Forssen et al, 1996) and there are issues of long-term physicochemical stability (reviewed by Gregoriadis, 1995). Some of these difficulties have been overcome with the addition of a poly (ethylene glycol) (PEG) coating. These liposomes, known now as Stealth® liposomes, have improved stability and reduced recognition by the RES and thus have longer circulation times (Papahadjopoulos et al, 1991). Liposomes can be prepared to have a large range of sizes and therefore size can be tailored as required.

DaunoXome® and Caelyx®/Doxil® are licensed medicines and their liposomes contain daunorubicin and doxorubicin respectively. DaunoXome® consists of a liposome of approximately 100nm diameter prepared from distearoylphosphatidylcholine : cholesterol in a molar ratio of 2 : 1 and daunorubicin. It is licensed for first line therapy of HIV-associated Kaposi's sarcoma at a dose of 40mg/m$^2$ of daunorubicin every 2 weeks (reviewed by Forssen, 1997). In a Phase III trial against HIV-associated Kaposi's sarcoma DaunoXome® displayed comparable response rates and survival time to the standard treatment (ABV – doxorubicin, bleomycin, vincristine) but showed a significant reduction in side effect profile (Gill et al, 1996). Caelyx®/Doxil® is a liposome with a diameter of around 100nm containing doxorubicin, mainly in the central core. Drug is contained within a lipid bilayer of phosphatidylcholine: cholesterol: methoxyPEG(MPEG)-distearolylglycerol-phosphoethanolamine in a ratio of 3: 1: 1. The presence of the MPEG designates this

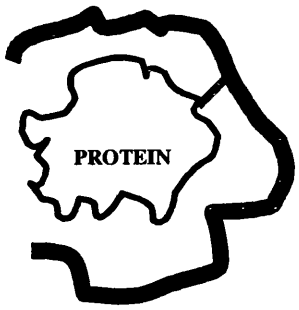
formulation as being a Stealth® liposome with benefits as previously outlined. Caelyx®/Doxil® (50mg/m$^2$ every three weeks) was administered in a Phase II study to 35 patients with ovarian cancer who did not respond to paclitaxel or platinum-based regimens. Nine responses were seen and the treatment was well tolerated (Muggia et al, 1997). Both Caelyx®/Doxil® and DaunoXome® produced decreases in the incidences of anthracycline-associated side effects in clinical trial (see Section 1.5.1 for these side effects). However Caelyx®/Doxil® caused palmar-plantar erythrodysesthesia (or hand-foot syndrome), which was also observed in the past after continuous infusion of doxorubicin. This is caused by extravasation of liposomes from peripheral capillaries with subsequent local release of anthracycline (Gabizon et al, 1994). Other liposomal systems containing anticancer drugs are being developed (reviewed by Allen & Moase, 1996) but a full review of these systems is beyond the scope of this thesis.

Although both antibodies and liposomes have been researched widely over a long period of time there are still relatively few products on the market and both suffer from a number of disadvantages as outlined above. Another family of macromolecular carriers developed over the last two decades are the water soluble polymer-drug conjugates (Duncan & Kopecek, 1984; Duncan, 1992; Putnam & Kopecek, 1995; Duncan et al, 1996; Kopecek et al, 2000 and Duncan, 2000). This approach was originally followed as polymers have widespread biomedical use e.g. as artificial prostheses, contact lenses, plasma expanders and pharmaceutical excipients (reviewed in Polymers: Biomaterials and Medical Applications, 1989), and thus they have been proven to be non-immunogenic and to cause no or minimal adverse effects in the body (reviewed by Duncan, 1992).

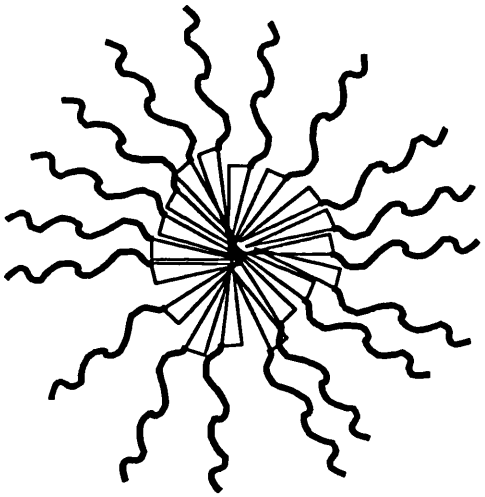
## 1.3 Polymer therapeutics

Polymer therapeutics have been defined as polymeric drugs, polymer protein conjugates, polymeric micelles and polymer drug conjugates (reviewed by Brocchini & Duncan, 1999) and see Figure 1.1. These classes of polymer therapeutic will be briefly reviewed before focusing on polymer-drug conjugates as anticancer agents, the topic of this study.

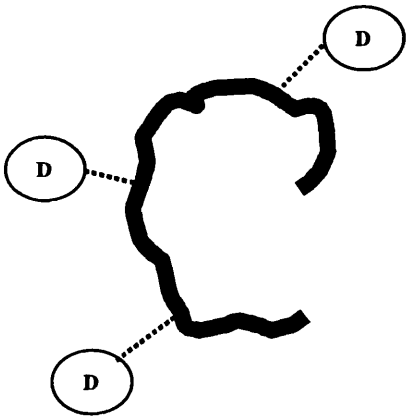
**Polymeric drug**




**Polymer-protein conjugate**



**Polymeric micelle**




**Polymer-drug conjugate**

**Figure 1.1:** Schematic of "polymer therapeutics".

*1.3.1* Polymeric drugs

As mentioned above polymers are widely used in biomedical applications and also as polymeric implants for use in cancer chemotherapy (Section 1.2.1). Recently some soluble polymers with intrinsic biological activity have been developed as chemical entities in themselves. For example Copaxone® (a random copolymer of L-alanine, L-glutamate, L-lysine and L-tyrosine units of a molecular weight of 4-13,000Da also known as glatiramer acetate) has recently been approved by Regulatory Authorities for use in the treatment of multiple sclerosis (Blumhardt, 2000). In cancer chemotherapy no polymeric drugs are licensed as anticancer agents. However some show anti-tumour activity. Pluronic F68 PEG suppresses growth of induced (using azoxymethane) colonic tumours in rats and mice (Parnaud et al, 2001). Additionally it has long been known that polyanions can display immunomodulatory properties (Ottenbrite et al, 1983) which give them potential as anticancer and antiviral agents.

*1.3.2* Polymer-protein conjugates

Proteins have widespread potential in the treatment of cancer and many other conditions. The major difficulties associated with the use of proteins as drugs has been their immunogenicity, poor stability, biodegradability, and also, in common with small molecular weight agents, poor localisation at the target site (reviewed by Mc Cafferty & Glover, 2000). Polymer-protein conjugation has been developed as a means of 'shielding' the protein from the immune system, reducing degradation and also to prolong blood circulation times. In addition certain polymer-protein conjugates show tumour localisation by means of the enhanced permeability and retention (EPR) effect which will be discussed in Section 1.4.3. The most common polymer component is PEG and PEGylation is used primarily as a means of increasing blood circulation times. Proteins which seem to have benefited from conjugation to PEG include granulocyte-macrophage colony stimulating factor, various interferons and interleukins (reviewed by Bailon & Berthold, 1998). In addition PEG-asparaginase (Oncaspar®) is an anticancer conjugate which is in clinical use. Asparaginase depletes asparagine levels, upon which certain leukaemic cells rely for survival. The use of free asparaginase as an anticancer treatment has been hampered by its limited half-life and the high incidence of allergic reactions. PEG modification increases asparaginase half-life (20-fold) and decreases asparaginase immunogenicity (reviewed by Nucci et al, 1991). An increasing number of PEG-protein conjugates are being developed but a full review of this field is beyond the scope of this thesis.

One other polymer-protein conjugate is worthy of mention. This is styrene maleic acid conjugated to the anti-tumour protein neocarzinostatin (SMANCS). This conjugate had a blood circulation half-life 10-fold higher than neocarzinostatin alone. More significantly the conjugate gave a tumour to blood ratio of 5 within 19h of administration and such a level was not achieved for neocarzinostatin alone demonstrating passive tumour targeting of this conjugate by the EPR effect (Matsumura & Maeda, 1986). SMANCS is approved in Japan for the treatment of primary hepatocellular carcinoma. Intra-arterial administration in the lipid contrast agent Lipoidal® in patients with unresectable hepatoma showed remarkable anti-tumour activity (95% reduction in tumour size and prolonged survival) with minimal incidence of side-effects (Konno et al, 1983).

*1.3.3* Polymeric micelles

Micelles can be defined as colloidal dispersions consisting of amphiphilic molecules. These amphiphiles aggregate in solution to form micelles when present at a concentration above the critical micelle concentration and many micelle-utilising formulations are in pharmaceutical use as they solubilise poorly water-soluble drugs (reviewed by Jones & Leroux, 1999). Polymeric amphiphiles form micelles in the same way if the polymer consists of at least two blocks of different hydrophobic/hydrophilic character. Drugs are incorporated, depending on their hydrophilicity, into either the hydrophobic core or the outer shell of the polymeric micelle or they can be covalently attached to the polymer (e.g. in the case of imaging agents for example [$^{111}$In]indium (Trubetskoy & Torchilin, 1995)).

The most widely used polymeric micelles are the Pluronic®s which are triple copolymers of hydrophilic ethylene oxide units with hydrophobic propylene oxide units. Several Pluronic® micelles have been shown to improve drug efficacy against multidrug resistant cells *in vitro*. The mechanism of this action has not been fully clarified but two possibilities have been suggested. Either the Pluronic® interacts with the cellular membrane directly (reviewed by Erukova et al, 2000) or they interact with multidrug resistance p-glycoprotein (Alakhov et al, 1996). However both mechanisms may be involved in combination. A number of anticancer drugs have been incorporated into Pluronic® systems. For example a doxorubicin-containing Pluronic® showed increased survival and decreased toxicity in a number of animal models (Batrakova et

al, 1996) and increased *in vitro* cytotoxicity against multidrug resistant cells (Venne et al, 1996).

A polymeric micelle consisting of a combination of bound and entrapped drug is poly(ethylene oxide-aspartate) block copolymer doxorubicin, first proposed by Yokoyama et al (1990). In this case a block copolymer of poly(ethylene oxide) linked to poly(aspartate) was used to form a micelle with doxorubicin bound to the poly(aspartate) via amide linkages. These micelles also contain free doxorubicin which is closely associated with the bound doxorubicin. In biodistribution experiments in C26 colon adenocarcinoma-bearing CDF1 mice this construct was shown to produce a 10-fold increase in tumour accumulation and a 4-fold decrease in peak cardiac levels compared to free doxorubicin (important because of anthracycline cardiotoxicity see Section 1.5.1) (Kwon et al, 1994). Anti-tumour activity has recently been reported in the same tumour model with significant tumour volume reduction compared to doxorubicin alone and no evident toxicity (Kataoka et al, 2000).

Both the Pluronic® doxorubicin micelles (Supratek website, 2001) and the poly (ethylene oxide)-poly (aspartate)-doxorubicin micelles (Nakanishi et al, 2001) are currently in early clinical testing.

### *1.3.4* Polymer-drug conjugates

The concept of conjugating low molecular weight anticancer drugs to a polymeric backbone was first proposed by Ringsdorf (1975). It was suggested that drug would be bound via a suitable linker to the polymer backbone and additionally targeting moieties could be present (Figure 1.2). Since then there has been a lot of interest in this field, evident from the increasing number of polymer-drug conjugates which have entered clinical Phase I/II testing (reviewed by Duncan, 2000). The three main components of a polymer-drug conjugate will now be discussed.

#### 1.3.4.1 Polymeric backbone:

The polymeric backbone is the major component of a polymer-drug conjugate and must perform a number of important functions. The ideal polymeric backbone would:

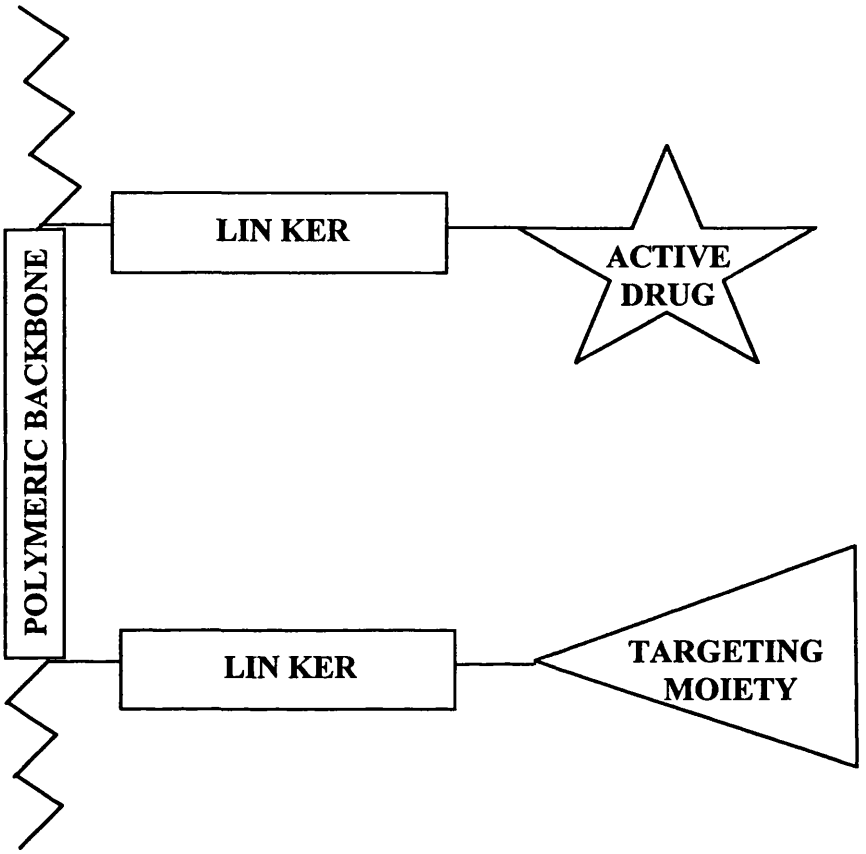



**Figure 1.2:** Schematic of a polymer-drug conjugate.

1. Be soluble in physiological conditions and capable of solubilising poorly water-soluble drugs
2. Possess functionality which allows conjugation of sufficient quantities of drug
3. Be monodisperse
4. Not be toxic and would have non-toxic breakdown products (if biodegradable)
5. Not be immunogenic and would be capable of 'shielding' immunogenic drugs
6. Be biodegradable or be of a size which would allow excretion from the body
7. Meet industrial constraints such as ease and cost of production and ease of characterisation (reviewed by Duncan et al, 1996)

The importance of these individual factors varies depending on the drug of interest and its characteristics. Solubility and solubilisation characteristics of the polymer are important because the majority of anticancer agents have very poor aqueous solubility, particularly those of natural origin (reviewed by Cragg et al, 1997). The polymer should possess functionality allowing drugs to be conjugated and it is important also that it can carry a sufficient drug load to be active, hence also the importance of drug potency.

Synthetic polymers typically exist as a range of molecular masses (Figure 1.3) and this is characterised by their polydispersity. Polydispersity is expressed as $M_w/M_n$. Weight-average molar mass ($M_w$) is defined as the sum of the products of the molar mass of each fraction multiplied by its weight fraction. Number-average molar mass ($M_n$) is defined as the sum of the products of the molar mass of each fraction multiplied by its mole fraction. Therefore the closer the polydispersity is to 1.0 the more uniform the polymer molecular weight (Young & Lovell, 1996).

The polymer backbone should either be of such a size that it can be excreted from the body, usually by glomerular filtration in the kidneys, or be biodegradable to such an extent. This size characteristic very much depends on the polymer in question but for example HPMA copolymers display a renal threshold of ~45kDa (Seymour et al, 1987).

A large number of polymers have been explored as carriers for the delivery of therapeutic agents (Table 1.2). Many of these polymers are non-biodegradable and therefore the molecular weight which can be safely used is limited by renal clearance. However, a number of polymers have been synthesised to contain biodegradable elements in the backbone, particularly using low molecular weight PEGs (Pechar et al,

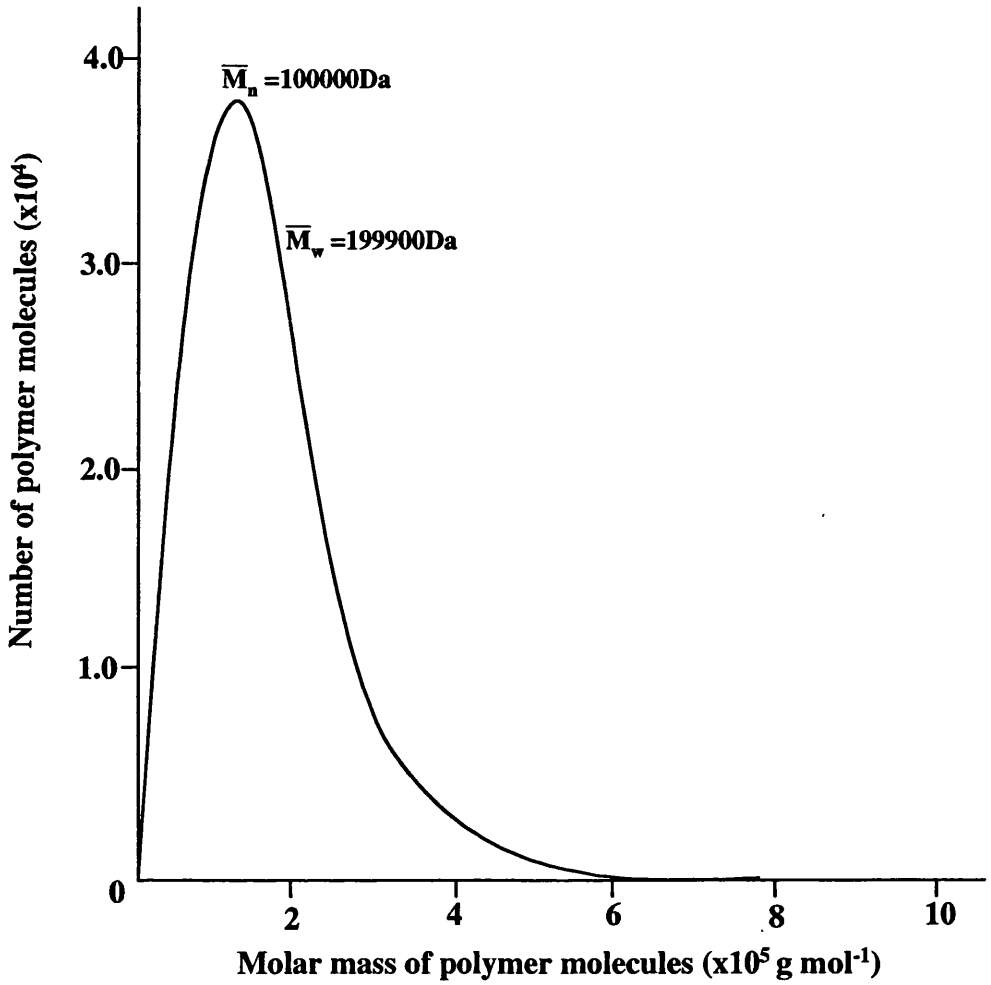



**Figure 1.3:** Graph showing $M_w$ and $M_n$ for a polymer.

**Note:** In this case the polydispersity would be 199900/100000 = 1.999.

**Table 1.2:** Polymers used to prepare polymer-anticancer conjugates

| Polymer | Source | Potentially Biodegradable | Drug carrying capacity | References |
|---|---|---|---|---|
| dextran | Natural | Yes | Good | reviewed by Mehvar, 2000 |
| chitosan | Natural | Yes | Good | reviewed by Dodane & Vilivalam, 1998 |
| poly (L-lysine) | Semi-synthetic | Yes | Good | Shen & Ryser, 1981 |
| poly (L-glutamic acid) | Semi-synthetic | Yes | Good | Hirano et al, 1979 |
| poly (amidoamines) | Synthetic | Yes | Good | Ranucci et al, 1991 |
| HPMA copolymers | Synthetic | No | Good | Duncan, 1992 |
| poly (styrene-co-maleic acid) | Synthetic | No | Poor | Matsumura & Maeda, 1986 |
| PEG | Synthetic | No | Poor | reviewed by Nucci et al, 1991 |
| Divinylether maleic acid copolymers (DIVEMA) | Synthetic | No | Good | Przybylski et al, 1978 |

2000) in order to prepare block copolymers.

The means by which the drug is conjugated to the polymer is also of vital importance. Most drugs must escape from the lysosomal compartment to exert their biological activity. This release from the conjugate is essential for activity (reviewed by Brocchini & Duncan, 1999). Many different types of polymer-drug linkage have been used to allow cleavage by enzymes or other local physiological conditions (e.g. hydrolysis at low pH) (reviewed by Soyez et al, 1996) and these will be reviewed next.

1.3.4.2 Linkers for drug conjugation

Careful consideration must be given to the linker design of an anticancer conjugate. The linker should be stable in the bloodstream and only release the drug intratumourally at a rate optimal for its mechanism of action. A number of linker chemistries have been used (Table 1.3) to benefit from the tumour environment. Linkers have been used which hydrolyse to liberate drug (e.g. esters and carbonates) (reviewed by Greenwald, 1997), for example the poly(glutamic acid)-paclitaxel conjugate which has recently entered Phase I clinical trial (Sludden et al, 2001).

The tumour extracellular environment is acidic in nature and acid-mediated cleavage can also be useful within the endocytic pathways of the cell (endosomes and lysosomes; see Section 1.4.4). A number of linkers have been developed to capitalise on this phenomenon (e.g. cis-aconityl and hydrazone linkers). An HPMA copolymer conjugate containing doxorubicin bound via a *cis*-aconityl linkage released 60% of the doxorubicin over 40h in a buffer at pH5 (Choi et al, 1999).

Linkers which release drug under the reductive conditions found in the centre of solid tumours and in the endocytic pathway of tumour cells have also been used. Ryser & Shen (1978) synthesised methotrexate-poly(L-lysine) conjugates using a disulphide linkage to allow drug release under reductive conditions.

The most widely investigated method of conjugation has been the use of peptidic linkers (Duncan et al, 1983 and Subr et al, 1992). The Gly-Phe-Leu-Gly (GFLG) spacer used to prepare HPMA copolymer-anticancer conjugates (see Section 1.3.4.2) was developed to be cleaved by cathepsin B, one of the thiol-dependent proteinase enzymes present in the lysosome (reviewed by Dean, 1977). Cathepsins (in particular cathepsins B and D (reviewed by Schwartz, 1995)) have been implicated in

**Table 1.3:** Examples of biodegradable linkers utilised in drug conjugates for anticancer therapy.

| Linker | Cleavage conditions | References |
|---|---|---|
| peptidic | cathepsin B | Subr et al, 1992 |
| carbamate | ß-lactamase | Senter et al, 1995 |
| *cis*-aconityl | acid hydrolysis | Shen & Ryser, 1981 |
| hydrazone | acid hydrolysis | Firestone et al, 1996 |
| esters | generalised hydrolysis | Li et al, 1998 |
| urea bonds | generalised hydrolysis | Ohya et al, 1991 |
| disulphide | reductive conditions | Ryser & Shen, 1978 |

tumour metastasis and spread and are also present in the extracellular compartment of many tumours. Therefore polymer-drug conjugates which contain this linker could potentially liberate free drug both within the cell and extracellularly in a tumour. The HPMA copolymer GFLG-doxorubicin conjugate (PK1) is in Phase II clinical trial (Vasey et al, 1999) and combinations of these GFLG linkers and terminal ester bonds (Caiolfa et al, 2000) or hydrazone bonds (Etrych et al, 2001) have been used to link anti-tumour agents to HPMA copolymers.

A number of polymer-drug conjugates have progressed into clinical trial and many are in pre-clinical development also. These will be reviewed before moving on to focussing on the conjugates of HPMA copolymers, which are the subject of this thesis.

1.3.4.3 Polymer-drug conjugates in clinical trial and development

Those polymer-drug conjugates in either pre-clinical development or Phase I/II clinical trial are listed in Table 1.4. Many of the first conjugates tested clinically contained doxorubicin. In a Phase I trial, a conjugate of dextran (70kDa molecular weight) and doxorubicin (Figure 1.4) was given as a single dose i.v. administered every 3 to 4 weeks. The conjugate MTD was 40mg/m$^2$ (doxorubicin-equivalent) (Danhauser-Reidl et al, 1993) indicating that this conjugate was more toxic than free doxorubicin, which is typically used at a clinical dose of 60-80mg/m$^2$ (Martindale, 1993).

In contrast HPMA copolymer-doxorubicin conjugates (PK1 and PK2; Figure 1.5) were less toxic than doxorubicin. PK1 (FCE28068) is now in Phase II testing (Vasey et al, 1999). PK1 consists of an HPMA copolymer backbone (28kDa) conjugated to doxorubicin via a GFLG linker and contains 8.5%w/w of doxorubicin. Dose limiting toxicities in the Phase I clinical trial were febrile neutropenia and mucositis (typical side-effects of doxorubicin administration) (Martindale, 1993). The MTD of PK1 given every three weeks was 320mg/m$^2$ (doxorubicin-equivalent) and this is 4-5 times higher than for free doxorubicin. Cardiotoxicity is the major toxicity of doxorubicin (see Section 1.5.1) and cumulative doses are limited to 450-550mg/m$^2$. However, PK1 was administered at cumulative doses of up to 1680mg/m$^2$ with no evidence of cardiotoxicity. PK1 also showed anti-tumour activity with two partial and two minor responses observed in the cohort of 36 patients. The patients entered the trial with histologically confirmed solid tumours that were refractory to conventional treatments.

**Table 1.4:** Polymer-drug conjugates developed for cancer chemotherapy and their stage of development.

| Polymeric backbone | Attached drug | Stage of development | Reference |
|---|---|---|---|
| poly (L-glutamic acid) | doxorubicin | T/C in mice similar to doxorubicin alone vs L1210 leukaemia | Hoes et al, 1993 |
| | paclitaxel | Phase I | Sludden et al, 2001 |
| Dextran | camptothecin analogue | 10 times more active against Walkers 256 sarcomas in rats than analogue alone | Okuno et al, 2000 |
| | doxorubicin | Phase I | Danhauser-Riedl et al, 1993 |
| | mitomycin C | Significant decrease in Walker 256 sarcoma growth in rats compared to mitomycin C alone | Nomura et al, 1998 |
| PEG | podophyllotoxin | Significant increase in activity against drug alone in P388 murine leukaemia model | Greenwald et al, 1999 |
| | doxorubicin | Delay in growth of colorectal carcinoma C26 in mice better than doxorubicin alone | Pechar et al, 2000 |

| | | | |
|---|---|---|---|
| PEG (contd.) | paclitaxel | Improved activity and reduced toxicity against P388 leukaemia model in mice | Pendri et al, 1998 |
| Chitosan | 5-fluorouracil | Improved anti-tumour activity against P388 leukaemia model in mice | Ohya et al, 1991 |
| | doxorubicin | Conjugate showed increase in activity against P388 leukaemia model in mice | Zunino et al, 1987 |
| DIVEMA | cyclophosphamide | Almost 3 times increase in survival in L1210 leukaemia model in mice | Hirano et al, 1980 |
| HPMA copolymers | doxorubicin | Phase I/II | Vasey et al, 1999 |
| | daunorubicin | Significant Walker sarcoma growth delay in rats | Cassidy et al, 1989 |
| | paclitaxel | Phase I | Meerum Terwogt et al, 2001 |
| | camptothecin | Phase I | Caiolfa et al, 2000 |
| | melphalan | Similar reduction in Walker sarcoma tumours in rats to melphalan alone | Duncan et al, 1991 |

| | | | |
|---|---|---|---|
| HPMA copolymers (contd.) | mesochlorin $e_6$ | Shows enhanced tumour accumulation in OVCAR-3 ovarian carcinoma xenograft in nu/nu mice | Shiah et al, 1999 |
| | emetine | Similar T/C value to drug alone in B16F10 melanoma model in mice | Dimitrijevic & Duncan, 1998 |
| | platinate | Phase I | Gianasi et al, 1999 |
| | 5-fluorouracil | Shows drug release from the polymer | Putnam & Kopecek, 1995 |

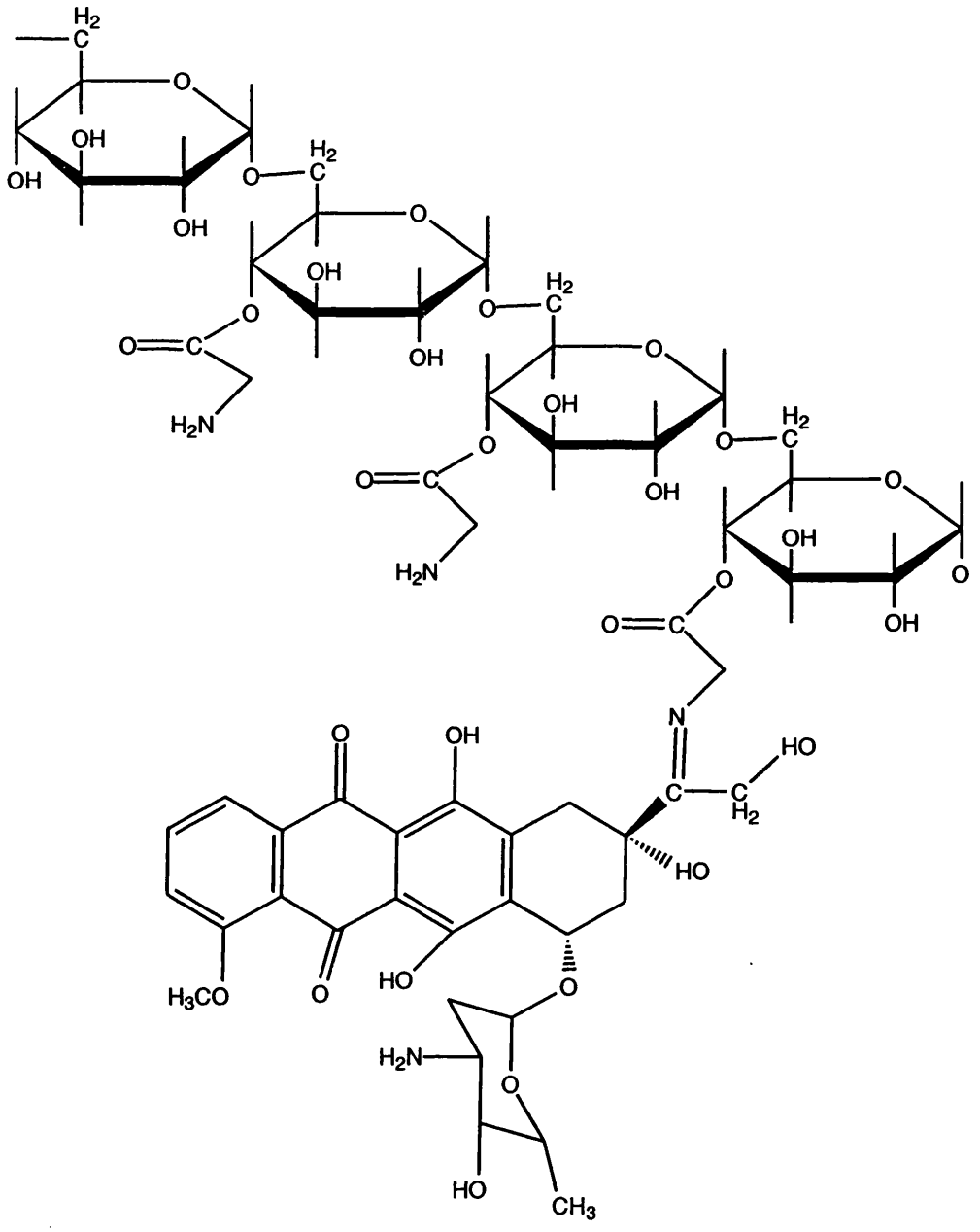
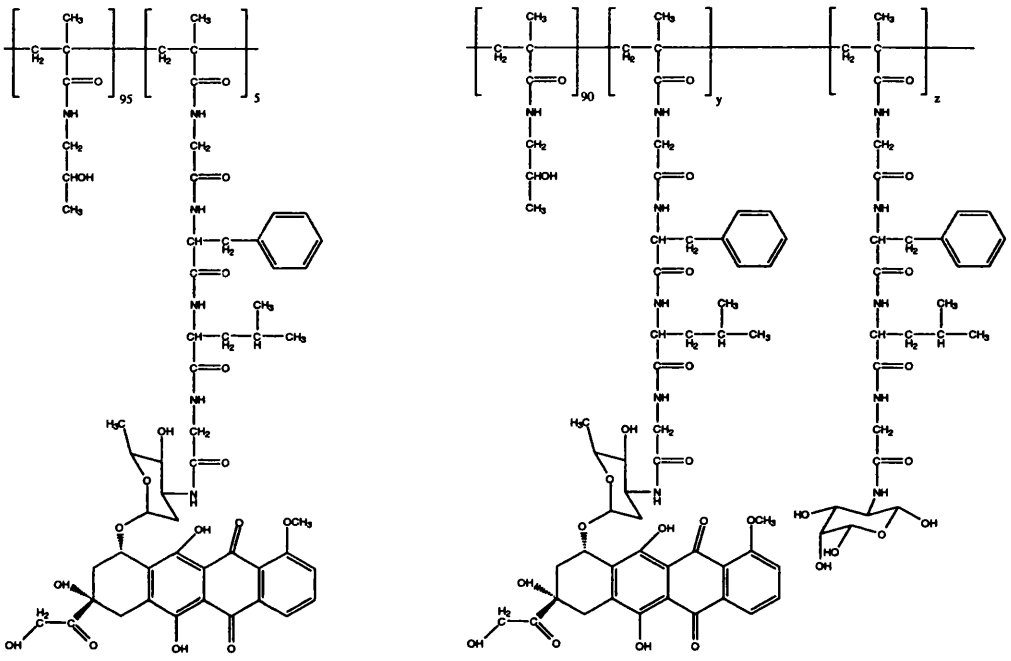


**Figure 1.4:** The structure of dextran doxorubicin.



A B

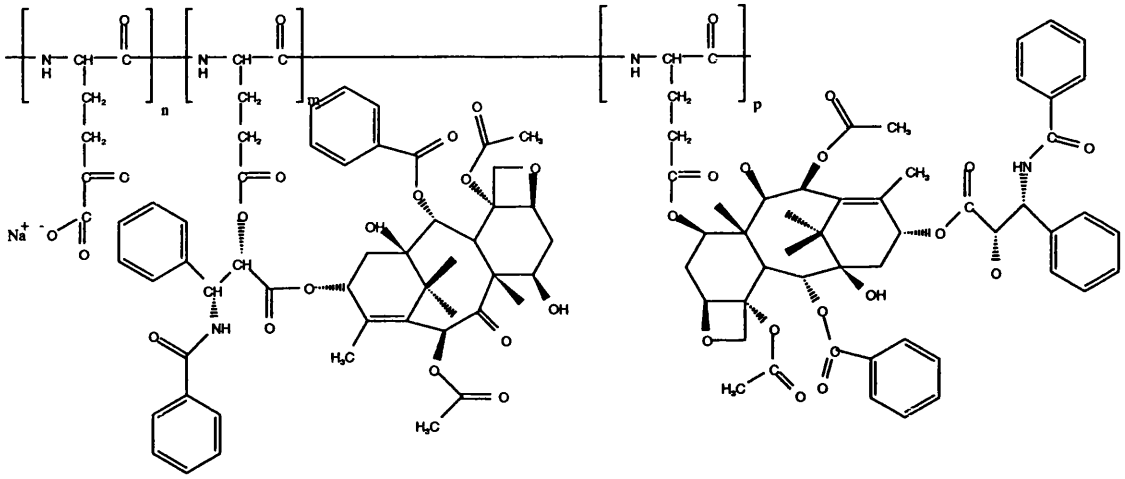
**Figure 1.5:** Structures of PK1 (A) and PK2 (B).

A similar conjugate with additional galactosamine residues (PK2 / FCE28069) incorporated to promote liver targeting via the asialoglycoprotein receptor has also undergone Phase I clinical testing in patients with primary or secondary liver cancer for whom conventional treatments had failed (Palmer et al, 2001). In this case dose limiting toxicities were febrile neutropenia, fatigue and mucositis (typical side-effects of doxorubicin administration) (Martindale, 1993). When given every three weeks by short infusion the MTD of PK2 was 160mg/m$^2$ (doxorubicin-equivalent) again with no incidence of cardiotoxicity. PK2 administration produced three partial responses and eleven patients had stable disease in the cohort of 23 patients. A gamma camera imaging analogue of PK2 confirmed significant liver targeting, when patients were imaged using single photon emission computed tomography (Julyan et al, 1999).

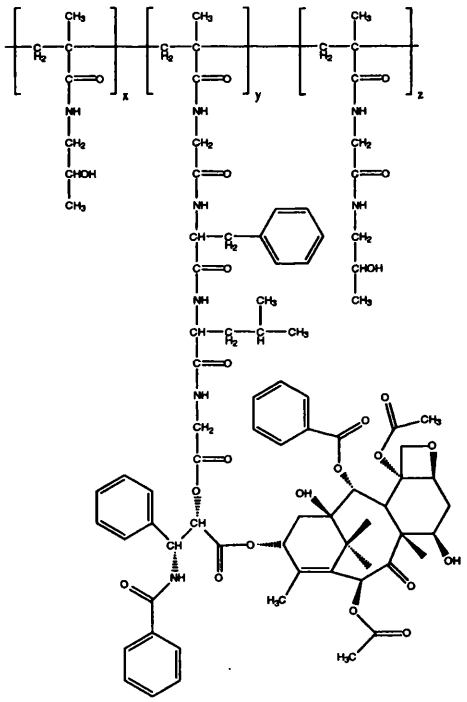
HPMA copolymer-paclitaxel (PNU166945) (Figure 1.6) (20,000 times more water soluble than paclitaxel alone) has also entered Phase I trial (Huinink et al, 1998). No dose limiting toxicities were noted up to a dose of 196mg/m$^2$ (paclitaxel-equivalent), the usual paclitaxel dose being 250mg/m$^2$ (Martindale, 1993). However this study was halted prematurely as a parallel study in rats demonstrated severe neurotoxicity of the conjugate compared to that of paclitaxel alone and during the trial also there was a case of grade 3 neurotoxicity observed (Meerum Terwogt et al, 2001). One partial response was observed in this study.

Another conjugate containing paclitaxel in early clinical testing is based on poly (L-glutamic acid) (Sludden et al, 2001; Figure 1.6). The poly (L-glutamic acid)-paclitaxel (Mw 36kDa) uses a simple ester linkage for drug conjugation and has a drug content of 21%w/w. It is considerably more water soluble than paclitaxel alone (Li et al, 1998). Doses of up to 266mg/m$^2$ (paclitaxel-equivalent) have been administered but there was evidence of serious neutropenia at this dose. Generally the conjugate was reported to be well tolerated and the trial is ongoing.

Most recently HPMA copolymer conjugates containing a camptothecin and a platinate have entered Phase I evaluation (Figure 1.7). HPMA copolymer-camptothecin (consisting of polymer conjugated to camptothecin via a terminal ester linkage showed significant *in vivo* anti-tumour activity against a HT29 human colon carcinoma xenograft model (growth inhibition coupled with long term survivors where none were seen in the group treated with camptothecin alone (Caiolfa et al, 2000)).



A



B

**Figure 1.6:** Structures of poly (L-glutamic acid)-paclitaxel (A) and HPMA copolymer paclitaxel (B).

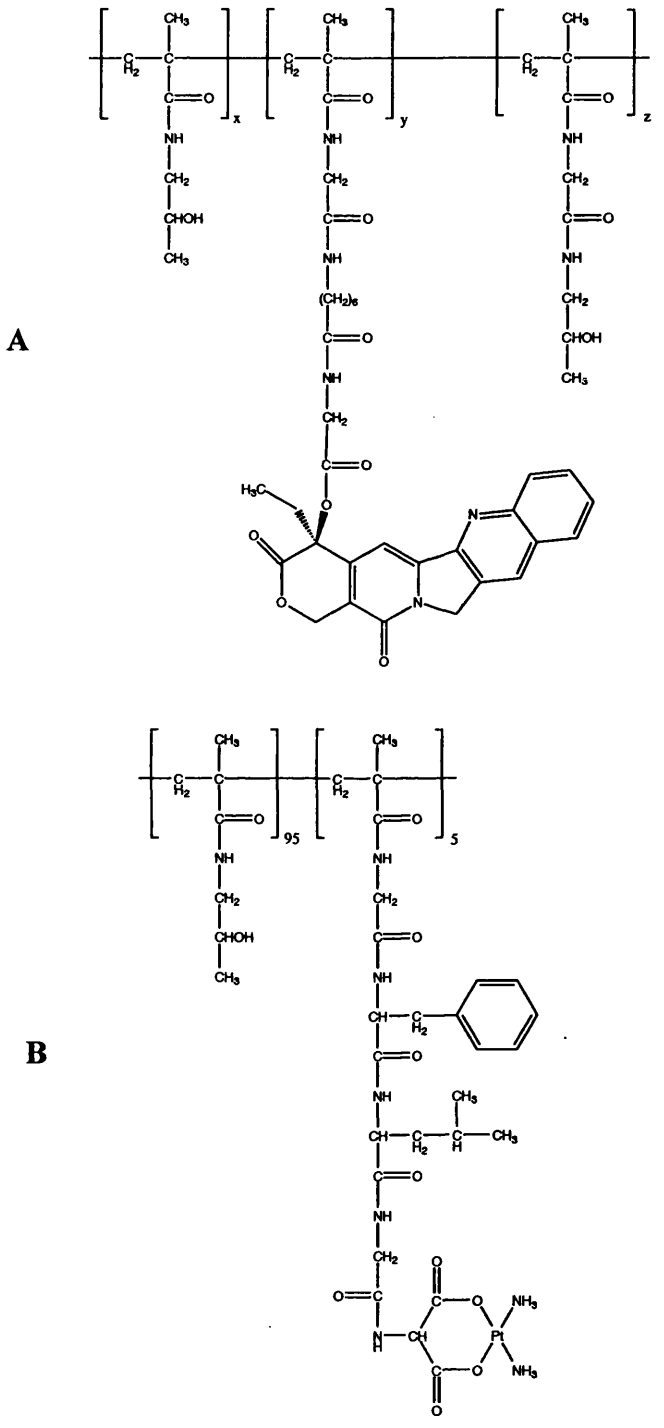


**Figure 1.7:** Structures of HPMA copolymer camptothecin (A) and HPMA copolymer platinate (B).

An HPMA copolymer-platinate conjugate also recently started Phase I clinical trial (Gianasi et al, 1999). This conjugate contains an aminomalonato chelate of *cis*-diammineplatinum (II) conjugated to HPMA copolymer GFLG and containing 7.5%w/w of platinum equivalence (Stewart et al, 2000). A number of HPMA copolymer-platinate conjugates were described which showed up to 60-fold higher levels of platinum compared to cisplatin in a B16F10 s.c. tumour in mice also demonstrating anti-tumour activity in this model. A recent press release describing the ongoing Phase I clinical trial with this conjugate stated that doses of up to 1440mg/m$^2$ (platinum equivalent) had been administered with no dose limiting toxicities observed. This is 22 times more platinum than the usual administered dose of cisplatin (Access Pharmaceuticals Inc. website, 2001).

The early promise of those polymer-drug conjugates that have entered clinical development bodes well for the future development of second generation conjugates. The clinical results seen for PK1 and PK2 agree with preclinical animal studies in terms of pharmacokinetics and reduced drug toxicity. As can be seen in Table 1.4 most of the polymer-drug conjugates which have progressed to Phase I/II clinical trial utilise the HPMA copolymer backbone. This is why this polymer was chosen here to prepare novel polymer-natural product conjugates. The advantages of HPMA copolymer conjugates and the biological rationale for their design is briefly discussed below.

## 1.4 Biological rationale for the design of HPMA copolymer-anticancer conjugates

It is important to better understand how those conjugates in development exert anti-tumour activity in order to design, synthesise and optimise new conjugates. Polymers used for drug conjugation must be safe, target drug selectively to the tumour and release active drug intratumourally usually following endocytic uptake. Here polymer-drug conjugate biodistribution and tumour targeting will be described and the mechanisms of endocytic uptake of conjugate discussed.

### *1.4.1* Biocompatibility and pharmacokinetics of HPMA copolymers

As HPMA copolymers are not biodegradable conjugates of molecular weight of > 45kDa would accumulate in the body (Seymour et al, 1987). Therefore the HPMA copolymer-drug conjugates taken into clinical trial were all of a molecular weight of approximately 30kDa (Duncan, 2000). However, as outlined earlier HPMA

copolymers do have many of the characteristics of an ideal polymeric carrier. They are very water soluble and consequently are capable of solubilising poorly-water soluble agents such as doxorubicin and paclitaxel. Neither PK1 nor PK2 caused antibody production when administered i.v., intraperitoneal (i.p.) or orally to two inbred strains of mice. Doxorubicin bone marrow toxicity was also considerably decreased by polymer conjugation as shown by the colony-forming unit-spleen assay (Rihova et al, 1989). Additionally HPMA copolymers did not activate either the classical or the alternative complement pathways at therapeutic concentrations (Simeckova et al, 1986). No evidence of HPMA copolymer-related toxicity was seen during clinical evaluation.

The Phase I trials of PK1 and PK2 have also demonstrated that these HPMA copolymer conjugates carry a sufficient loading of doxorubicin to produce anticancer activity in chemotherapy refractory patients.

### *1.4.2* Optimisation of linker design

As discussed previously a number of linkers have been used in the design of polymer-drug conjugates (Section 1.3.4.2). The peptides have been the most widely examined and were designed to be cleaved in the lysosomal compartment of the cell. All the HPMA copolymer-anticancer conjugates in clinical development utilise a peptidic linker, in most cases the GFLG linker, designed to be cleaved by cathepsin B. A series of HPMA copolymer peptide linkers terminated with *p*-nitroanilide (NAp) were tested for the stability of the linker in rat serum and plasma, monitored by release of NAp. None of the linkers tested released more than 5% in either serum or plasma and the GFLG linker released less than 2% in either test solution (Rejmanova et al, 1985). While the susceptibility of the terminal bond may depend on the structure of the conjugated drug, this is an indication that the bond will be stable in plasma and serum as release upon injection would be undesirable.

A number of di-, tri- and tetra-peptidic linkers linker were then selected to synthesise a series of HPMA copolymer-doxorubicin/daunorubicin conjugates which were examined for their liberation of drug when co-incubated with tritosomes (rat liver lysosomes) and for their activity in a mouse tumour model (i.p. L1210 leukaemia model) (Subr et al, 1992). A GLFG linker showed the most complete release (~100%/50h) but was toxic to mice in the anti-tumour experiment. The GFLG linker showed good release (~70%/50h) and showed some anti-tumour activity. There was no evidence of inappropriate release of doxorubicin in the Phase I clinical trials of PK1 and PK2 as such release would be expected to manifest itself as cardiotoxicity. Also

some anti-tumour activity was observed in these trials. For these reasons the GFLG linker was selected as a suitable linker for this study.

*1.4.3* The Enhanced Permeability and Retention Effect

HPMA copolymer-anticancer conjugates have been shown to accumulate in tumours by the EPR effect. PK1, for example, was administered to C57 black mice with s.c. B16F10 melanoma at 5mg/kg (doxorubicin-equivalent) (Seymour et al, 1994). PK1 showed a greater than 15-fold increase in doxorubicin concentration in the tumour 48h after i.v. administration compared to free doxorubicin administered at the same dose. As PK1 can be administered at higher doses than free doxorubicin, because of reduced cardiotoxicity, a higher dose of 18mg/kg was administered in this same study and resulted in a greater than 70-fold increase in doxorubicin in the tumour, compared to the 5mg/kg free doxorubicin dose. The EPR effect will now be discussed.

The EPR effect was first described by Matsumura and Maeda (1986). They observed that the polymer protein conjugate SMANCS accumulated in tumour tissue more than neocarzinostatin alone. Furthermore tumours lack a functional lymphatic system. They proposed that this effect could be similar for other macromolecules and they would extravasate into tumour tissue passively and be retained there because of this poor lymphatic drainage. The tumour vascular system has many structural and functional abnormalities (Konerding et al, 1999). Structural abnormalities include the abnormal vessel wall, abnormal architecture and abnormal vascular density due to unregulated angiogenesis (reviewed by Carmeliet & Jain, 2000). The underlying functional abnormalities result in this increased vascular permeability, altered flow characteristics and altered morphological characteristics. It has been known for some time that neovascularisation in tumours results in distorted blood vessels (reviewed by Rubin and Casarett, 1966) and that these blood vessels are sometimes incomplete with large intracellular gaps. These gaps allow macromolecules to pass into the tumour interstitium whereas in normal vasculature this would not be possible due to the tight continuous intracellular nature of the normal blood vessel (reviewed by Jain & Baxter, 1988) (Figure 1.8). The EPR effect is mediated by imbalances in the level of many endogenous factors such as vascular endothelial growth factor (VEGF), bradykinin, tumour necrosis factor (TNF) as well as a host of cytokine and related factors such as the hypoxic character of tumours (Maeda et al, 1992). In non-tumour tissue if

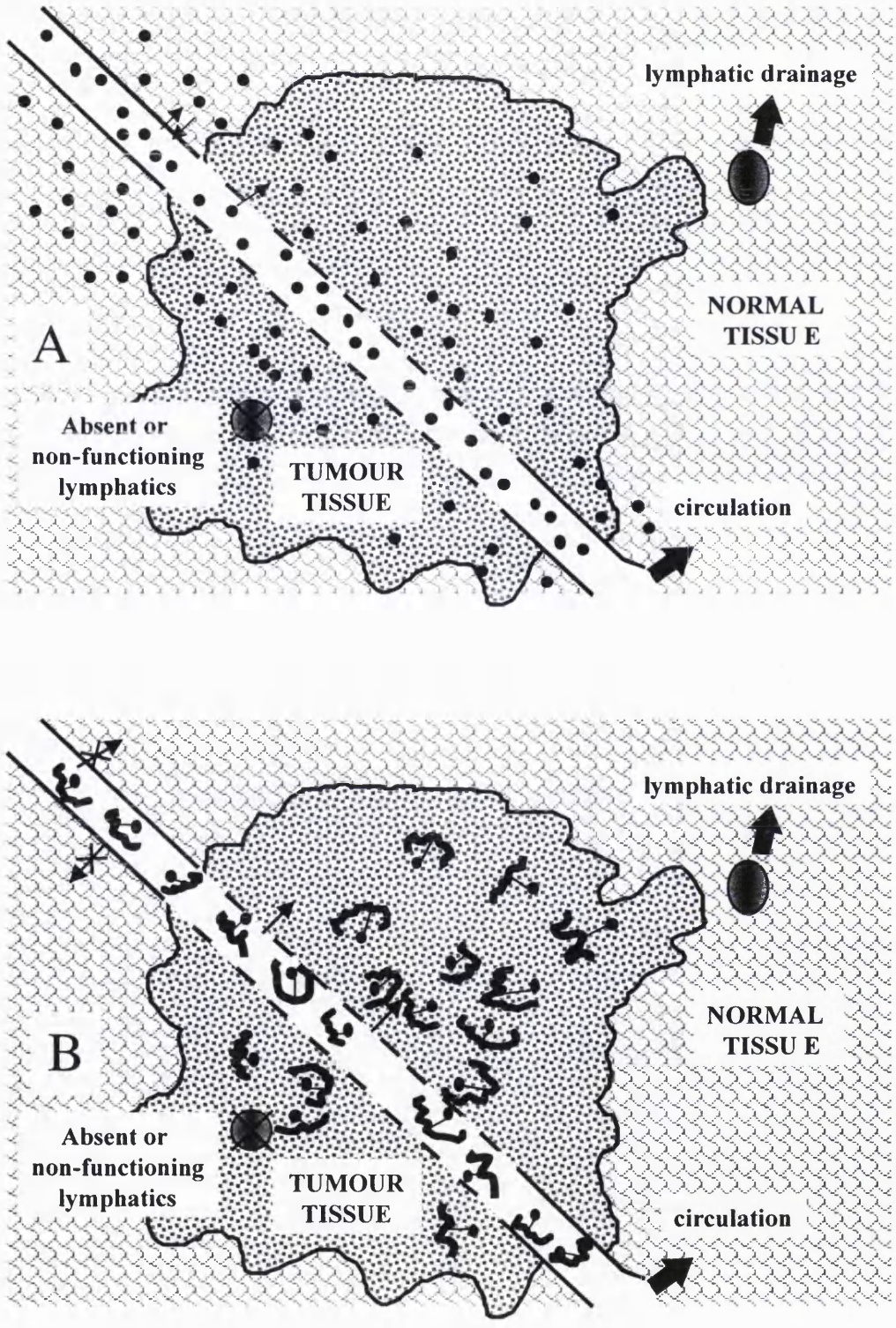



**Figure 1.8:** The EPR effect showing non-selective extravasation of small molecules (A) contrasted with tumour accumulation of a polymer-drug conjugate (B).

macromolecules extravasate they are brought back to the general circulation by drainage mediated by the lymphatic system. Lymphatic drainage from tumours is severely restricted or absent and therefore once a macromolecule has been captured in a tumour by the EPR effect it remains within the tumour interstitium (Maeda & Matsumura, 1989).

Macromolecular drug delivery systems can take advantage of these unique physiological circumstances found in tumours to passively target them to the tumour by the EPR effect. What is interesting in the case of the HPMA copolymers is that it has been shown that their EPR-mediated targeting is governed solely by their plasma concentration across a wide range of molecular weights (15–800kDa) (Seymour et al, 1995). The HPMA copolymer drug conjugates in clinical development have been shown to be of a physical diameter of around 6-8nm (Duncan et al, 1996). They are thus smaller in dimension than the liposomal systems. Many liposomal systems, however, show poor penetration into the tumour mass, with even those liposomes in clinical use, of a diameter of approximately 90nm, showing marked restriction in penetration beyond the close proximity of the blood vessels (Uster et al, 1998). The size dependence of tumour extravasation is being examined (Yuan et al, 1995) as liposomes of up to 400nm have been shown to extravasate in a human colon adenocarcinoma LS174T model but those of 600nm don't. It is thought that these localised deposits of liposomes containing drug then act as a slow drug release system (Yuan et al, 1994). The extent to which polymer-drug conjugates extravasate has never been characterised in terms of intratumoural distribution and this will be examined using PK1 as a model polymer-drug conjugate in Chapter 6 of this thesis.

After the HPMA copolymer-anticancer conjugate has been passively retained in the tumour the cytotoxic agent needs to be liberated in order to be active. For those polymer-drug conjugates containing the linkers which are susceptible to cathepsin B, described in Section 1.3.4.2, the conjugate needs to be taken up by the cell and trafficked to the lysosome in order to be exposed to cathepsin B and release active drug. Therefore the process of endocytosis will now be described as it applies to polymer-drug conjugates.

*1.4.4* Intracellular pharmacokinetics

Low molecular weight anticancer agents show a widespread distribution in the body following systemic administration (Figure 1.8). This is also true at the cellular level and as most anticancer agents tend to be lipophilic they can traverse the cell membrane non-specifically. Macromolecular drug delivery systems, however, are incapable of traversing the cell membrane due to their size and are therefore reliant on the process of endocytosis to enter the cell (De Duve et al, 1974). Endocytosis can be broadly described as two mechanisms of uptake by cells of extracellular fluid. Phagocytosis can be defined as the process by which specialised cells such as macrophages engulf foreign matter for processing, usually by the RES, and is related to large particles (>500nm diameter). Pinocytosis can be defined as the process of engulfing small portions of the surrounding fluid and processing it through endosomes and into lysosomes and is usually initiated by clathrin-coated pits and is common to almost all cell types (reviewed by Mellman, 1996).

Figure 1.9 shows the differences in subcellular localisation of low molecular weight and a polymer-drug conjugate. Polymer-drug conjugates have to go through a number of stages before the cytotoxic agent can get to its site of action, if that site is intracellular which in the case of many anticancer agents is the nucleus. They firstly need to be taken up by the cell and therefore need to be of a physical diameter which is not too large (Mellman, 1996). The attached anticancer agent needs to be stable in the acidic milieu of the intracellular compartment (Ohkuma & Poole, 1978) and also needs to be not broken down by the enzymes it encounters, of which the lysosome is thought to contain up to 70 types (Dean, 1977). In the case of the HPMA copolymer-anticancer conjugates the drug needs to be cleaved from the polymeric backbone in order to escape from the lysosome to reach its intended target by crossing the lysosomal membrane. In most cases it will need to be a free drug, without any residual linker in order to do so and to exert its effect within the cell (reviewed by Lloyd, 2000).

The reliance of polymer-drug conjugates on endocytosis also provides a means of circumventing the common resistance mechanism of p-glycoprotein-mediated expulsion of anticancer agents back into the extracellular milieu which is a common mechanism of resistance to conventional anticancer agents (reviewed by Krishna & Mayer, 2000). PK1, for example, has shown cytotoxic activity towards human ovarian carcinoma cells which are resistant to doxorubicin both *in vitro* (Minko et al, 1998) and

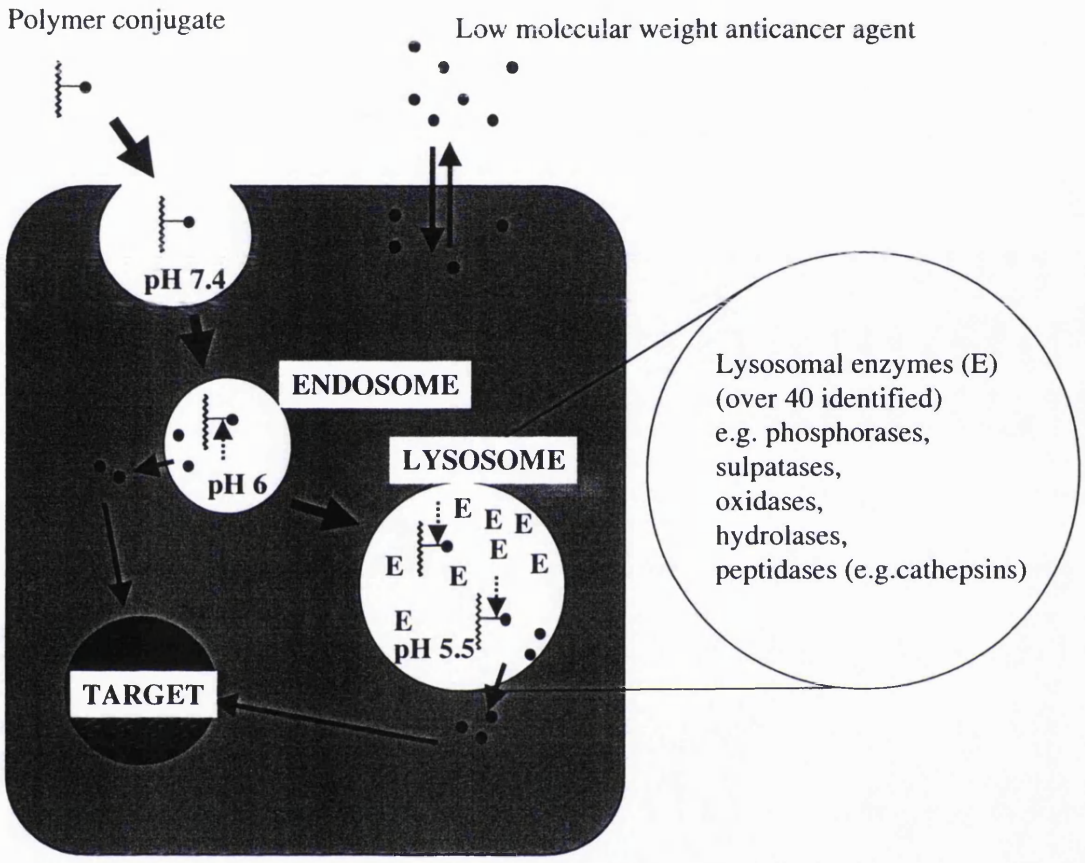



**Figure 1.9:** Schematic of intracellular release of pendant drugs with respect to a low molecular weight anticancer drug.

*in vivo* in animal models (Kopecek et al, 2000). These results demonstrate that polymer-drug conjugates offer the possibility of overcoming such resistance.

The choice of polymeric backbone and linker are thus governed by biological as well as physicochemical characteristics and both need to be taken into account in order to design novel conjugates. The aspects of polymer-drug conjugates described so far demonstrate the suitability of HPMA copolymer GFLG conjugates as the system of choice for novel conjugates. The next section describes those natural products which show anticancer activity and the reason why an analogue of ellipticine, namely APE, was chosen for conjugation to HPMA copolymer GFLG in this thesis.

## 1.5 Natural products in Cancer

Natural products have always been at the fore in the development of anticancer agents. In the U.S.A (1994) more than 50% of anticancer drugs on the market were either of natural origin, derived from natural sources or were semi-synthetic natural products (reviewed by Cragg et al, 1997) (Figure 1.10). Natural products are also a widespread source of novel structures and, in the case of anticancer agents, of novel modes of action which might circumvent resistance, penetration and targeting issues described earlier (reviewed by Newman et al, 2000).

### *1.5.1* Natural products in clinical use

A number of natural products are used in cancer treatment and there are a number of classes of these compounds which have been widely studied. Some of the major interesting classes will now be described.

The anthracyclines are very commonly used chemotherapeutic agents as they exhibit a broad spectrum of activity against many solid tumours as well as lymphomas and leukaemias and include doxorubicin and daunorubicin (reviewed by De Vita et al, 1993). They are thought to have a number of potential mechanisms of anti-tumour activity but are usually described as being topoisomerase II inhibitors and to form damaging free radicals (reviewed by Gewirtz, 1999). This radical formation has been implicated in the major toxicity of the anthracyclines, notably cardiotoxicity. This cardiotoxicity results from cumulative dosing of doxorubicin which is usually given at a dose of 60-80mg/m$^2$ alone or 30-40mg/m$^2$ when given with agents having overlapping

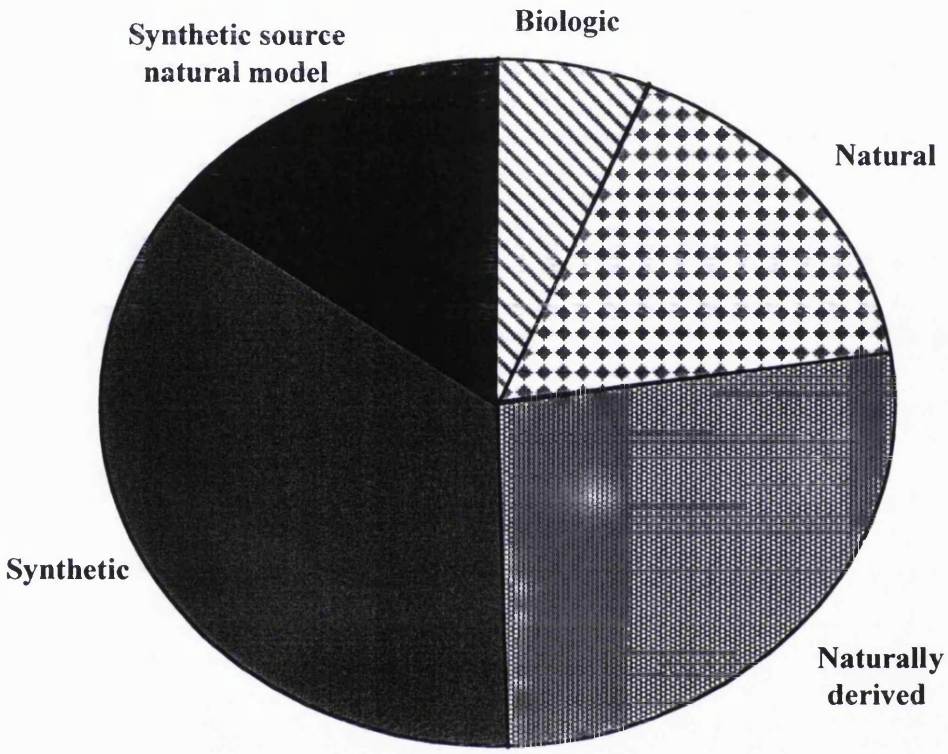



**Figure 1.10:** Anticancer drugs approved in the U.S.A. in 1994 and their origin (adapted from Cragg et al, 1997).

toxicity by fast running intravenous infusion every three weeks. The maximum recommended cumulative dosage is 450-550mg/m$^2$ as above this significant ventricular failure and dysrrhythmias commonly occur. Other common adverse events include myelosuppression, mucositis and local extravasation at the site of administration resulting in severe necrosis (Martindale, 1993). PK1 (Figure 1.5; Section 1.3.4.3) demonstrated the feasibility of conjugating anthracyclines to HPMA copolymers via a GFLG linker and this was the first polymer-drug conjugate to enter clinical development.

The vinca alkaloids are another class of clinically used natural products and vinblastine and vincristine are the best known and are in routine clinical use. Analogues of these compounds vinorelbine and vindesine are also approved for use in many European countries (reviewed by Newman et al, 2000). These agents act by binding to tubulin and inhibiting microtubule assembly thereby blocking the formation of the mitotic spindle resulting in cell division being suspended in mitosis and causing cell death by apoptosis (Lobert et al, 1996). So far there are no reports of polymer-drug conjugates of the vinca alkaloids, although vincristine encapsulated in a glucuronide-modified liposome showed significant tumour growth delay in a mouse model (Tokudome et al, 1996).

Paclitaxel is another agent that acts on the tubulin polymerisation process. Paclitaxel, however, acts in a different way to the other so-called spindle poisons in that it stabilises microtubules and prevents depolymerisation back to tubulin whereas the others, such as the vinca alkaloids, bind to tubulin and prevent the formation of microtubules (reviewed by Wall & Wani, 1995). As described in Section 1.3.4.3 both HPMA copolymer and poly (L-glutamic acid)-paclitaxel conjugates (Figure 1.6) have entered Phase I clinical trial (Meerum Terwogt et al, 2001 and Sludden et al, 2001 respectively). Both conjugates show a remarkable increase in paclitaxel solubility. However the Phase I trial involving the HPMA copolymer-paclitaxel conjugate was halted prematurely because of excessive neurotoxicity; worse than that of paclitaxel alone. This could have been caused by the unstable nature of the ester linkage leading to premature release of paclitaxel at unexpected sites producing toxicity. Paclitaxel is a potent and clinically useful drug and therefore further investigation of polymer-paclitaxel conjugates is justified and indeed the clinical trial of the poly (L-glutamic acid) conjugate is ongoing.

Another class of natural products of importance are the camptothecins. Camptothecin was initially discovered in 1958 but only became of interest when its specificity for the topoisomerase I enzyme was discovered and it underwent clinical trials in the 1960's. Some anti-tumour activity was reported but severe side-effects halted further trials (reviewed by Potmesil, 1994). Further analogues were developed and the analogues topotecan and irinotecan are now in routine clinical use with a number of other camptothecin analogues in clinical development (Newman et al, 2000). As described earlier a HPMA copolymer-camptothecin construct (Figure 1.7) has recently entered Phase I/II clinical trial (Caiolfa et al, 2000 and Zamai et al, 2001).

A number of biologic agents are also in clinical use in cancer therapy such as the colony stimulating factors used in adjuvant therapy (e.g. Filgrastim), interleukin-2 (a lymphokine which stimulates proliferation of lymphocytes) and interferons which can have an anti-proliferative effect (Martindale, 1993).

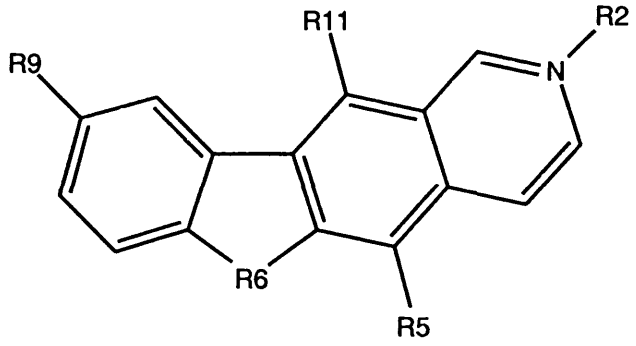
Natural products are a useful source of novel therapeutics and new natural products are constantly entering clinical trial. An example of a novel natural product which recently underwent Phase I clinical trial is dolastatin 10. This is a peptidic drug and binds to tubulin at a different site to the other antimitotic agents previously outlined causing inhibition of tubular polymerisation and leading to apoptosis. The initial Phase I trial was promising and Phase II trials are currently in progress (Madden et al, 2000). Combretastatin A4 is isolated from the South African tree *Combretum caffrum* and has been shown to have a high affinity for tubulin causing destabilisation of the tubulin polymers in the cytoskeleton (Woods et al, 1995). Where this compound has been shown to have activity is as an antivascular agent. The disodium phosphate derivative of this drug has been shown to induce vascular shutdown very rapidly in experimental tumours and has recently entered Phase I clinical trial in the U.K. (Tozer et al, 1999). The disodium phosphate modification of combretastatin is a means of ensuring activation in the tumour where alkaline phosphatase activity is increased and the drug will be liberated locally. This is a novel mechanism of action for an anticancer drug and it has been shown not to induce resistance in a number of *in vitro* and *in vivo* models (Kerbel, 1997). Therapies with unique mechanisms of action, such as this, are very important for the development of future anticancer therapies and in particular for combination chemotherapy.

*1.5.2* Ellipticines

The majority of the polymer-drug conjugates which have been developed so far have utilised anticancer agents which are already on the market (e.g. doxorubicin, paclitaxel, mitomycin C and platinates, Table 1.4). However the potential candidates for polymer conjugation should not be limited to these widely used anticancer agents. The approach, now validated with these known compounds, could be applied to other potent cytotoxic agents which initially failed early pre-clinical and clinical testing. The candidate compound chosen for study in this thesis is from one such family of compounds, the ellipticines, which were identified as interesting compounds some time ago. Many of the ellipticine analogues failed early preclinical or clinical testing due to poor formulatory properties and severe toxicities.

Ellipticines (5, 11-dimethyl-6H-pyrido(4,3-b)carbazoles; Figure 1.11) are anti-tumour alkaloids isolated from the *Ochrosia elliptica* tree (Goodwin et al, 1959). The potential of these agents as anti-tumour agents was first identified in 1967 (Dalton et al, 1967). The mechanism of action of ellipticine and its subsequent analogues is multifactorial. However most analogues act as DNA intercalators and topoisomerase II inhibitors, supported by their cross-resistance to other topoisomerase II inhibitors (Charcosett et al, 1988). It is interesting to note that a derivative of ellipticine, termed T-215, was the first anticancer drug shown to induce apoptosis of tumour cells in connection with mutant p53 protein. Another derivative, 9-hydroxyellipticine, also promotes apoptosis by inhibiting phosphorylation of mutant p53 protein (reviewed by Ohashi & Oki, 1996).

Celliptium (elliptinium acetate; Figure 1.11) in a Phase II study showed activity in advanced breast cancer and is marketed as second line treatment for advanced breast cancer (Rouesse et al, 1993). Datelliptium (2-diethylaminoethyl-9-hydroxyellipticine, Figure 1.11) is also marketed and showed activity in a Phase I study against breast cancer (Khayat et al, 1992). Celliptium was shown in previous studies to cause serious immune-mediated haemolytic reactions (Rouesse et al, 1985) but this was abrogated in the reported Phase I study by changing the dosing schedule. These compounds have only entered clinical use relatively recently given how long ellipticines have been studied. A large series of derivatives of ellipticine have been synthesised in this time and a number of these have entered pre-clinical and clinical development (Table 1.5).




| Compound | R2 | R5 | R6 | R9 | R11 |
|---|---|---|---|---|---|
| Ellipticine | H | H | NH | H | $CH_3$ |
| Celliptium | $CH_3$ | $CH_3$ | H | OH | $CH_3$ |
| Datelliptium | $H_2N(C_2H_5)_2$ | $CH_3$ | H | OH | $CH_3$ |
| APE | H | $CH_3$ | $NCH_2CH_2CH_2NH_2$ | H | $CH_3$ |

**Figure 1.11:** General structure of the ellipticines showing some of the common sites of derivatisation and the structures of ellipticine, Celliptium, Datelliptium and APE.

**Table 1.5:** Ellipticine derivatives which have undergone pre-clinical and/or clinical trial but are not yet marketed.

| Compound | Status | Results | Reference |
|---|---|---|---|
| Retelliptine | Phase I | Dose limiting cardiac toxicity | Cappelaere et al, 1993 |
| Pazellipticine | Pre-clinical | Needs bioactivation for activity | Balosso et al, 1991<br>Vilarem et al, 1986 |
| Elliprabin | Phase I | Terminated due to hepatic toxicity | Ohashi & Oki, 1996 |
| RPI-6 | Pre-clinical | 1000-fold as active as doxorubicin against some lung cancer cell lines | Ruckdeschel & Archer, 1989 |
| S-16020 | Phase I | Still ongoing. Selected as a particularly active derivative | Le Mee et al, 1998 |

The major toxicity of the ellipticines has been cardiotoxicity. They can cause an initial tachycardia and hypotensive shock on administration, followed by long term bradycardia linked with an increased force of contractility. Haemolysis is also a common adverse event (reviewed by Ohashi & Oki, 1996), and there has been evidence of hepatotoxicity (Donato et al, 1992) and renal toxicity (Raguenez-Viotte et al, 1988). An immune response, observed as anti-elliptinium immunoglobulin G-type antibodies, has also been seen after administration of Celliptium (Alberici et al, 1986). A number of these toxicities are exacerbated by the problem of poor solubility of the ellipticines in aqueous solutions.

Ellipticine and its analogues therefore seemed to be good candidates for polymer-drug conjugation. It was hoped that conjugation to HPMA copolymers would improve water solubility, improve tumour targeting by the EPR effect and moreover reduce non-specific toxicity as seen in the case of PK1 (Vasey et al, 1999). The ellipticine derivative APE was selected because of its potent *in vitro* cytotoxicity (Inhibitory concentration for 50% cell killing ($IC_{50}$) of $3.2x10^{-7}M$ in the National Cancer Institute (NCI) screen (NCI website, 2001). APE was also selected because of its chemical suitability for synthesising an HPMA copolymer-APE conjugate using an HPMA copolymer precursor and a simple aminolysis reaction which will be described in Chapter 3.

**1.6 Aims of the study**

When this project started it had become clear that PK1 was already showing clinical potential and that the approach of HPMA copolymer conjugation might be applicable to other (novel and more interesting) anticancer agents. The broad aims of this thesis were therefore to investigate further the mechanism of the EPR effect by understanding better the extravasation characteristics of PK1. This was studied using an *in vivo* visualisation system of a tumour (rat dorsal window chamber model; Chapter 6).

The most important aim was to try to design an HPMA copolymer-natural product conjugate that could progress into early clinical trial. The ellipticine derivative APE was chosen as the model for this work. First it was necessary to synthesise and characterise HPMA copolymer-APE conjugates using two peptidyl spacers (GG, thought to be lysosomally non-degradable and GFLG chosen as a degradable linker).

To enable study of the effect of drug loading compounds were synthesised containing 1-6.1%w/w APE (Chapter 3). First the *in vitro* physicochemical and biocompatibility (cytotoxicity and haematocompatibility) were studied and the effect of drug loading on the rate of drug liberation by lysosomal enzymes was studied (Chapter 4). Then selected conjugates were evaluated *in vivo* using murine and xenograft tumour models to examine the therapeutic index (Chapter 5).

# Chapter Two

## *Materials and General Methods*

## 2.1 Materials

### *2.1.1* Chemicals

All chemicals were obtained from Sigma-Aldrich Ltd. (Dorset, U.K.) or Fisher Chemicals Ltd. (Leicestershire, U.K.) and were of analytical or reagent grade unless otherwise stated. HPMA copolymer-GG-*p*-nitrophenol (ONp) containing approximately 5mol% peptidyl side-chains and HPMA copolymer-GFLG-ONp of approximately 1, 5 and 10mol% peptidyl side-chains were supplied by Polymer Laboratories (Church Stretton, U.K.). APE was kindly donated by the NCI (Maryland, U.S.A.). Daunomycin hydrochloride was kindly donated by Rhone Poulenc (France). PK1 and doxorubicin hydrochloride were kindly donated by Pharmacia (Milan, Italy). All solvents designated dry were dispensed from stock containers under argon gas. All solvents used in High Pressure Liquid Chromatography (HPLC) and Gel Permeation Chromatography (GPC) applications were filtered through a 0.22μm filter and subsequently sonicated for 5-10min before use.

### *2.1.2* Cell culture

B16F10 murine melanoma cells were kindly donated by Prof. Ian Hart (St. Thomas' Hospital, London, U.K.). CORL23 human non small cell lung carcinoma cells were purchased from ECACC (European Collection of Cell Cultures, Centre for Applied Microbiology & Research, Salisbury, Wiltshire, U.K.). Tissue culture grade dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and trypan blue were purchased from Sigma (Dorset, U.K.). Foetal Calf Serum (FCS), trypsin with ethylenediaminetetraacetic acid (EDTA) and RPMI 1640 media were purchased from Gibco BRL Life Technologies (Paisley, U.K.). Phosphate buffered saline (PBS) was obtained from Oxoid Unipath Ltd. (Hampshire, U.K.) and 0.9% NaCl (normal saline) was prepared using sodium chloride from BDH (Dorset, U.K.) and distilled water and both these solutions were autoclave sterilised before use in cell culture work.

## 2.2 Equipment

### *2.2.1* Analytical equipment

The Ultra Violet (UV)-visible spectrophotometers used were a UV-1601 purchased from Shimadzu Scientific Instruments (Milton Keynes, U.K.) and a Cary 1G UV-visible spectrophotometer purchased from Varian Ltd. (Surrey, U.K.). 717plus Autosampler and C18 reverse phase μBondapak columns were from Waters Ltd. (Hertfordshire, U.K.). Mobile phase was delivered using a PU-980 Intelligent HPLC pump from Jasco Inc. (Essex, U.K.). Fluoromonitor III fluorescence detector fitted with fluorescence lamp (excitation 480nm, emission 560nm) was from LDC/Milton Roy (U.K.) for doxorubicin determination. For APE determination a Dynamax-60Å Cn column from Varian Analytical Instruments Ltd. (California, U.S.A.) was used. A Spectroflow 783 UV detector from Kratos Analytical (Milton Keynes, U.K.) and an LC 1255 fluorodetector from GBC Scientific Equipment (Illinois, U.S.A.) were used as detectors. All chromatography data was recorded and analysed using PowerChrom hardware and software (version 2.0.7).

### *2.2.2* Cell culture equipment

The 96 well plate spectrophotometers used were from Titertek Multiskan Plus (EFLAB, Finland) and a Tecan Sunrise instrument (Tecan Ltd., Berkshire, U.K.). All cell culture work was carried out in a Class II biological safety cabinet.

### *2.2.3 In vivo* equipment and suppliers

L.I.P. Services and Equipment Ltd. (West Yorkshire, U.K.) supplied heparin/lithium blood tubes. All nu/nu mice were housed in an isolator unit (Isolator type 12162 (M20)) which was purchased from Harlan Ltd. (Oxon, U.K.). Other procedures involving nu/nu mice were carried out on a laminar flow bench. Needles and syringes were from Becton Dickinson (Oxford, U.K.). All animals were purchased from Bantin and Kingman Ltd. (Hull, U.K.) or Harlan UK Ltd. (Bicester, Oxon, U.K.). Gases were from BOC Ltd. (Surrey, U.K.) and isoflurane was obtained from Abbott Laboratories Ltd. (Kent, U.K.).

### *2.2.4* Miscellaneous and general equipment

The freeze-drying system for lyophilising samples was a Flexi-Dry freeze drier from FTS Systems (New York, U.S.A.) attached to a double stage high vacuum pump

from Javac Ltd. (Melbourne, Australia). Dialysis membranes Spectra/Por® (Cellulose ester) were from Spectrum Laboratories Inc. (California, U.S.A.) with a molecular weight cut-off of 3,000Da. All low speed centrifuge work was carried out using a Varifuge 3.0RS from Heraeus Instruments (Osterode, Germany). All other centrifuge work was carried out using an Allegra 6KR, Avanti J-25 centrifuge or an Optima LE-80K ultracentrifuge from Beckman Coulter (California, U.S.A.).

## 2.3 General methods

### *2.3.1 In vitro* methods

Cell line usage was conducted based on the principles outlined in the UKCCCR Guidelines for the use of Cell Lines in Cancer Research (2000). All cell manipulations, except centrifugation steps, were carried out in a Class II biological safety cabinet. Any equipment brought into the cabinet was sterile and sprayed with a solution of ethanol (70%) and water. All solutions in contact with cells were heated to 37°C in a water bath.

#### 2.3.1.1 Growing cells from frozen vials

Both cell types (B16F10 and CORL23) were grown from frozen by heating vials to 37°C in a water bath and as soon as they had defrosted they were added to 9ml of RPMI-1640 media containing 10% FCS in a sterile container. This cell suspension was then centrifuged at 1,000g for 5min at room temperature and the supernatant liquid discarded to remove any residual DMSO. 10ml of the same media was then added and the cells were disaggregated using a syringe and needle added to a 75cm$^2$ tissue culture flask and this was then put into a 37°C incubator with a humidified atmosphere and 5% $CO_2$.

#### 2.3.1.2 Day to day maintenance of cells

Both cell types were maintained in the same media as outlined before in a 75cm$^2$ vented tissue culture flask. Cells were examined every day for confluence and at 75-95% confluence were split 1:10 as follows. The old media was removed and the cells washed with 10ml of sterile PBS twice. Trypsin (0.25%)/EDTA (1mM) solution (1ml) was then added and the cells returned to the incubator for 3-5min until the cells had detached from the surface of the flask and disaggregated. Fresh media (9ml) was

added to this cell suspension. Subsequently 9ml of this cell suspension was removed and 9ml of fresh media was added to complete a 1:10 split. The 9ml of cell suspension removed was used to produce further flasks of cells by adding 1ml to a fresh flask containing 9ml of fresh media.

2.3.1.3 Cell counting

To count cells 50µl of a cell suspension was taken and added to 50µl of a solution of 0.2% trypan blue. This suspension was left for 2-3min, then pipetted onto a haemocytometer with a cover slide. A cell count was made by counting the cells, which were not blue, in 4 of the grids on the haemocytometer and averaging the result. This value was then doubled to allow for the dilution by trypan blue and the number of cells calculated by multiplying this value by $1x10^4$ to get a final value of viable cells per ml of a suspension. Any cells which did not exclude trypan blue and were therefore blue were not counted and assumed to be non viable as intact cells should not take up this dye.

2.3.1.4 Freezing cells

Cells were periodically frozen as follows. Cells were trypsinised as detailed previously made up to 10ml with media and centrifuged at 1,000g for 5min at room temperature. Cells were also counted. After centrifugation the cells were made up to a concentration of $1x10^6$cells/ml in a freezing media (90%FCS with 10%DMSO) and immediately frozen to -80°C and either continually stored at this temperature or transferred to a liquid nitrogen cell bank until required.

2.3.1.5 Growth curve

Cells were trypsinised as described in Section 2.3.1.2 and made up to a volume of 10ml with fresh media. A 50µl sample was taken and the cell number counted as described in Section 2.3.1.3. The rest of the suspension was centrifuged at 1,000g for 5min at room temperature. The supernatant liquid was decanted and the cells made up to a concentration of $1x10^5$cells/ml using media. Aliquots of this cell suspension (100µl) were added to each well of a 96 well plate which was then returned to the incubator overnight to allow the cells to attach to the surface and form a monolayer. The next day, and on each subsequent day, 20µl of MTT (5mg/ml in sterile PBS and filter sterilised through a 0.22µm filter) was added to each well in one row of wells and the cells were incubated for 5h at 37°C. Viable cells transform MTT, in the

mitochondria, to an insoluble formazan derivative in a time and cell number dependent manner (Sgouras and Duncan, 1990 and Figure 2.1). After 5h all cell debris and media were removed and the formazan crystals dissolved in 100µl of spectrophotometric grade DMSO at 37°C for 30min. The absorbance (550nm) for each well was measured using a micro-titre plate spectrophotometer reader. The DMSO, containing formazan crystals, was then removed from each well. Fresh media or PBS was then added to each of these wells to prevent the DMSO from evaporating and causing toxicity to the remaining cells.

2.3.1.6 Cell cytotoxicity using the MTT assay

To determine drug cytotoxicity cells were plated into 96 well micro-titer plates in the same manner as for a growth curve. After 24h, when the cells had adhered, media was removed and the drug to be tested, dissolved in media, was added. The cells were returned to the incubator for a further 67h. After this time MTT solution (20µl; 5mg/ml in PBS sterile filtered) was added to each well and the plates re-incubated for a further 5h. The formazan crystals were dissolved in DMSO as before and read at 550nm using a micro-titer plate reader. Cells grown in media alone were used as a reference for 100% viability. The absorbance of the other wells was calculated against the average absorbance of the reference wells and calculated as a percentage of this value.

2.3.1.7 Cell preparation for s.c. injection into mice

Injection (s.c.) of B16F10 and CORL23 cells as cell suspensions was used to establish tumours in C57Bl (B16F10) or nude mice (CORL23). Prior to injection cells were prepared by trypsinising and counting as described above. The cells were then made up to the appropriate volume ($1x10^6$cells/ml for B16F10 or $1x10^7$cells/ml for CORL23) in sterile saline solution.

*2.3.2* Evaluation of anti-tumour activity

All *in vivo* work involving tumours was carried out according to the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia (1998). In all of these experiments the animals were allowed food and water *ad libitum*.

The B16F10 cell line grows s.c. upon injection of $1x10^5$cells into the nape of the neck of C57BL black male mice. After injection the tumours were allowed to establish

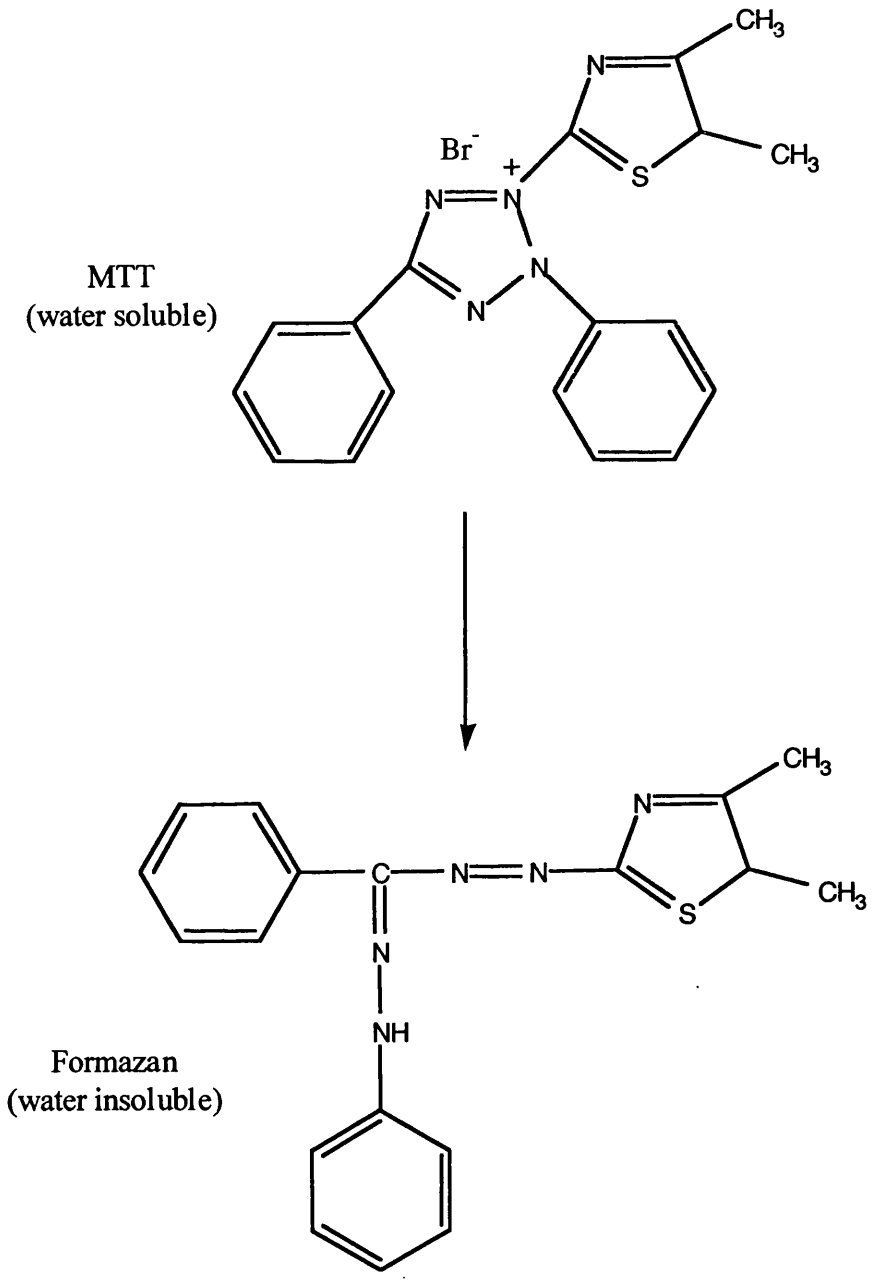



**Figure 2.1:** Metabolism of MTT by viable cells.

to a palpable size of between 25-50mm$^2$ as determined by the measurement of the two longest diameters. The animals were then randomised by assigning each mouse to a separate group, when the tumours reached the correct size, and then sequentially adding each mouse to the next group until each group contained at least 5 mice. The test drugs or saline, as a control, were injected i.p. into the animals as a single dose on the day the tumour reached the correct size. On each subsequent day each animal was weighed and the tumour measured. If the animal had lost more than 20% of body weight or was obviously sick (lethargic, unkempt and unresponsive) or the tumour had reached 289mm$^2$ the animal was humanely killed by $CO_2$ asphyxiation. All organs and tumours were retained, weighed and examined for gross morphological changes.

The CORL23 cell line also grows s.c. upon injection of 1x10$^6$cells into the nape of the neck of nu/nu male mice. Nude mice were housed in an isolator at all times. For manipulations, such as tumour measurement or injections, the animals were removed in a box with a filter on top to a laminar flow bench. All items added to the isolator, such as food and water, were purchased sterilised by irradiation and the exterior sprayed with a virucidal solution. After injection of the cell suspension tumours were allowed to establish to a palpable size of between 25-50mm$^2$ as determined by the measurement of the two longest diameters. The animals were then randomised in the same manner as for the C57Bl mice above. The test drugs or saline, as a control, were injected i.p. into the animals as a single dose on the day the tumour reached the correct size. On each subsequent day each animal was weighed and the tumour measured. If the animal had lost more than 20% of body weight or was obviously sick (lethargic, unkempt and unresponsive) or the tumour had reached 289mm$^2$ the animal was humanely killed by $CO_2$ asphyxiation. All organs and tumours were retained, weighed and examined for gross morphological changes.

### *2.3.3* Analytical methods

#### 2.3.3.1 HPLC determination of APE

A HPLC method was used to determine the free APE content of conjugates as well as APE released in tritosome release studies. All solutions were kept on ice throughout the course of the experiment to minimise potential degradation and original test solutions were in polypropylene tubes. A solution of 0.1mg/ml was used to make various standard concentrations of APE (0-1μg/ml) in distilled water. Conjugates for

analysis were dissolved in distilled water at a known concentration. Samples from tritosome release studies were used as the 100µl taken at each timepoint as described in Section 2.3.4. Two methods were used in these studies as it was found that the first method did not counteract the buffering capacity of proteins in tritosome release studies. The first method was used for analysis of conjugates while the second method was used for all biological samples.

Extraction and HPLC analysis of non-biological samples:

To the test solutions 100µl of doxorubicin (0.6mg/ml) was added as the internal standard and they were then made up to 1ml using distilled water. To this a small amount (usually 5µl) of 0.1M NaOH was added to make the solution pH10. Then 5ml of 4:1 chloroform : isopropanol was added to each tube. The organic layer was subsequently removed into fresh polypropylene tubes. This organic solution was evaporated by blowing nitrogen gas at the surface of the liquid. The residue was dissolved in 200µl of methanol : distilled water (60 : 40v/v) which was also the mobile phase used. 20µl of this solution was injected either manually or using an autosampler onto a C18 µBondapak reverse phase column with mobile phase delivered by a HPLC pump delivering mobile phase at 1ml/min. Detection was by means of a UV absorbance detector set to 296nm. Data were recorded and analysed using the PowerChrom system.

Extraction and HPLC analysis of biological samples:

To the test solutions 200µl of quinaldine red (0.1mg/ml in methanol) as the internal standard was added and they were made up to 1ml using distilled water. To this 100µl of ammonium formate 1M at pH8.5 was added. This ammonium formate was used to counteract any protein-mediated buffering. To these solutions 5ml of 4 : 1 dichloromethane : isopropanol was added. The tubes were then centrifuged at 2,000g for 10min at 4°C to aid separation. The organic layer was removed into glass test tubes which had been silanized by treatment with a solution of 93:7 toluene : dichloromethylsilane and then washed with ethanol and allowed to dry (as per the method of Fraier et al, 1995). This organic solution was evaporated by blowing nitrogen gas at the surface of the liquid. The residue was dissolved in 70% acetonitrile in distilled water which contained 0.035% trifluoroacetic acid, which was also the mobile phase. This solution (20µl) was injected using an autosampler onto a Dynamax-60Å Cn column with mobile phase delivered by a HPLC pump delivering the mobile

phase at 0.6ml/min. Detection was achieved using a UV absorbance detector set to 296nm and a fluorescence detector in series using wavelengths of 286nm excitation and 480nm emission. Data were recorded and analysed using the PowerChrom system. Figure 2.2 gives example chromatograms and Figure 2.3 shows standard curves from such HPLC determinations of APE

2.3.3.2 HPLC determination of free and total doxorubicin

This method was adopted from Wedge (1991) and was used to examine the free and total doxorubicin content of organs as well as the doxorubicin liberated from PK1 in tritosome release studies. Samples from these studies were stored in polypropylene tubes in the dark at 4°C before analysis to minimise degradation. On the day of extraction they were removed but stored on ice for the entire process of extraction to minimise any potential degradation. In the free doxorubicin method it is free doxorubicin and daunomycin which are being detected. In the total doxorubicin method it is aglycone doxorubicin and daunomycin, because the acid hydrolysis liberates these species, being detected and also the retention time is increased.

Determination of free doxorubicin content:

To each tube 25µl of a 10µg/ml solution of daunomycin (10µg/ml dissolved in distilled water) was added. The tubes were then vortexed to mix the contents and made up to 1ml with distilled water and revortexed. The solution was buffered by adding 150µl of a 1M solution of ammonium formate adjusted to pH8.5 with ammonium hydroxide and the solution revortexed. To each tube 5ml of a 4 : 1 solution of chloroform : propan-2-ol was added and the solutions mixed. These were then centrifuged at 1,000g for 30min at 4°C and the organic layer removed and evaporated under nitrogen gas to dryness. The residue was redissolved in 100µl of methanol and injected, using an autosampler, onto a C18 reverse phase µBondapak column with a mobile phase delivered at 1ml/min of propan-2-ol 29% in distilled water adjusted to pH3.2 using *o*-phosphoric acid. Detection was by a fluorescence detector (excitation 480nm; emission 560nm) and data were analysed using PowerChrom system.

Determination of total doxorubicin content:

To each tube 25µl of a 10µg/ml solution of daunomycin (10µg/ml dissolved in distilled water) was added. The tubes were then vortexed to mix the contents and made up to 1ml with distilled water and revortexed. To each solution 1ml of a 2M solution of

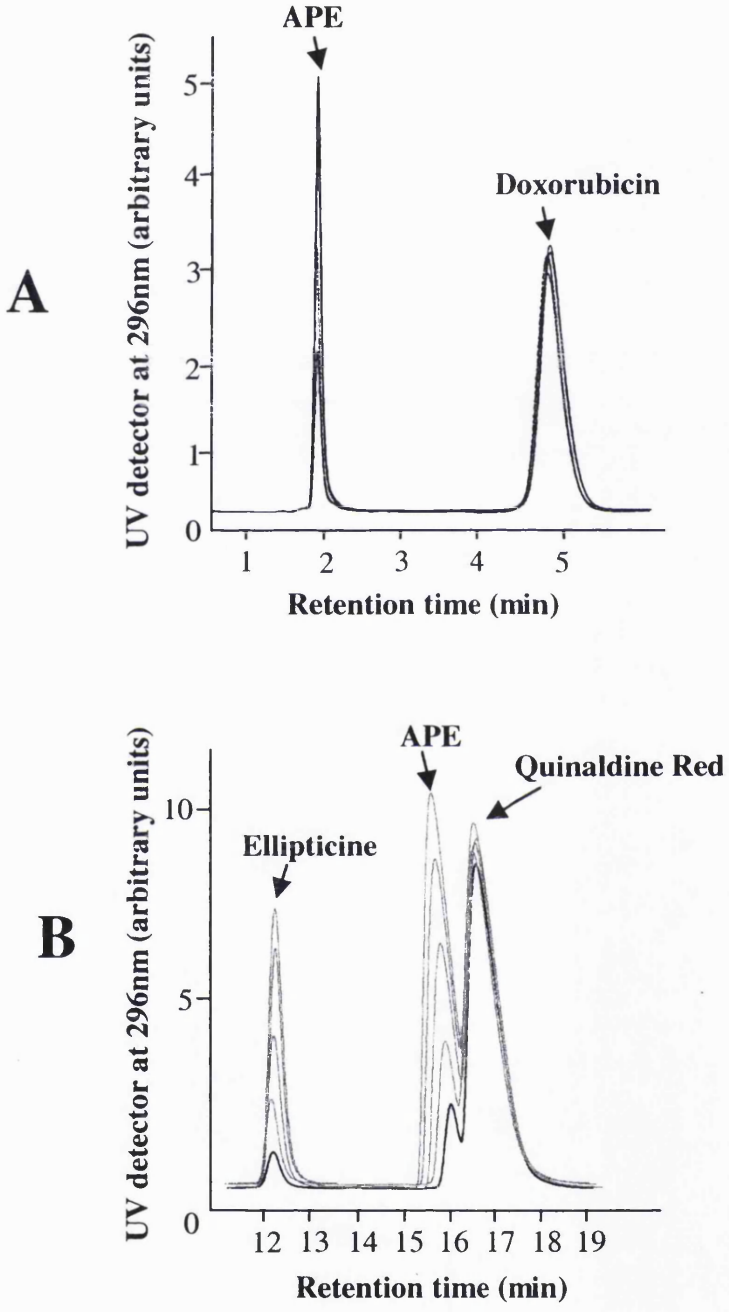



**Figure 2.2:** Typical HPLC chromatograms obtained during APE quantitation using method 1(A) and method 2 (B).

**Note:** In both cases the internal standard (Doxorubicin (60µg/ml) (A) and Quinaldine Red (20µg/ml) (B)) are at a set concentration with increasing amounts of APE (0-4 µg/ml for A and 0-1µg/ml for B) and/or ellipticine (0-1µg/ml).

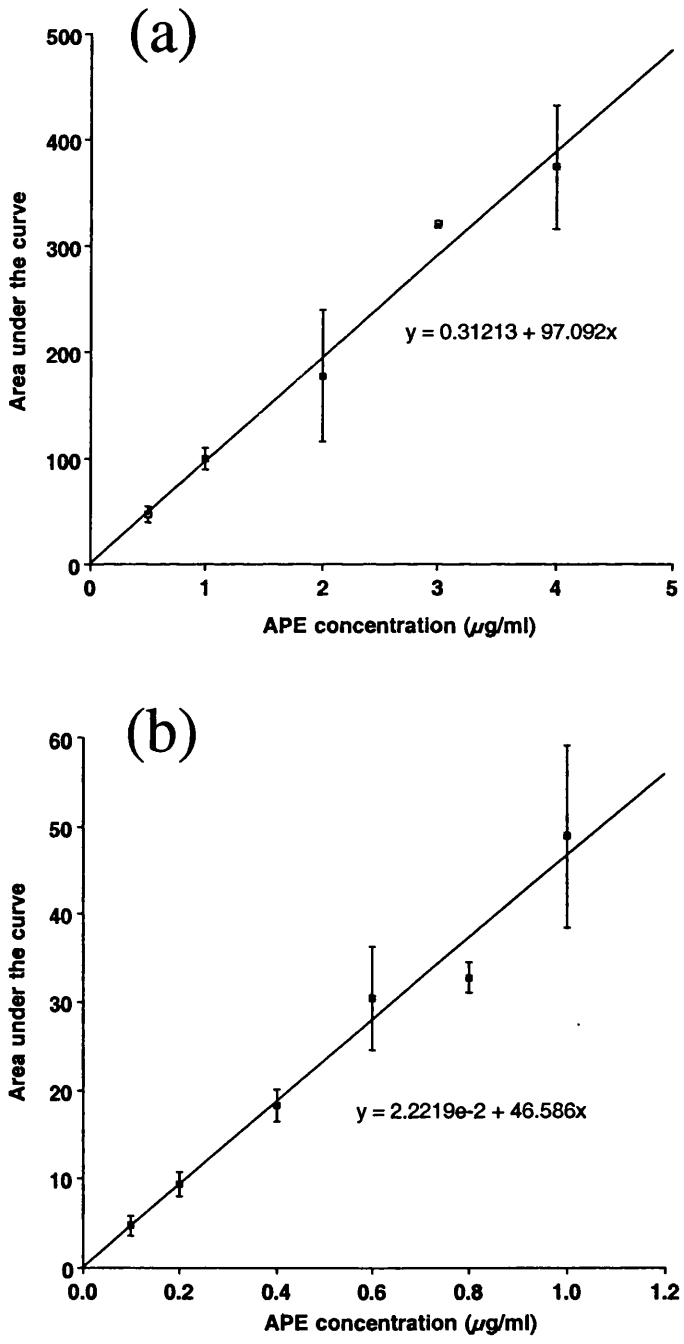



**Figure 2.3:** A standard curve of determination of APE in the HPLC system.

**Note:** Panel a is from a study using Method 1 whilst b is from a study using Method 2. Data represent mean ±SD (n=3).

hydrochloric acid was added. These tubes were then heated to 80°C for 30min. These solutions were buffered by adding 1.5ml of a 1M solution of ammonium formate adjusted to pH8.5 and the solution revortexed. 1ml of 2M sodium hydroxide was then added to each tube to neutralise the acid. To each tube was added 5ml of a 4 : 1 solution of chloroform : propan-2-ol and the solutions mixed. These were then centrifuged at 1,000g for 30min at 4°C and the organic layer removed and evaporated under nitrogen gas to dryness. The residue was redissolved in 100µl of methanol and 20µl injected, using an autosampler, onto a C18 reverse phase µBondapak column with a mobile phase delivered at 1ml/min of propan-2-ol 29% in distilled water adjusted to pH3.2 using *o*-phosphoric acid. Detection was by means of a fluorescence detector as outlined in Section 2.2 above. Data were collected and analysed using the PowerChrom system. Sample chromatograms and standard curves from the HPLC determinations using each of these methods are shown in Figures 2.4 and 2.5.

### *2.3.4* General biochemical methods

#### 2.3.4.1 Isolation and standardisation of rat liver lysosomes (tritosomes)

As described in Section 1.3.4 the tetrapeptide linker GFLG is designed to be cleaved by cathepsin B, a lysosomal enzyme. To study release of drug from this linker it was necessary to isolate rat liver lysosomal enzymes, so-called 'tritosomes'.

Tritosomes were prepared using the detergent Triton WR1339 (Tyloxapol) to allow separation of lysosomes from other intracellular organelles of similar density (Trouet, 1974). Male Wistar rats (5 weighing 250-300g) were injected i.p. with tyloxapol (20% v/v in 0.15M NaCl) at a dose of either 1ml/100g of body weight of tyloxapol (Sigma grade) (batches RKT1-RKT5) or 0.5ml/100g of tyloxapol (SigmaUltra grade) (batches RKT6-8). The animals were then left for 4 days to allow the detergent to accumulate in the liver lysosomes. On day 3 rats were starved overnight before being humanely killed by $CO_2$ asphyxiation and the liver removed. For the remainder of the experiment all tissue was kept on ice.

The livers were crudely cut into small pieces using scissors and the pulp pushed through a sieve of mesh size of approximately $1mm^2$. The pulp was then suspended in 0.25M sucrose (5ml/g) and homogenised using a smooth-walled glass tube using a Citenco homogeniser attached to a teflon pestle with 0.019cm clearance from the glass

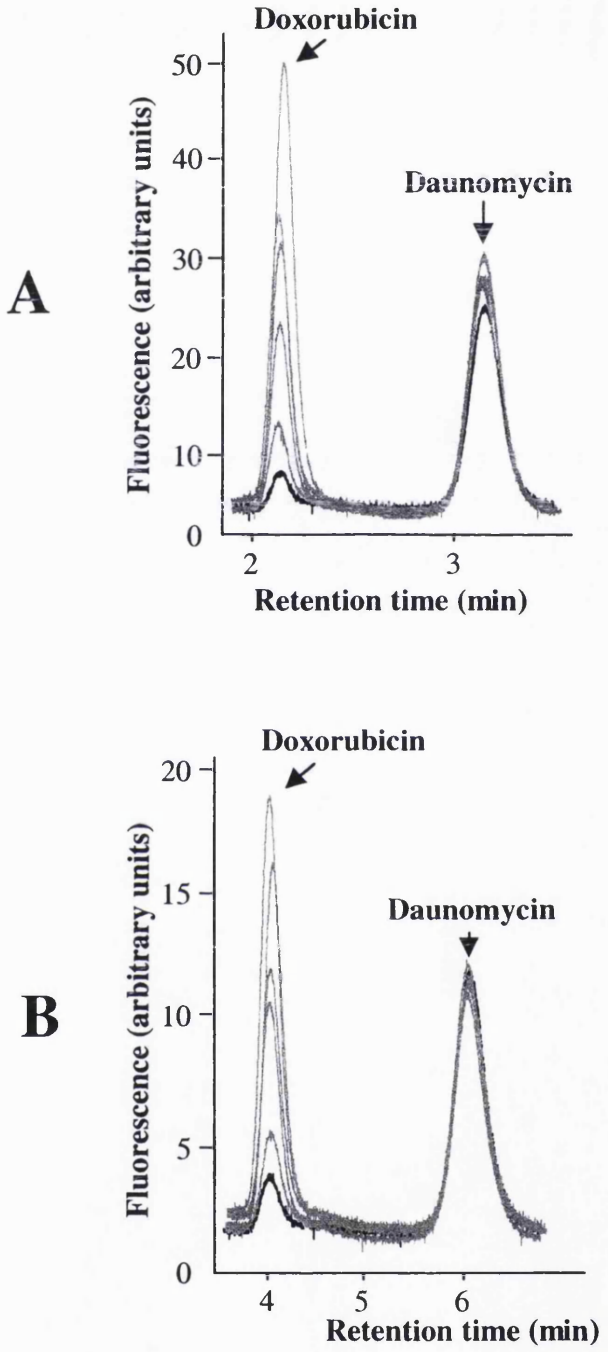



**Figure 2.4:** Typical HPLC chromatograms obtained during quantitation of doxorubicin using free (A) and total (B) methods.

**Note:** To determine free doxorubicin, daunomycin is used as an internal standard whilst for the determination of total doxorubicin (liberated from PK1) the daunomycin and doxorubicin are converted to the aglycone form. In both cases the internal standard (daunomycin) is used at a concentration of 250ng/ml. Doxorubicin concentrations shown are 20 to 500ng/ml.

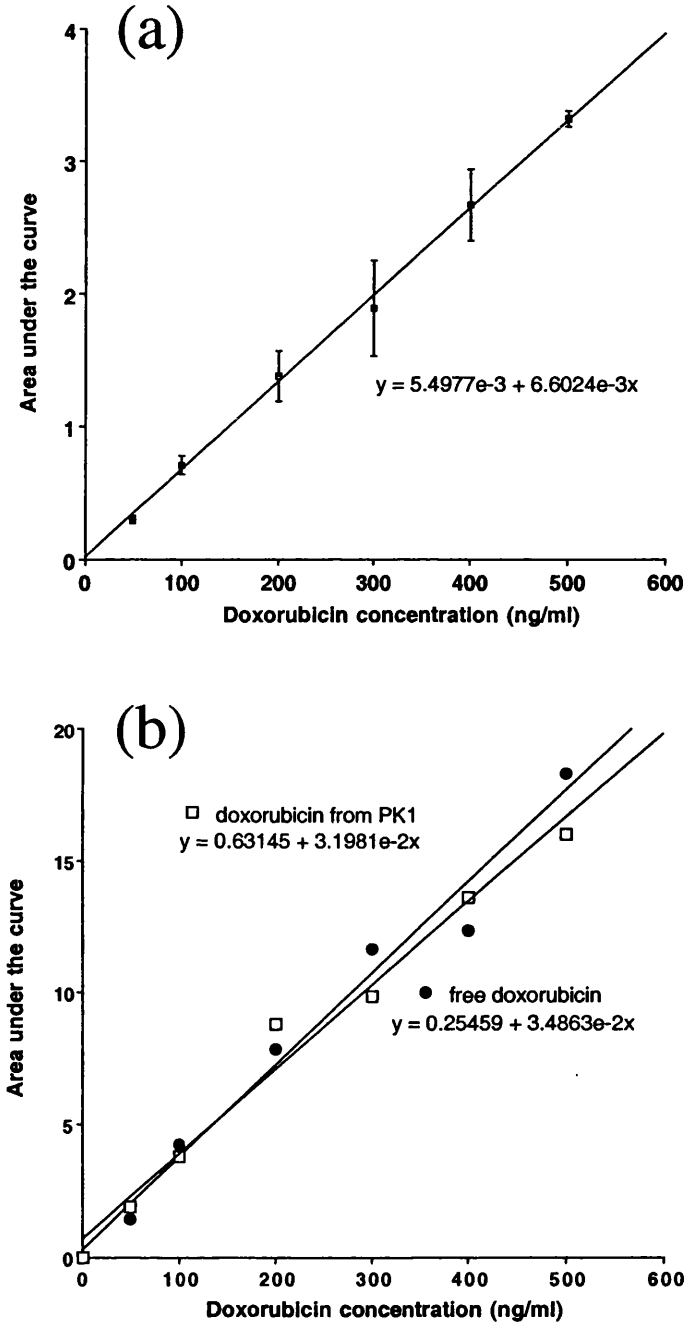



**Figure 2.5:** Typical standard curve used to determine doxorubicin using HPLC analysis.

**Note:** Panel a is a determination of free doxorubicin and b is a determination of total doxorubicin. Panel b shows standard curves determined using the hydrolysis method for free doxorubicin and PK1 at the same doxorubicin equivalent concentration. Data represent mean ±SD (n=3).

at approximately 300rpm. This procedure was carried out in order to separate and break open the liver cells. This suspension was then centrifuged at 700g for 10min at 4°C in 50ml centrifuge tubes (capped polycarbonate tubes). The supernatants were saved and the pellets were resuspended in 0.25M sucrose (3ml/g) and centrifuged in the same tubes at 500g for 10min at 4°C and again the supernatants were saved. These centrifugation steps removed debris such as intact cells and nuclei. The pooled supernatants were centrifuged at 14,000rpm for 10min at 4°C in fresh tubes and the supernatants discarded. The pellets were resuspended in 0.25M sucrose (5ml/g) and centrifuged again at 14,000rpm for 10min at 4°C and the supernatant discarded. This step was carried out to pellet the lysosomes and mitochondria which have similar density. The pellets were resuspended in a small volume (1-2ml) of 45% sucrose and the volume made up to 56ml with 45% sucrose (if 6 tubes were to be used) or 28ml (if 3 tubes were to be used). This suspension (9ml) was added to Ultra-Clear™ centrifuge tubes. On top of this layers of 34.5% sucrose (8ml) and 14.3% sucrose (4ml) were added sequentially to create a discontinuous density gradient. These tubes were then centrifuged at 21,000rpm for 2h 4min (the extra 4 minutes allowing the centrifuge to get to the correct speed) at 4°C and the tritosomes removed, using a pasteur pipette, from the interface of the 34.5% and 14.3% layers. The isolated lysosomes were separated into 1ml aliquots and then frozen until required. The isolated lysosomes (tritosomes) concentrate at this interface because of the increased buoyant density caused by tyloxapol. Tritosomes were characterised for their protein content and enzyme activity. As tritosomes are intact organelles it is necessary to rupture the membrane before use therefore triton X-100 (0.2%v/v) was used in the buffer of the following assays.

2.3.4.2 Protein determination using the bicinchoninic acid assay

Bicinchoninic acid is a chromogenic reagent for copper (I) and proteins are known to reduce copper (II) to copper (I) in a concentration-dependent manner (Smith et al, 1985). This method was used to determine the protein content of the tritosomes. First, BCA reagent was prepared by mixing 20ml of reagent A (bicinchoninic acid) with 400µl of reagent B (copper(II) sulphate pentahydrate 4%) and 40.8µl of triton X-100 and bovine serum albumin (BSA) (1mg/ml in distilled water) was used as a standard. Various concentrations of BSA (0-10µg/ml) or tritosomes (2, 5 and 10µl) were added to the wells of a 96 well flat-bottom plate. BCA reagent (200µl) was added to each well and the plate was incubated at 37°C for 20min. Using a UV plate reader

the absorbance was taken at 550nm for each well and the protein content of the tritosomes was determined from the BSA standard curve (Figure 2.6).

2.3.4.3 Standardisation of lysosomal enzyme activity in tritosomes

In order to standardise the thiol-protease (cathepsin B) activity in each batch of tritosomes a low molecular weight substrate (n-benzoyl-Phe-Val-Arg-NAp) was used as follows. Firstly, the bound NAp content of the substrate in the stock solution was determined by UV determination in a solution of citrate phosphate buffer containing 0.2%v/v triton X-100 as follows. Buffer was made up by adding a solution of 0.2M citric acid in distilled water to a 0.2M solution of disodium hydrogen orthophosphate 12 hydrate in distilled water until the pH was 5.5. The appropriate volume of triton X-100 was added and the pH rechecked. The bound content of NAp in the standard solution was determined using the molar extinction coefficient value of bound NAp (extinction coefficient $(\varepsilon)_{315nm}$ = 12,700). Then reaction solutions were made as follows. Each cuvette contained 100µl of a 10mM solution of EDTA in buffer to chelate divalent ions which are known to inhibit thiol proteases and 100µl of a 50mM solution of reduced glutathione (GSH) in buffer to activate the lysosomal thiol-dependent proteinases (Duncan, 1986). Substrate solution (30µl) and either 30µl of DMSO (for the blank) or 30µl of tritosomes (for the active cell) and the remainder as buffer were added with tritosomes added last to commence the reaction. These solutions were mixed and allowed to react for 5min at 37°C. The absorbance at 410nm was determined every 30sec over 15min at 37°C (Figure 2.7). The rate of NAp release was used to determine enzyme activity. Using this value, in combination with the protein content, thiol protease activity of tritosomes was expressed as NAp release in nmol/minute/mg protein. This allowed standardisation of the amount of tritosome activity used in the drug release studies.

2.3.4.4 Studying drug release from HPMA copolymer GFLG conjugates using tritosomes

As outlined previously the purpose of isolating tritosomes was to study *in vitro* the rate of release of drug from conjugates containing the GFLG linker. Citrate phosphate buffer (0.2M; pH5.5) containing 0.2%v/v triton X-100 was prepared as described before. EDTA (10mM) and GSH (50mM) solutions were also freshly prepared.

For the release studies 100µl of these solutions of EDTA and GSH were added

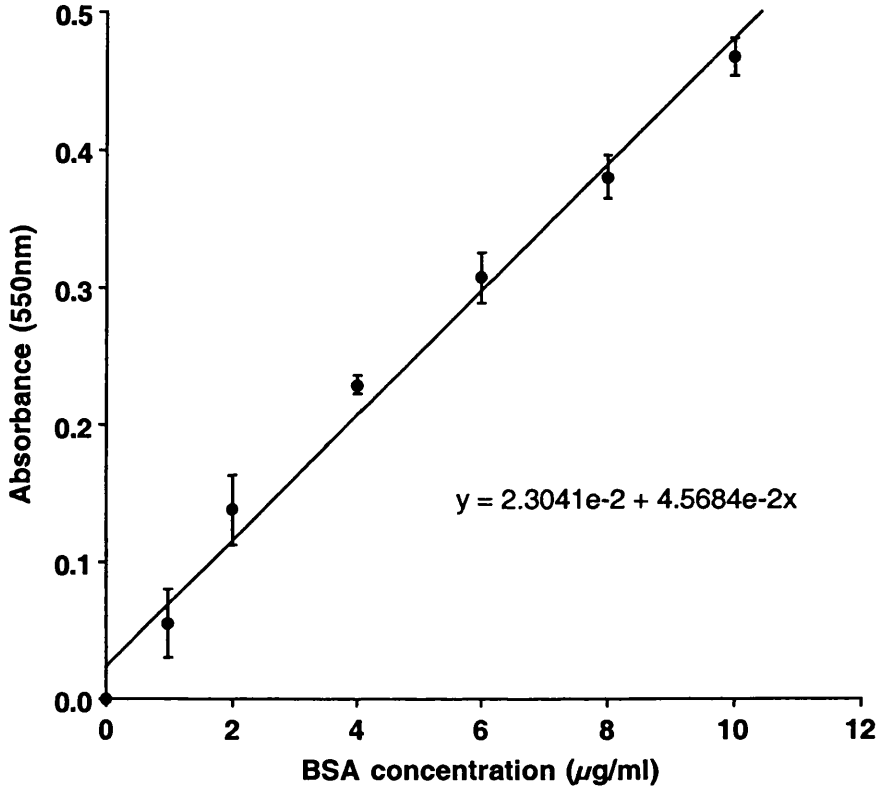



**Figure 2.6:** Typical BSA standard curve used for lysosomal protein content determination. The equation of the straight line is used to determine the protein content of tritosomes.

**Note:** Data represent mean ±SD (n=8).

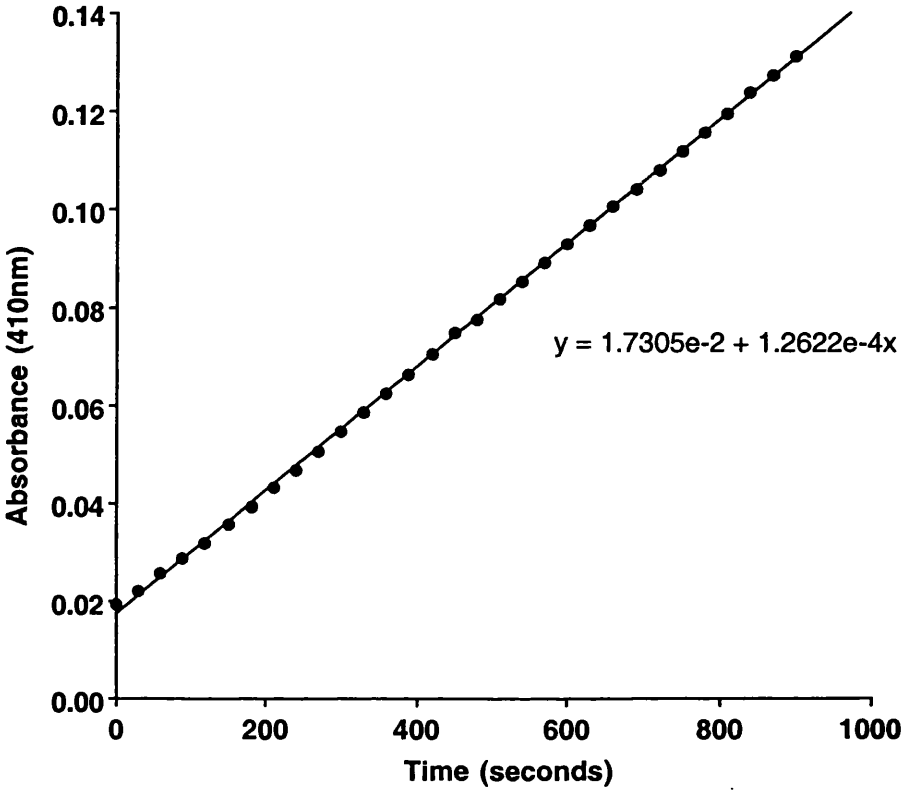



**Figure 2.7:** Typical example of NAp release from the low molecular weight substrate, n-Benzoyl-Phe-Val-Arg-NAp, in a tritosome activity assay. The equation is used to calculate the cathepsin B activity of the tritosomes.

**Note:** This release profile shows time commencing after 5min incubation as there is an intial lag phase in NAp liberation but only this later section of the release profile is used to determine enzyme activity.

to 15ml polypropylene tubes along with sample concentrations (usually 50μg drug equivalence) of HPMA copolymer-APE or PK1 all made up using the citrate phosphate buffer. To commence the reaction a specified activity of tritosomes was added to make the solution 1ml using the citrate phosphate buffer to adjust as necessary. Tritosomes were added last and this was taken as being time zero. At each time-point (0, 5, 15, 30minutes and then 1, 2, 3, 4 and 5 hours) 100μl of reaction mixture was removed using a pipette and frozen immediately in liquid nitrogen in a fresh polypropylene and kept in a freezer until analysis. The reaction tubes were incubated at 37°C for the duration of the experiment. Analysis was by extraction followed by HPLC determination as detailed in Section 2.3.3.1 for APE and 2.3.3.2 for doxorubicin samples.

### *2.3.5* Statistics

All error bars describing *in vitro* studies are the standard error of the mean (SE). The error bars in all other studies are standard deviation (SD) of the mean. Statistical significance was calculated using the students' t-test for small sample sizes (two tailed). Statistical significance was defined as a p value $<0.05$. All equations of a straight line were determined using Cricket Graph software (version 1.3.2). All calculations were performed using Microsoft Excel 98 software.

# Chapter Three

## *Synthesis and characterisation of HPMA copolymer-APE conjugates*

## 3.1 Introduction

Ellipticines (Figure 1.11) are potent cytotoxic agents as their $IC_{50}$ values are in the range of 0.3-0.8μM towards a variety of malignant cell lines *in vitro* (Devraj et al, 1996). As mentioned in Section 1.5.2 their mechanisms of action include DNA intercalation (Elcock et al, 1996), topoisomerase inhibition (Froelich-Ammon et al, 1995) and DNA alkylation (reviewed by Sainsbury, 1990). Early clinical development was limited by poor drug solubility and *in vivo* host toxicities including haemolytic activity, decreased heart rate and hepatotoxicity (Donato et al, 1992). Two ellipticine derivatives did, however, show interesting clinical activity against a number of solid tumours (Section 1.5.3). These observations led to the choice of ellipticines, and APE in particular, as interesting candidates for evaluation in the form of polymer-drug conjugates.

APE (NSC176328) is a derivative of ellipticine which retains cytotoxicity with an average $IC_{50}$ across the NCI anticancer screen of $3.2x10^{-7}$M. This is comparable to ellipticine (NSC71795; $7.5x10^{-7}$M), doxorubicin (NSC123127; $1.3x10^{-7}$M) and paclitaxel (NSC125973; $1.3x10^{-8}$M) in the same screen. APE showed a similar spectrum of activity to ellipticine being most toxic against cell lines derived from leukaemias, colon, lung and breast tumours (NCI website, 2001). APE is also particularly suited to conjugation to a HPMA copolymer precursor because of its free amine and the fact that this amine is removed from the main ellipticine structure (Figure 3.1) and therefore sterically amenable to conjugation by aminolysis.

Biocompatible, water-soluble polymers such as HPMA copolymers provide a useful platform for tumour-selective drug delivery (Section 1.3.4.3). Conjugation of doxorubicin, paclitaxel and camptothecin to HPMA copolymers has been shown to improve the drug water solubility and reduce drug toxicity. Additionally, after i.v. administration such conjugates selectively accumulate in solid tumour tissue by the EPR effect (described in Section 1.4.1). In this study a series of HPMA copolymer-APE conjugates were synthesised with the aim of improving the water-solubility of APE and also improving its therapeutic index.

Previously HPMA copolymer-drug conjugates have been synthesised by four routes:

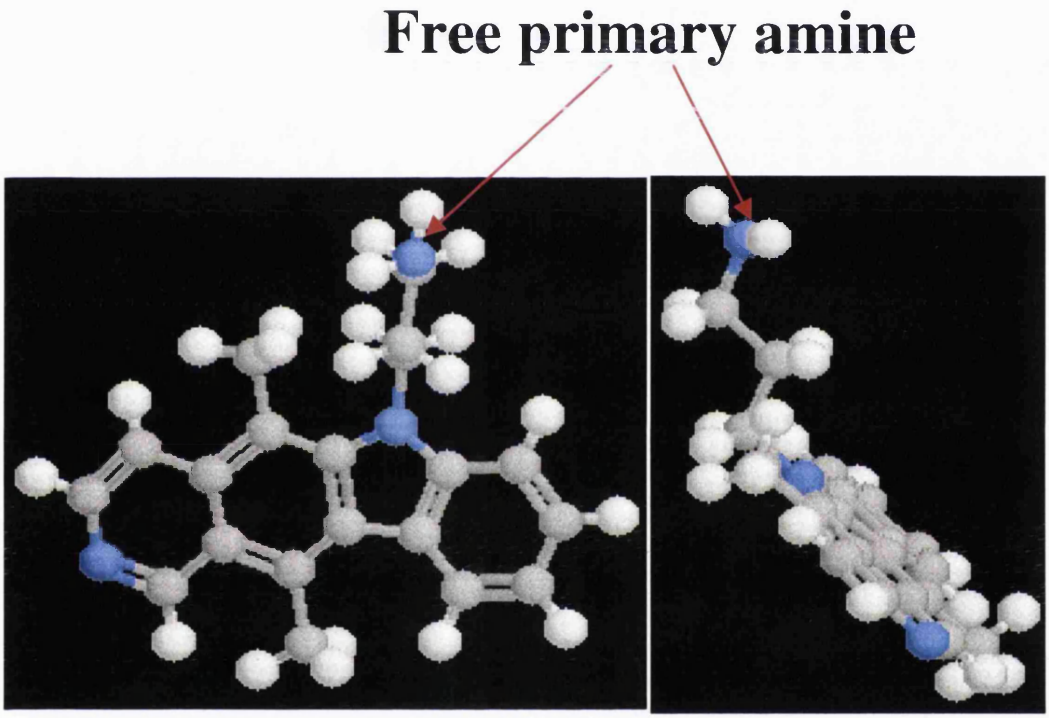



**Figure 3.1:** Three dimensional structure of APE (NSC176328).

1) Polymer analogous reaction using aminolysis of HPMA copolymer-ONp precursors

The most widely used method has been the use of an HPMA copolymer precursor containing HPMA copolymer bearing peptidyl spacers with a terminal ONp. Firstly the HPMA copolymer precursor is prepared by copolymerisation of HPMA and *N*-methacryloylated oligopeptide-ONp (Figure 3.2) using a free radical polymerisation method. Drugs containing an aliphatic amino group can displace ONp by aminolysis (Rejmanova et al, 1977; Figure 3.3) and this approach was used to prepare PK1 and PK2 (reviewed by Kopecek & Duncan, 1987). The advantages of this method are:

A. This is a simple aminolysis reaction and as long as the drug to be attached has only one accessible aliphatic group then cross-linking should not occur.

B. The polymer $M_w$ can be standardised and the 'universal precursor' used to prepare conjugates of different drug loading.

C. The 'universal precursor' can be used to prepare conjugates with drug and targeting groups by sequential aminolysis.

The difficulties with this method are:

A. In the case of poorly reactive drugs the drug needs to be stable in basic conditions as basic conditions are often required to facilitate the reaction.

B. There is a competing hydrolysis reaction leading to the generation of side products (Figure 3.4).

C. The conjugate is a random copolymer and therefore there could be an uneven distribution of drug along the polymer chain and between different polymer chains.

D. Free drug will always be present in the conjugate (even if this is a small contaminant).

Because of some of these difficulties other methods of preparation have been explored.

2) Copolymerisation of methacryloylated drug-bearing monomers

St'astny et al (1996) described the preparation of HPMA copolymer GFLG-doxorubicin conjugates by first synthesising a monomer of methacryloylated-GFLG-doxorubicin and then copolymerising this with HPMA using free radical polymerisation to produce a conjugate of similar structure to PK1 (Figure 3.5). This synthetic

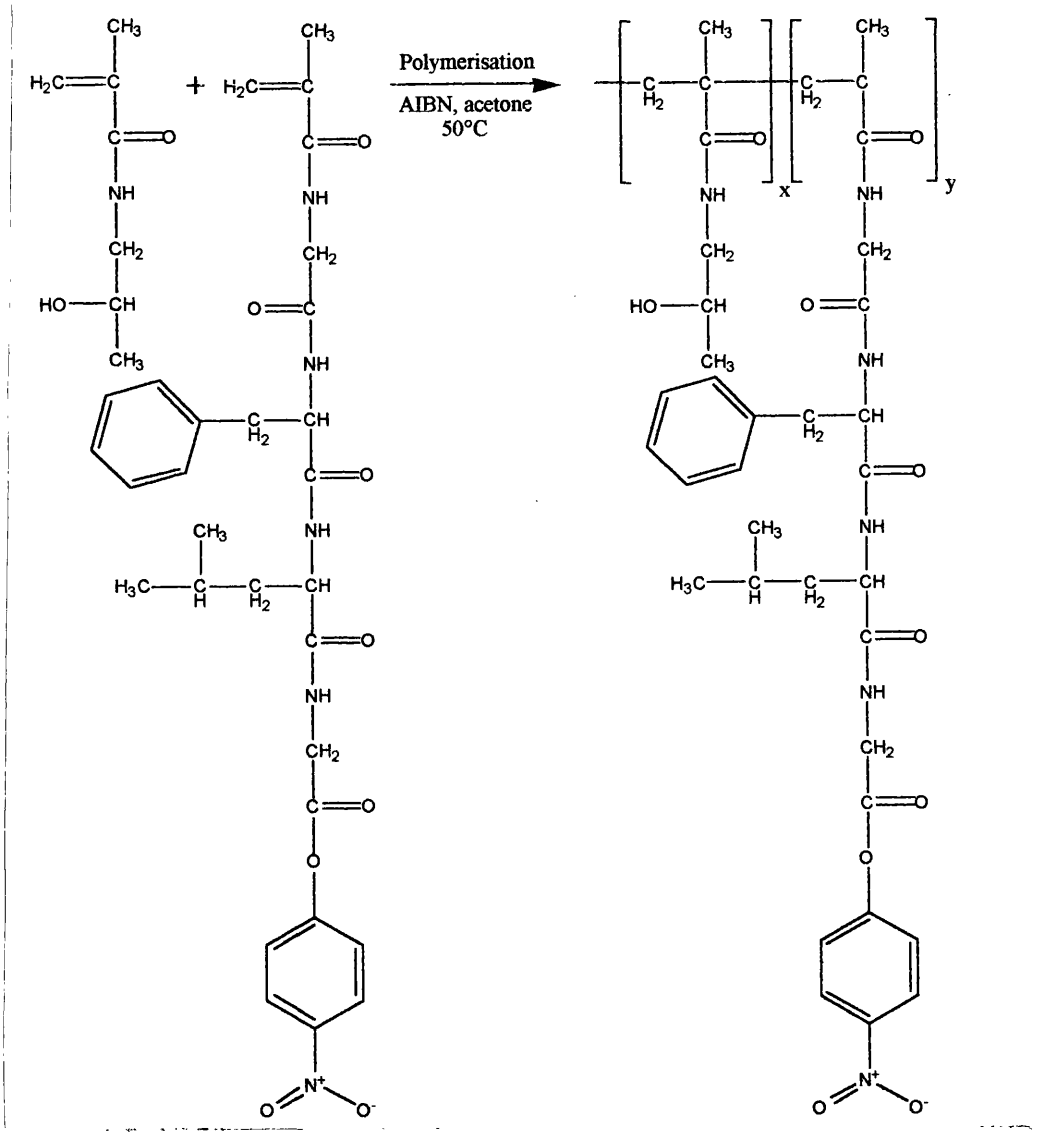



**Figure 3.2:** Synthesis of HPMA copolymer GFLG-ONp precursor by free radical polymerisation.

**Note:** The values of x and y are varied (e.g. for CPT-03 x=95.15, y=4.85; CPT-05 x=90.91, y=9.09 and CPT-09 x=99.07, y=0.93, Table 3.1). AIBN is azobisisobutyronitrile.

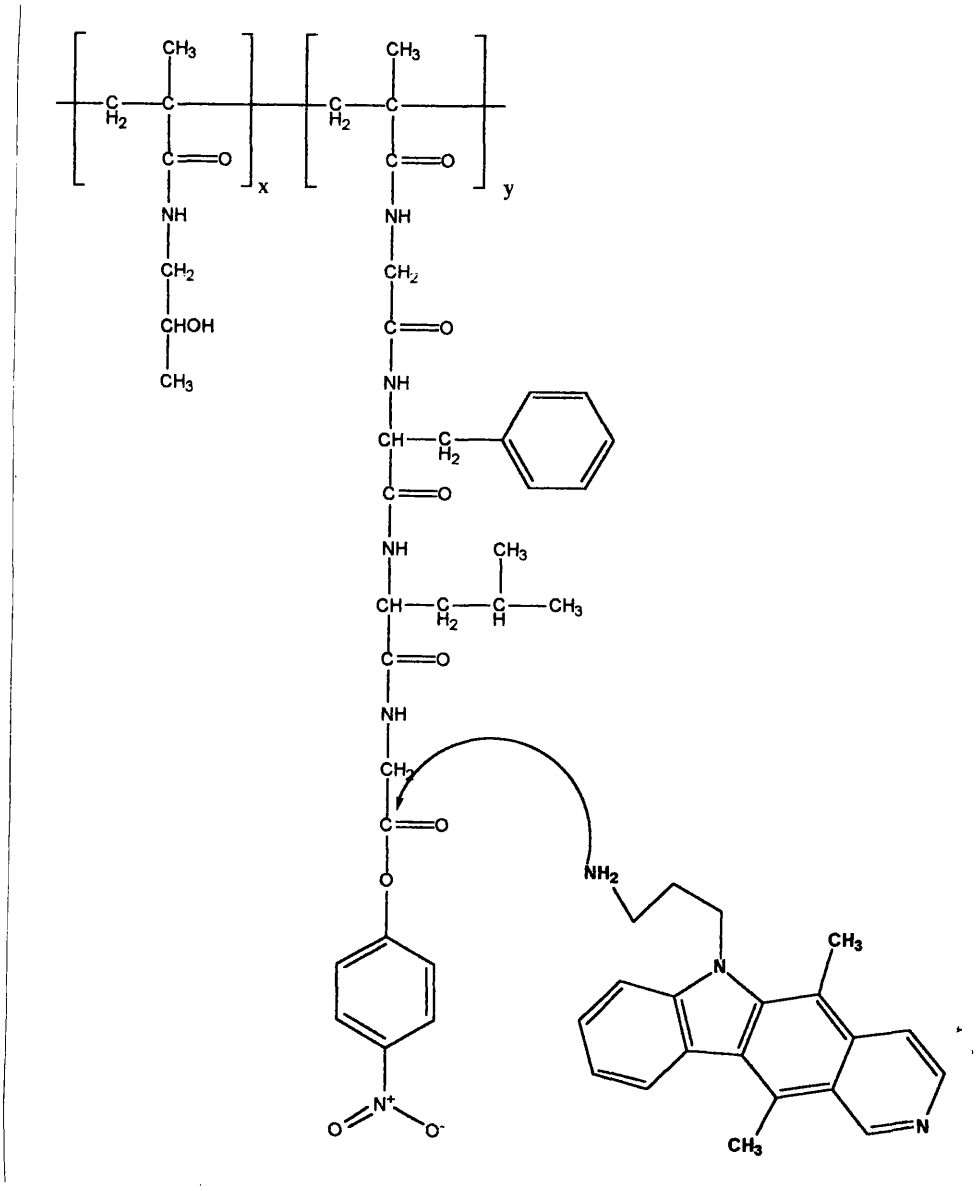


**Figure 3.3:** Aminolysis of the HPMA copolymer GFLG-ONp using APE as an example of a suitable drug with a free primary amine.

**Note:** The values of x and y are varied as described in Table 3.1.

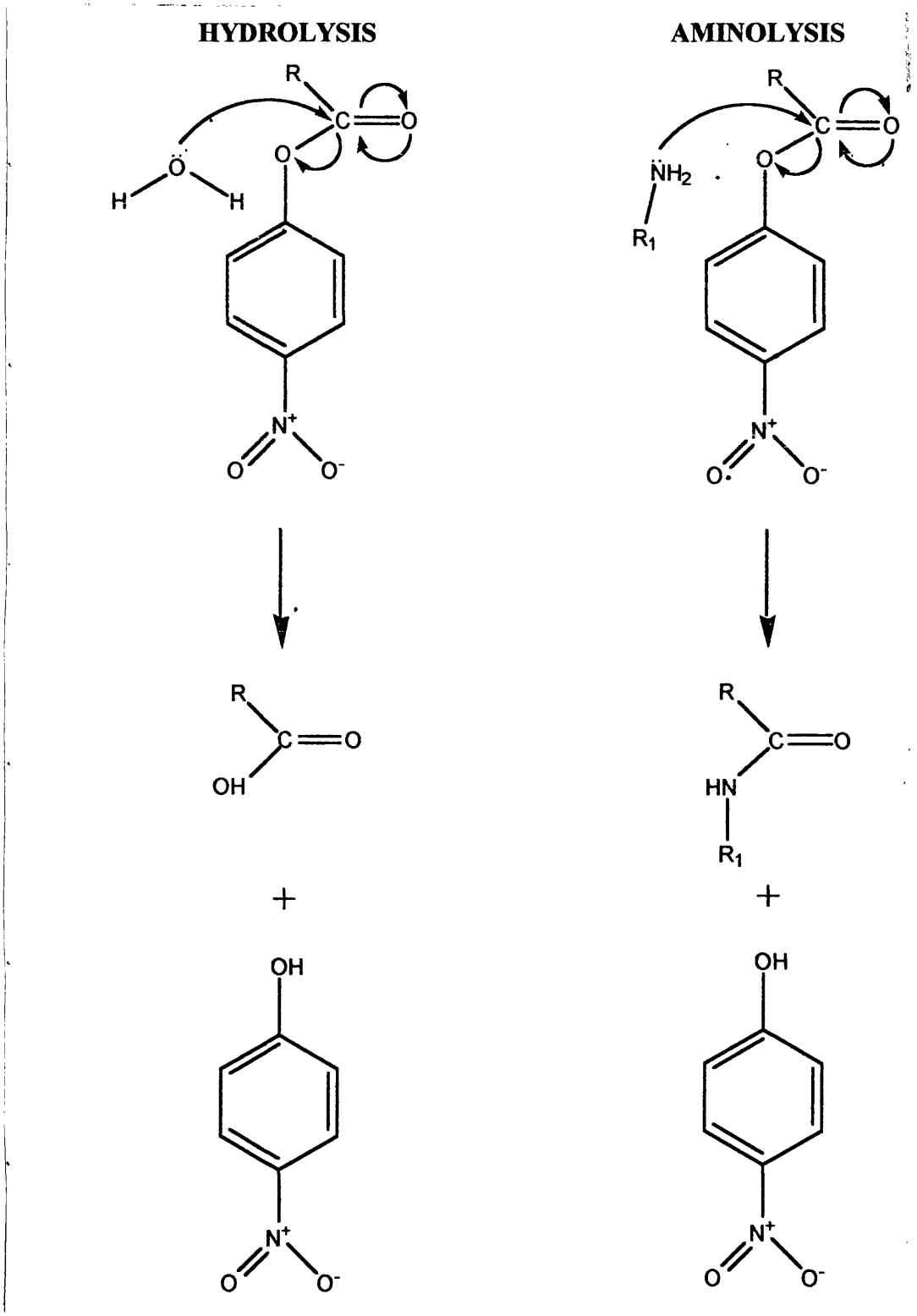



**Figure 3.4:** Reaction schemes showing both the undesired (hydrolysis) reaction and the desired (aminolysis) reactions where R is HPMA copolymer-GFLG and $R_1$ is a reactant with a free amine e.g. APE or doxorubicin.

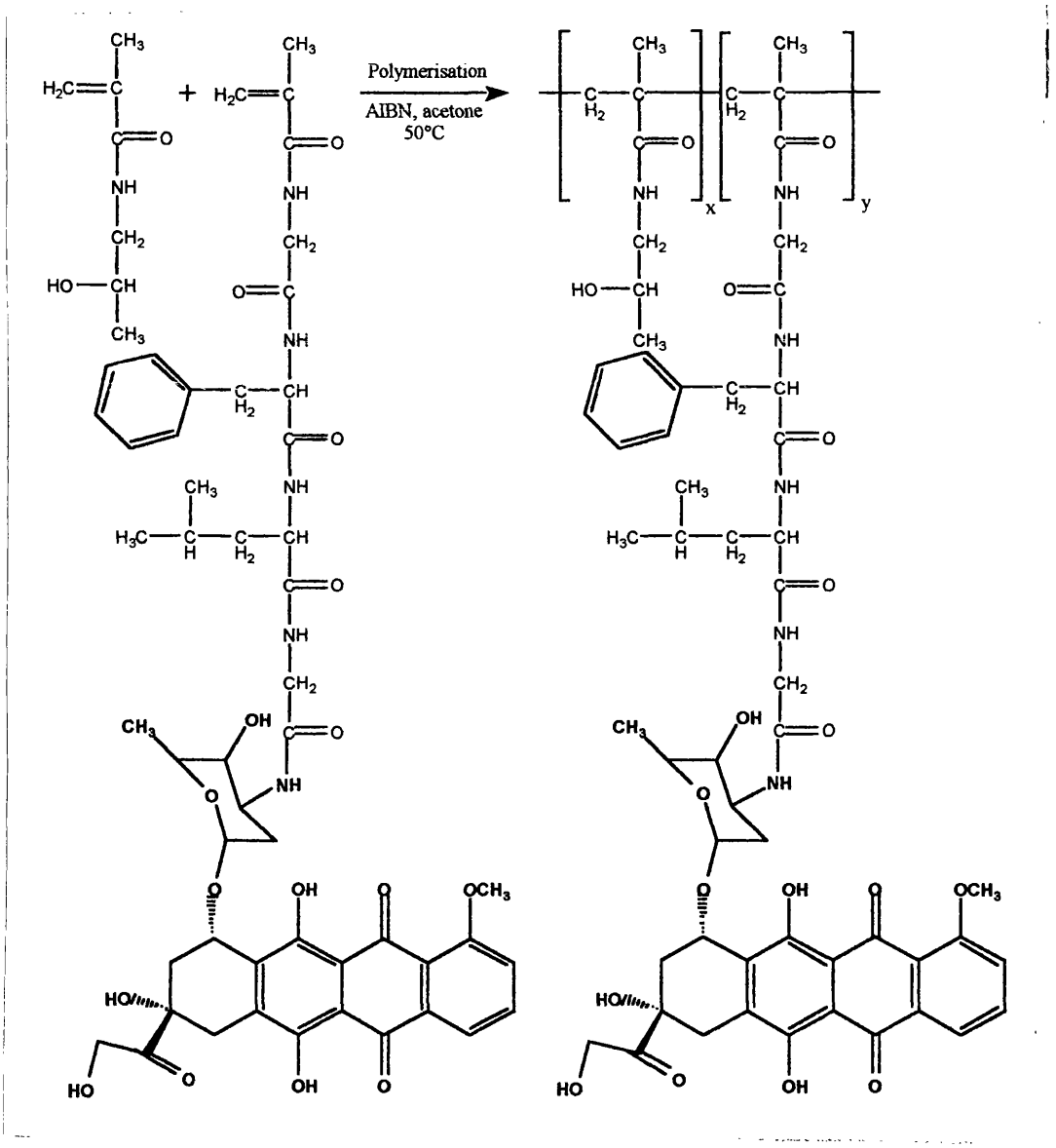



**Figure 3.5:** Reaction scheme showing free radical polymerisation of HPMA with methacryloylated-GFLG-doxorubicin monomers to produce a conjugate similar to PK1 (St'astny et al, 1996; Section 3.1).

approach has also been utilised to obtain cross-linked HPMA copolymer GFLG conjugates containing both doxorubicin and antibodies for targeting (Ulbrich et al, 2000). This synthetic route avoids high levels of free drug in the product (as the potential contaminant is methacryloylated GFLG-doxorubicin) but poor control during polymerisation leads to high polydispersity (approximately 2-3). When this method was used to conjugate antibodies there was evidence of high molecular weight species containing higher amounts of antibody (Ulbrich et al, 2000).

3) Use of 'MAG' precursors

An alternative method has been used by Pharmacia to prepare HPMA copolymer-anticancer conjugates and the HPMA copolymer-G-$C_6$-G-camptothecin conjugate recently brought into Phase I clinical trial (Zamai et al, 2001). The synthesis involves firstly the preparation of a peptidyl camptothecin derivative which is then bound by aminolysis to an HPMA copolymer-G-ONp (called a 'MAG') precursor (Figure 3.6). It has been suggested that these conjugates have a low free drug content and that the drug is equally distributed across high and low molecular weight species present in the conjugate (Rizzo, 2000). However the polydispersity is higher than seen for PK1 (1.5-1.7) and there are free peptidyl-drug species (e.g. FLG-camptothecin) in the final product (Caiolfa et al, 2000).

4) Synthesis of a universal precursor of low polydispersity

Recent work by Godwin et al (2001) has sought to overcome the problem of HPMA copolymer conjugate polydispersity by developing a new polymerisation method. All the methods above utilise free radical polymerisation while this method uses an atom-transfer radical polymerisation (ATRP) method. This method produces an activated homopolymer which can then be used to prepare polymer-drug conjugates (Figure 3.7) with a polydispersity of 1.1-1.2, thus potentially giving better defined HPMA copolymer-drug conjugates more suitable for regulatory approval.

The potential disadvantages of this method are again free linker drug species in the final product similar to those seen in the use of 'MAG' precursors outlined above. Also incomplete reaction of the polymer precursor with drug (or side reactions / hydrolysis) would lead to poorly-defined polymer species.

As outlined in Section 1.5.3 ellipticines are thought to act mainly on targets within the nucleus of the cell (reviewed by Sainsbury, 1990) and therefore intracellular

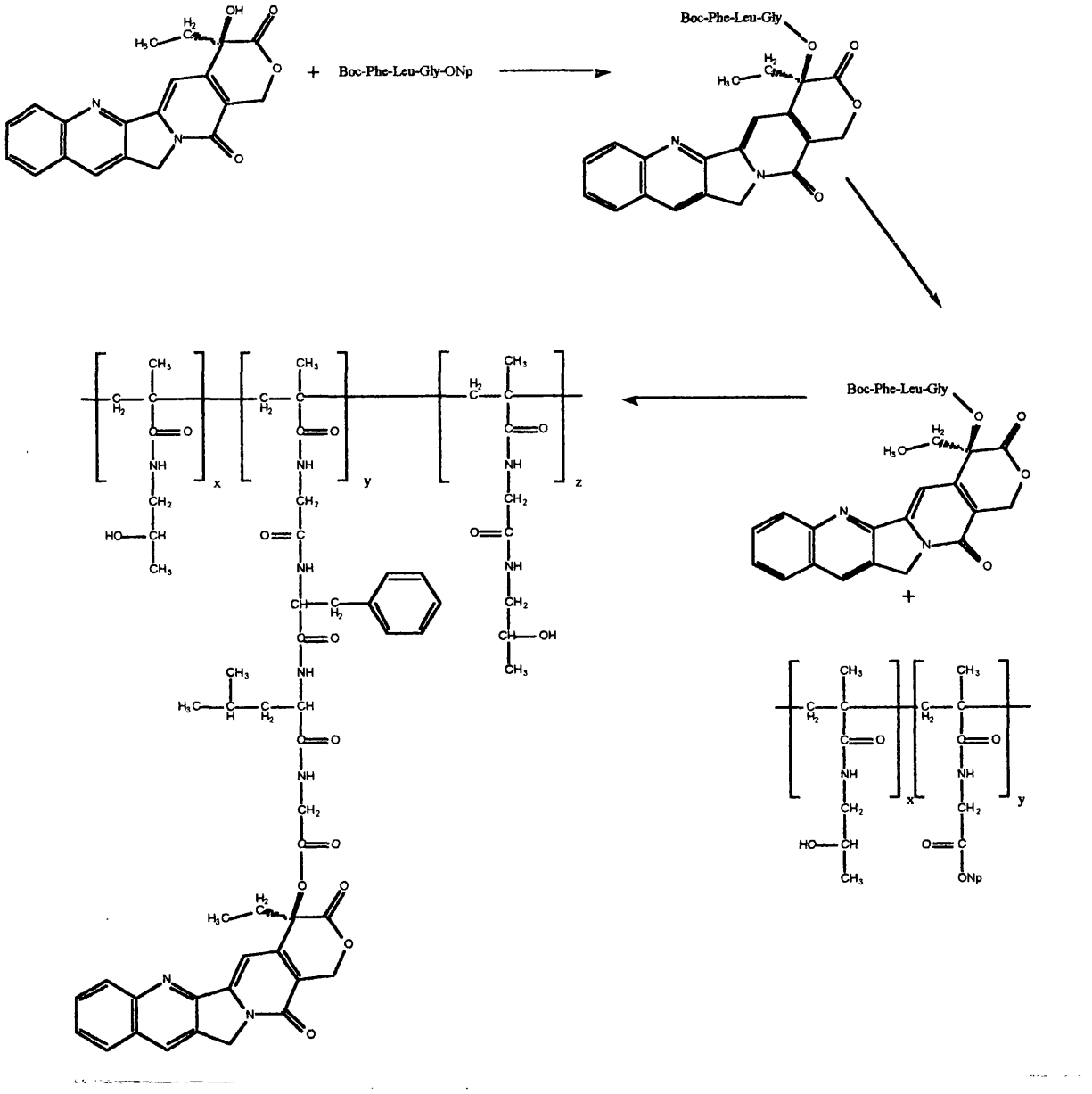



**Figure 3.6:** Method used by Caiolfa et al (2000) to synthesise an HPMA copolymer GFLG-camptothecin conjugate.

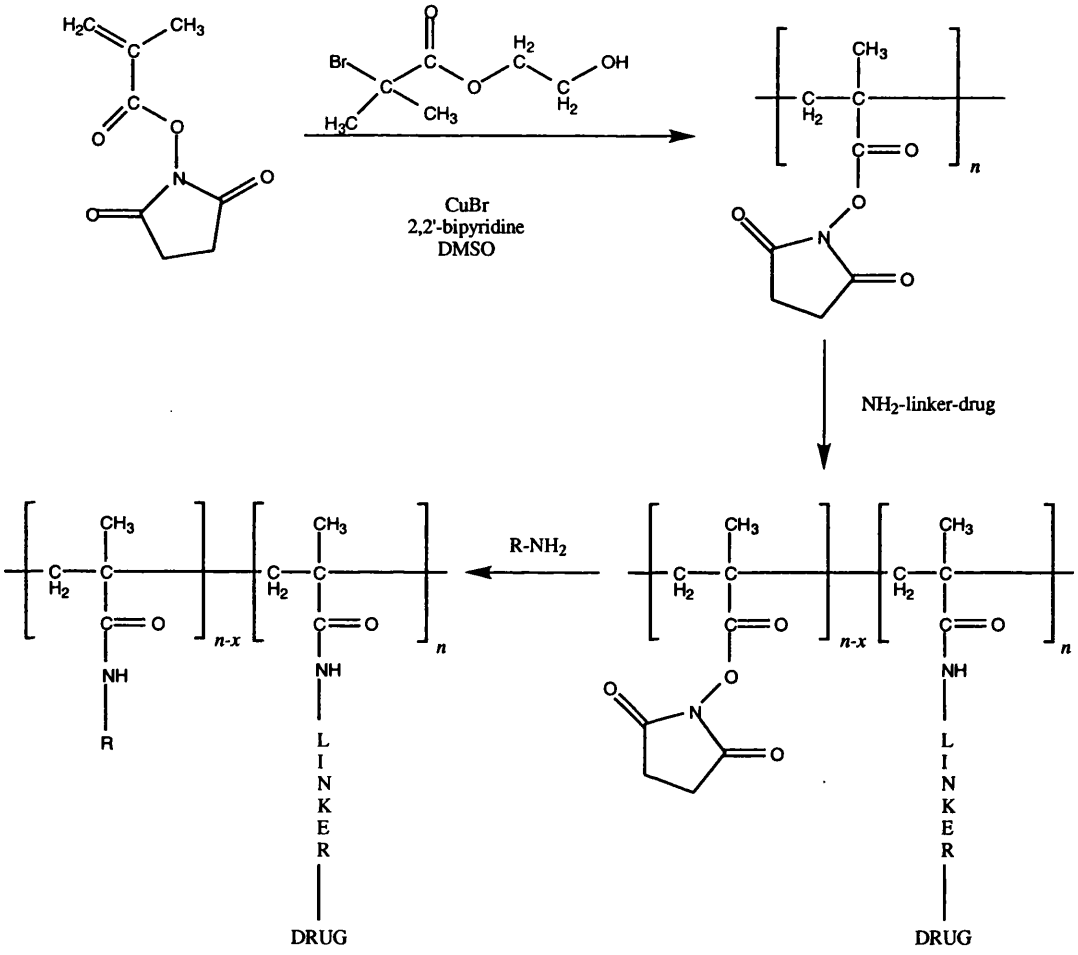



**Figure 3.7:** ATRP-mediated copolymerisation of activated HPMA monomers to produce a 'universal' precursor (Godwin et al, 2001).

release from the HPMA copolymer backbone would be essential to their activity. For this reason it seemed reasonable to hypothesise that the GFLG linker used to produce PK1 (which is degraded by lysosomal thiol-dependent proteases (Section 1.3.4.2)) would be most suitable for an HPMA copolymer-APE conjugate. HPMA copolymer precursors containing 1 and 10mol% linker content on a backbone of a similar molecular weight were also selected as a means of synthesising conjugates with varying degrees of drug loading. Varying the drug loading of conjugates would be expected to affect various characteristics of the constructs due to the chemical nature of the components and this feature would need to be optimised to select a lead candidate for future development. Conjugates containing a diglycyl linker were also synthesised as this linker is usually not degraded by lysosomal enzymes when a bulky substituent is present as the terminal group (Subr et al, 1992) and is potentially a useful comparator, particularly in drug release studies.

## 3.2 Methods

### *3.2.1* Synthesis of HPMA copolymer-APE conjugates

It was considered important to first carefully standardise the aminolysis reaction to determine APE reaction kinetics. During drug conjugation ONp can also be displaced non-specifically by simple hydrolysis (Figure 3.4). This is particularly true when a strong base like triethylamine is added. Basic conditions greatly accelerate both aminolysis and hydrolysis so it is very important to use dry solvents. DMSO, in particular, can contain a high percentage of water. To quantitate non-specific hydrolysis an HPMA copolymer precursor (CPT-03; Table 3.1) was dissolved (5mg/ml) in either water or a mix of dimethyl formamide (DMF)/DMSO in the presence and absence of triethylamine. The change in UV absorbance (400nm) with time was measured to determine how much ONp was liberated. Free ONp species absorb at this wavelength. To quantitate the total possible ONp liberated an excess of 1-amino-2-propanol was added. Release of ONp was expressed as a percentage of the total ONp present.

The HPMA copolymer-APE conjugates were synthesised essentially using the method outlined in Searle et al (2001). The following is a typical synthesis.

HPMA copolymer GFLG-ONp (200mg; CPT-03, Table 3.1) was dissolved in dry DMF (5ml). APE.2HCl (18mg) was dissolved to 5mg/ml in dry DMSO (which would be equivalent to a maximum of 75% displacement of ONp from the precursor). A sample (5μl) of each solution was retained for thin layer chromatographic (TLC) analysis. The APE solution was added to the solution of HPMA copolymer and mixed. Aliquots of a 1/100v/v solution of triethylamine in dry DMSO were added over 30min (1.9ml total volume) with further 5μl aliquots of reaction mixture being retained at various time-points for TLC analysis. TLC was performed using chloroform : methanol : triethylamine 8 : 1 : 1 by volume as the mobile phase. Any unreacted APE could be seen at a retention factor (Rf) value of 0.4 while bound APE remained at the origin. Spots were visualised using a UV lamp. During the reaction the absorbance (400nm) was also measured (taking 10μl of the reaction mixture and diluting to 1ml with PBS) to monitor ONp release (as in Section 3.2.4). The reaction was allowed to run overnight in a dark cupboard and the next day an absorbance reading was taken as before at 400nm. 1-Aminopropan-2-ol (1/100 v/v) in DMF (440μl) was then added to react with any remaining ONp groups.

Conjugates containing different drug loading were prepared using HPMA copolymer GFLG-ONp precursors, CPT-09 and CPT-05. Conjugates containing the GG linker were synthesised using CPT-04 (Table 3.1). In each case the same weight of precursor (200mg) and sufficient APE to displace 75% of the bound ONp were used. Otherwise the synthesis was identical to that outlined above. After completing the reaction with 1-aminopropan-2-ol the absorbance at 400nm was again determined.

The organic solvents were evaporated in a glass flask heated to approximately 30°C using a paraffin bath attached to a high vacuum pump with a liquid nitrogen trap to retain all evaporated solvents. The residual film was dissolved in approximately 20ml of distilled water and added into a SpectraPor® cellulose dialysis membrane with a molecular weight cut-off of 2,000Da, pre-soaked in distilled water to remove any residual sodium azide which is used as a preservative agent. This was then dialysed against approximately 5l of distilled water with frequent water changes for three days to remove any low molecular weight impurities including unbound APE. Finally the conjugate solution was poured into a round-bottomed flask, frozen using liquid nitrogen and then lyophilised to a constant mass as outlined in Section 2.2.4.

**Table 3.1**: Characteristics of the HPMA copolymer precursors used in the synthesis of HPMA copolymer-APE conjugates.

| **Precursor** | **Peptidic linker** | **ONp content (mol%)**[†] |
|---|---|---|
| CPT-03 | GFLG | 4.85 |
| CPT-04 | GG | 5.05 |
| CPT-05 | GFLG | 9.09 |
| CPT-09 | GFLG | 0.93 |

[†] These values were supplied by Polymer Laboratories and were determined by them using a UV absorbance method.

*3.2.2* Determination of APE content in conjugates

The HPLC method used to determine the free APE content of the conjugates was described in Section 2.3.3.1. To determine total APE content a simple UV assay was used. The UV spectrum of APE shows a maximal absorbance at 296nm which is not interfered with by any UV absorbance of the HPMA copolymer precursors (Figure 3.8). A standard curve was prepared by determining the absorbance at 296nm of APE samples (0-5μg/ml) in PBS using a quartz cuvette (Figure 3.9). Conjugates were dissolved in PBS and similarly absorbance (296nm) was determined. Values were compared to the standard curve and the total APE content in the conjugate determined.

*3.2.3* Gel Permeation Chromatography

HPMA copolymer-APE molecular weight was assessed by comparison with poly(ethylene oxide) standards (18.3 to 124.7kDa) but ideally HPMA copolymer-APE standards should be used. GPC analysis was performed using two TSK-gel columns in series (G3000 PW followed by G2000 PW) with a guard column (Progel$^{TM}$ PWXL) and differential refractometer and UV-visible detectors connected in series. Tris buffer (Tris 0.05M, NaCl 0.5M) was used as the mobile phase (1ml/min). Conjugates (20μl of a 5mg/ml solution in eluant buffer) were injected onto the columns using a 20μl loop and manual injection. PowerChrom software was used for recording the detector output signal and for analysis of the data.

*3.2.5* Determination of solubility of free APE and HPMA copolymer-APE

Firstly to assess the solubility of APE a solution of 0.1M PBS was prepared and

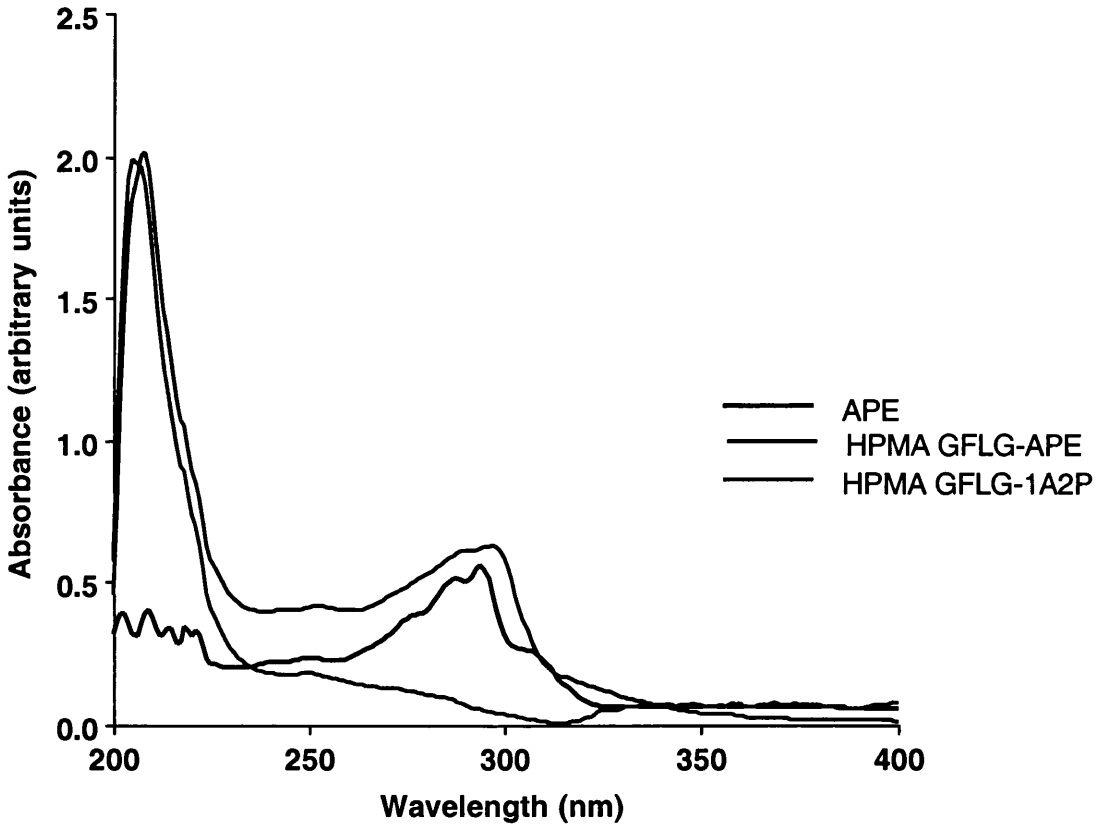



**Figure 3.8:** The UV spectra of APE, HPMA copolymer GFLG-1-amino-2-propanol and HPMA copolymer GFLG-APE (RK3).

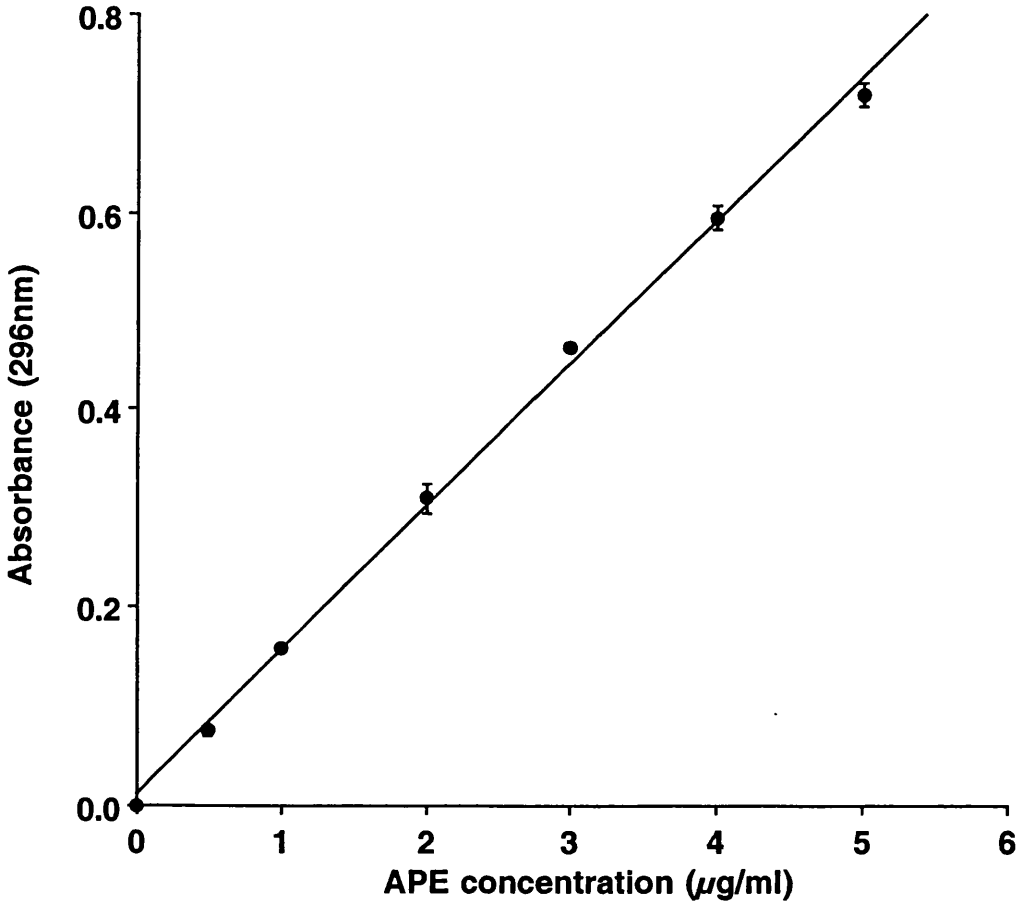



**Figure 3.9:** Standard curve for APE in PBS measured using UV absorption at 296nm.

**Note:** Data represent mean ± SD (n=3).

heated to 37°C. A standard (0.1mg/ml) solution of APE in PBS was also prepared and a standard curve constructed using a UV-visible spectrophotometer taking triplicate measurements at a wavelength of 296nm as in Figure 3.9. PBS (5ml) was added to a glass container accurately. Powdered APE was added until the solution became a dark colour. The pH of this solution was adjusted to 7.4 using small volumes of a 0.1M NaOH solution. The resulting solution was allowed to equilibrate at 37°C for 10min and the pH was taken again to ensure that the APE.2HCl had not changed the solution pH. This solution was filtered using a syringe and 0.45μm filter. The resulting filtrate (10μl) was diluted to 1ml with PBS at 37°C and absorbance at 296nm measured, in triplicate.

To assess the solubility of HPMA copolymer GFLG-APE 2ml of PBS at 37°C was measured accurately into a glass container. Approximately 50mg of HPMA copolymer GFLG-APE (Conjugate RK3; Table 3.2) as a powder was added to the PBS solution and allowed to remain at 37°C for 10min. The pH of this solution was adjusted to 7.4 again using small volumes of 0.1M NaOH. This solution was filtered as before (despite there being no visible particles in the solution) to allow for equivalent losses. This solution (10μl) was made up to 1ml with PBS as before (37°C) and the absorbance at 296nm measured in triplicate.

## 3.3 Results

### *3.3.1* Conjugation characteristics of HPMA copolymer conjugates

It was found that considerable care had to be taken during drug conjugation by aminolysis due to the competing hydrolysis reaction (Figure 3.4; Figure 3.10). For this reason dry solvents dispensed under argon (DMF/DMSO) were always used in the synthesis of HPMA copolymer-APE conjugates. Triethylamine, a strong base, has been used before to activate the doxorubicin hydrochloride giving the free base necessary for preparation of PK1 (Rihova et al, 1989). As APE is also supplied as a hydrochloride salt triethylamine was also used to generate the free base needed for the synthesis of HPMA copolymer-APE conjugates. Unfortunately triethylamine also accelerates the competing hydrolysis reaction, re-enforcing the need for dry solvents (Figure 3.10).

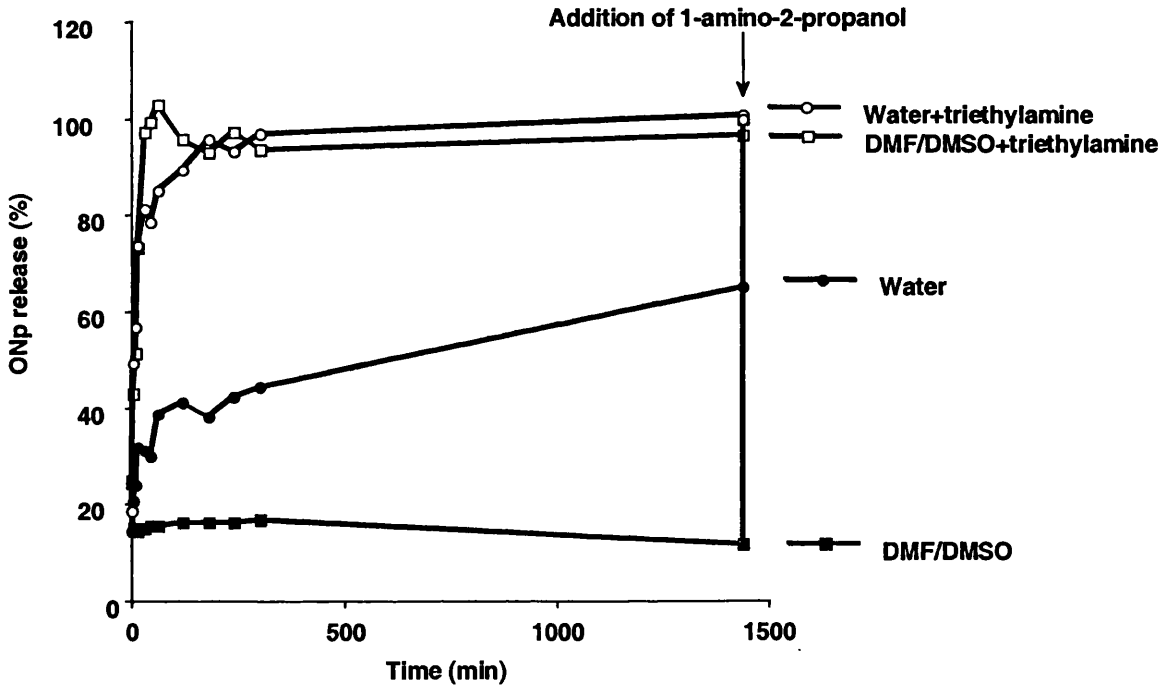



**Figure 3.10:** Hydrolysis of the HPMA copolymer GFLG-ONp (CPT-03).

*3.3.2* Conjugation of APE to HPMA copolymers

During the reaction the disappearance of free APE and appearance of conjugate was followed by TLC. Free APE had an Rf of 0.4; bound APE remained at the origin while ONp had an Rf of 0.9 (Figure 3.11). It was found in a previous study that a molar ratio of 1:0.75 of ONp:APE gave the most efficient conjugation (Searle et al, 2001). Therefore all conjugates were synthesised using these conditions. Using the HPMA copolymer precursors (Table 3.1) three batches (synthesised using 200mg of HPMA copolymer precursor) of each conjugate were prepared to ensure the reproducibility of the reaction (Table 3.2). The recovery of conjugates at the end of the synthesis was between 110mg and 150mg giving an overall recovery/yield compared to initial mass of conjugate of approximately 65%.

In addition the conjugates contained a small quantity of ellipticine (<0.1%w/w of conjugate). This impurity is a theoretical breakdown product of APE. The HPMA copolymer-APE conjugates (Table 3.2) contained less than 0.1%w/w free APE (the exception to this was the batch RK6 where there were problems during purification and it contained a high percentage of free APE).

*3.3.3* GPC analysis of HPMA copolymer-APE conjugates

GPC was used as a method of size determination using poly(ethylene oxide) standards. Table 3.3 shows the apparent molecular weight of the conjugates when compared to poly(ethylene oxide) standards. These are clearly incorrect based on the molecular weight of the precursors used and the possible reasons for this will be discussed in Section 3.4. The conjugates were shown to consist of an apparently single species using this method. The chromatograms of the HPMA copolymer-APE conjugates suggest a lack of cross-linked species, as expected given the nature of the aminolysis reaction with the only potential source of cross reactivity being a drug with two free amines. Figure 3.12 shows an example of one such GPC trace and shows two HPMA copolymer species, namely a HPMA copolymer GG-APE (RK4) and a HPMA copolymer GG-aminopropanol (where the HPMA copolymer precursor has been aminolysed using 1-amino-2-propanol). The HPMA copolymer GG-aminopropanol showed no UV absorbance (296nm) as described earlier (Figure 3.8) and gave a refractive index profile similar to that of the HPMA copolymer GG-APE conjugate (RK4) which showed a UV peak at 296nm. This UV peak is of a similar profile to the refractive index (RI) trace and this indicates that the APE is bound across the different

**Table 3.2:** Characteristics of the HPMA copolymer-APE conjugates synthesised in this study.

| Code | Precursor | Total APE (%w/w)[†] | APE (mol%) | Free APE (%w/w total APE)[††] | Impurities (%w/w conjugate) | |
|---|---|---|---|---|---|---|
| | | | | | APE[††] | ellipticine[††] |
| RK1 | CPT-03 | 3.07 | 1.7 | 0.043 | 0.001 | 0.005 |
| RK2 | CPT-03 | 4.34 | 2.4 | 0.148 | 0.006 | 0.042 |
| RK3 | CPT-03 | 3.79 | 2.1 | >0.017 | >0.001 | 0.009 |
| RK4 | CPT-04 | 4.27 | 2.2 | 0.017 | 0.001 | 0.035 |
| RK5 | CPT-04 | 4.62 | 2.3 | 0.051 | 0.002 | 0.024 |
| RK6 | CPT-04 | 4.90 | 2.5 | 12.176 | 0.597 | 0.088 |
| RK7 | CPT-09 | 1.07 | 0.5 | 7.742 | 0.082 | 0.011 |
| RK8 | CPT-09 | 1.20 | 0.6 | 7.653 | 0.092 | 0.012 |
| RK9 | CPT-09 | 1.21 | 0.6 | 4.321 | 0.052 | 0.011 |
| RK10 | CPT-05 | 6.10 | 3.7 | 0.077 | 0.005 | 0.042 |
| RK11 | CPT-05 | 6.01 | 3.7 | 0.092 | 0.007 | 0.035 |
| RK12 | CPT-05 | 5.85 | 3.5 | 0.058 | 0.003 | 0.051 |

[†] Determined using UV analysis (Section 3.2.2)

[††] Determined using extraction and HPLC analysis (Section 2.3.3.1)

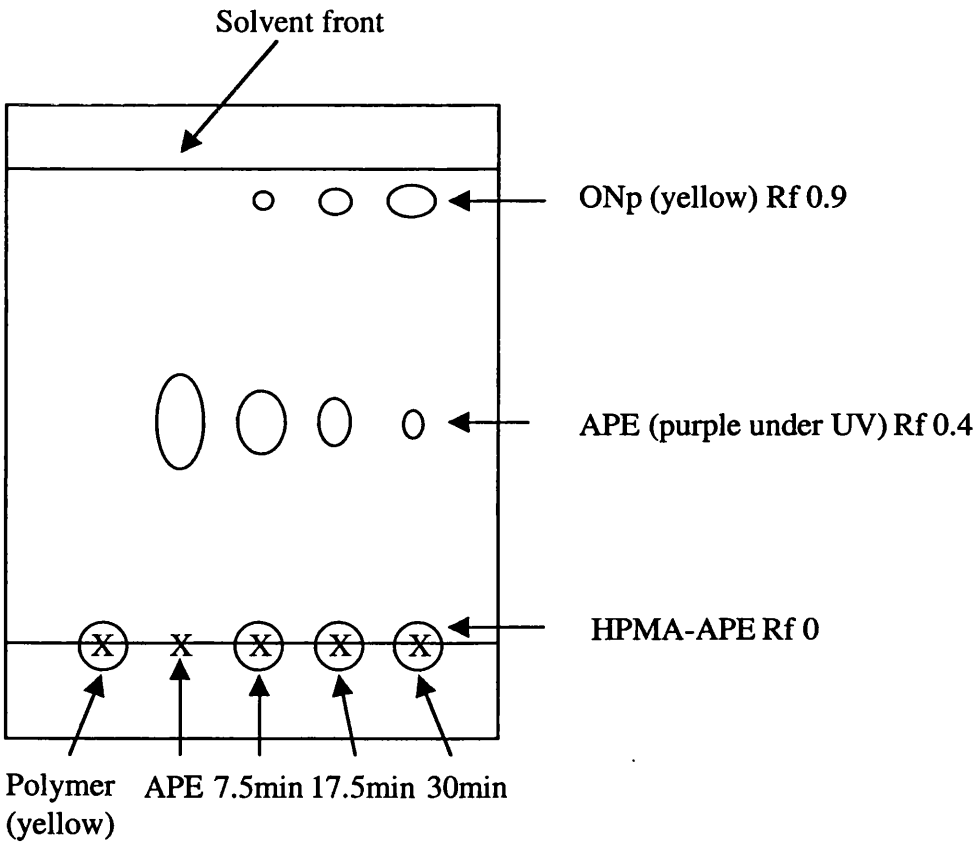



**Figure 3.11:** TLC of a typical HPMA copolymer-APE reaction mixture.

**Table 3.3:** Molecular weight of HPMA copolymer-APE conjugates compared to poly(ethylene-oxide) standards.

| Conjugate | %w/w APE | Mw (Da) | Polydispersity |
|---|---|---|---|
| HPMA GFLG-APE (RK8) | 1.20 | 32068 | 2.2 |
| HPMA GFLG-APE (RK3) | 3.79 | 974 | 1.4 |
| HPMA GFLG-APE (RK10) | 6.10 | 692 | 1.2 |
| HPMA GG-APE (RK4) | 4.27 | 9907 | 1.3 |

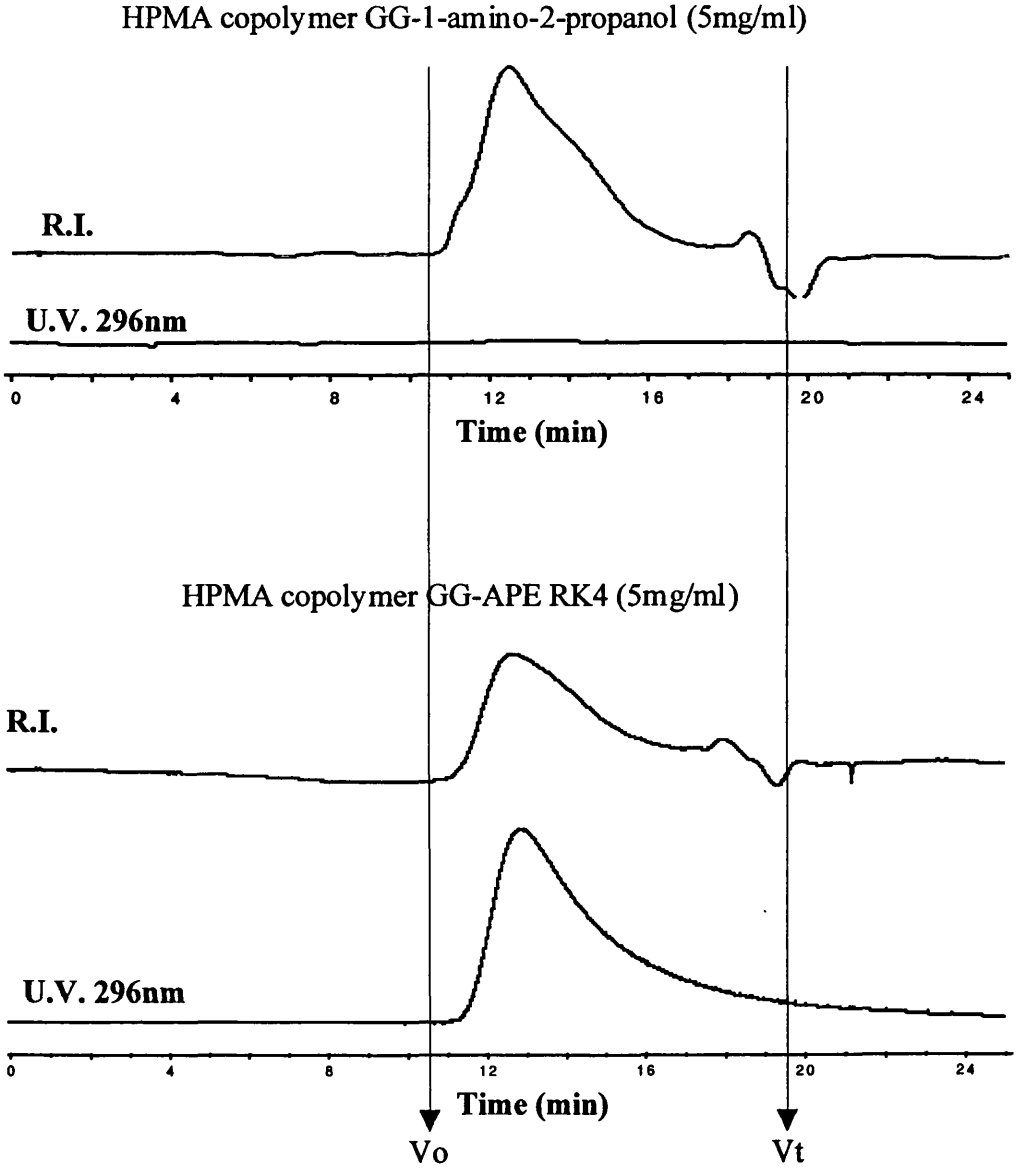



**Figure 3.12:** GPC chromatogram of an aminolysed HPMA copolymer and the equivalent HPMA copolymer-APE conjugate (RK4).

**Note:** The UV detector was placed in series with a differential refractometer. Vo indicates the exclusion volume of the columns and Vt the total elution volume.

sizes of conjugate species and is not preferentially binding to long or short species.

*3.3.5* Solubility of HPMA copolymer-APE conjugates

A solubility experiment was carried out on an HPMA copolymer-APE conjugate (RK3). It was found that, at physiological pH (7.4) and at 37°C in PBS, RK3 (625μg/ml) was at least ten-fold more soluble than APE alone (62μg/ml) in terms of equivalence of APE. This experiment demonstrates the ability of HPMA copolymer conjugates to solubilise poorly-water soluble drugs. This is an important obstacle to overcome in order to use such agents *in vivo* and therefore this was a useful benefit of conjugation to the HPMA copolymer.

**3.4 Discussion**

In this study HPMA copolymer GFLG and GG-APE conjugates were successfully synthesised using a standard aminolysis reaction (Rejmanova et al, 1977; Figure 3.3). The ease of conjugation was predictable as the 3 dimensional structure of APE (Figure 3.1) shows that the primary amino group is free from any steric hindrance, standing well outside the plane formed by the unsaturated rings. Conjugates were synthesised to contain a range of APE loadings (1.07-6.10%w/w APE; Table 3.2). Maintenance of high concentrations of reagents in dry solvents and dropwise addition of the triethylamine to free the 3'-amino group of APE slowly from its hydrochloride salt (such that the copolymer remained in excess during the reaction) was critical for successful conjugation. Also the use of an APE:ONp ratio of 75:100 gave good

reaction yields (55 to 80% of the maximum expected). A drug loading of 2.4mol% was achieved for APE, which is similar to that achieved for doxorubicin when synthesising PK1 (2.5mol%; Subr et al, 1992).

A number of methods of purification have been employed in the past to remove low molecular weight impurities from HPMA copolymer drug conjugates. These include gel filtration to remove free doxorubicin from PK1 (Rihova et al, 1989) and precipitation to remove peptidyl-camptothecin from HPMA copolymer-camptothecin (Caiolfa et al, 2000). In this study simple dialysis using a large volume of distilled water was able to remove most low molecular weight impurities. The high sensitivity of the HPLC analytical procedure showed that only small amounts of free APE and ellipticine were present in the conjugates (Table 3.2). Impurities detected represented < 0.1% of the conjugate by weight in most cases. Further optimisation of the reaction conditions is necessary to reduce the free APE content in the conjugates made from the precursor CPT05. This polymeric precursor has a small number of reactive ONp groups and therefore a greater percentage of reactive groups may be lost to any hydrolysis reaction mediated by water. Also the higher ratio of GFLG-ONp/APE to hydrophilic HPMA may have led to greater stearic hindrance in the reaction. The minimal free drug in most of the conjugates is particularly important for biological testing such as *in vitro* cytotoxicity and haematocompatibility (Chapter 4) where free drug has been shown to interfere in previous studies on PK1 and doxorubicin (Wedge, 1991).

GPC analysis has been shown to be difficult as a method of determining the molecular weight of HPMA copolymer-APE conjugates using the poly(ethylene-oxide) standards (Table 3.3). Interestingly the higher the drug loading the lower the apparent molecular weight of the conjugate and therefore perhaps drug loading is playing a role in this determination. The use of other standards may be helpful but ideally HPMA copolymer-APE standards would be used in such a study. However, GPC did show that APE was conjugated to the HPMA copolymers uniformly across the molecular weight range of the conjugates (Figure 3.12). Non-uniformity of conjugation has been reported previously with the HPMA copolymer-doxorubicin/antibody conjugates of Ulbrich et al (2000). GPC also showed that the HPMA copolymer-APE conjugates exist as single species which are not cross-linked.

A major problem encountered with previous ellipticine derivatives is their limited solubility (Djuric et al, 1992). In this study it was found that the maximum solubility of APE at room temperature and 37°C was 44.5μg/ml and 62.4μg/ml respectively. Drug conjugation to HPMA copolymers dramatically improved solubility (approximately 10-fold). This was not unexpected from the previous studies on HPMA copolymer-paclitaxel which was 20,000 times more water soluble than paclitaxel alone (Huinink et al, 1998). Paclitaxel alone is extremely water insoluble and therefore this dramatic increase in solubility is significant. The HPMA copolymer-APE conjugate (RK3) was still soluble at an APE-equivalent concentration of 625.2μg/ml. APE is not as water insoluble as paclitaxel, and is comparable to doxorubicin in its water solubility. Therefore a 10-fold increase in solubility of APE, similar to the 10-fold increase in doxorubicin solubility in PK1, was felt to be sufficient for the studies which were planned *in vitro* and ultimately *in vivo*.

Following the successful synthesis and characterisation of HPMA copolymer-APE conjugates in this study these conjugates need to be evaluated i*n vitro* for drug release and biocompatibility before they can be evaluated as anti-tumour conjugates. The effect of drug loading on these characteristics needs to be determined in order to identify a lead optimal loading for progression to *in vivo* studies. To do this firstly the conjugates will be compared for their solution properties before being evaluated for their ability to release free APE, haematocompatibility and *in vitro* cytotoxicity. These studies will be described in Chapter 4.

# Chapter Four

## *Effect of APE loading on physicochemical and biological properties of HPMA copolymer-APE conjugates in vitro*

## 4.1 Introduction

Following the synthesis of the HPMA copolymer-APE conjugates described in Chapter 3 it was necessary to evaluate their physicochemical and *in vitro* biological properties (APE release by lysosomal enzymes, haemolytic activity and cytotoxicity), focusing particularly on the effect of drug loading on these parameters before progressing to *in vivo* anti-tumour testing. Before evaluating the effect of drug loading on biological properties of the conjugates it was considered necessary to characterise the structure of conjugates in solution. Specific techniques were chosen to allow conjugate characterisation and also to determine the effect of APE loading on each parameter. The rationale for selection of the methods used is briefly given below.

### *4.1.1* Polymer conformation in solution

A number of methods have been utilised historically to study polymer conformation in solution. These include viscosimetry (Frenoy, 1986), potentiometric titrations (Pederson et al, 1971), calorimetry (Biltonen & Friere, 1978), Raman spectroscopy (Brown et al, 1972), circular dichroism (Mezo et al, 2000), flow light scattering (Lee & Muller, 1999) and fluorimetric determinations (Ringsdorf et al, 1991). Many of these methods (particularly viscosimetry, potentiometric titration and calorimetry) were developed to evaluate polymers as plastics and pharmaceutical excipients, therefore large samples are often required and these methods are not particularly sensitive to minor changes in polymer conformation. Raman spectroscopy, circular dichroism and flow light scattering are more sensitive techniques but are quite specific to certain chemical and structural changes which are used to elucidate polymer conformational changes and do not detect more general changes in conformation. For this reason it was decided to use a fluorescent probe to try and understand the conformation of HPMA copolymer-APE conjugates in aqueous solution.

The use of fluorimetric methods to determine conformation rely on the change of fluorescence of a fluorescent probe when sitting within the polymer coil or simply free in solution. Many studies have used the hydrophobic fluorescent probe pyrene (Figure 4.1) which is extremely water insoluble (approx 0.2mg/l; Gautier, 1995) but has a strong fluorescence signal, which consists of three major emission peaks when excited at a wavelength of 330nm (Figure 4.1). These peaks represent the diffuse excimer emission centred at 382nm and two vibrational bands at 372nm ($I_1$) and 392nm ($I_3$). The relative intensities of $I_1$ and $I_3$ are extremely sensitive to the local solution

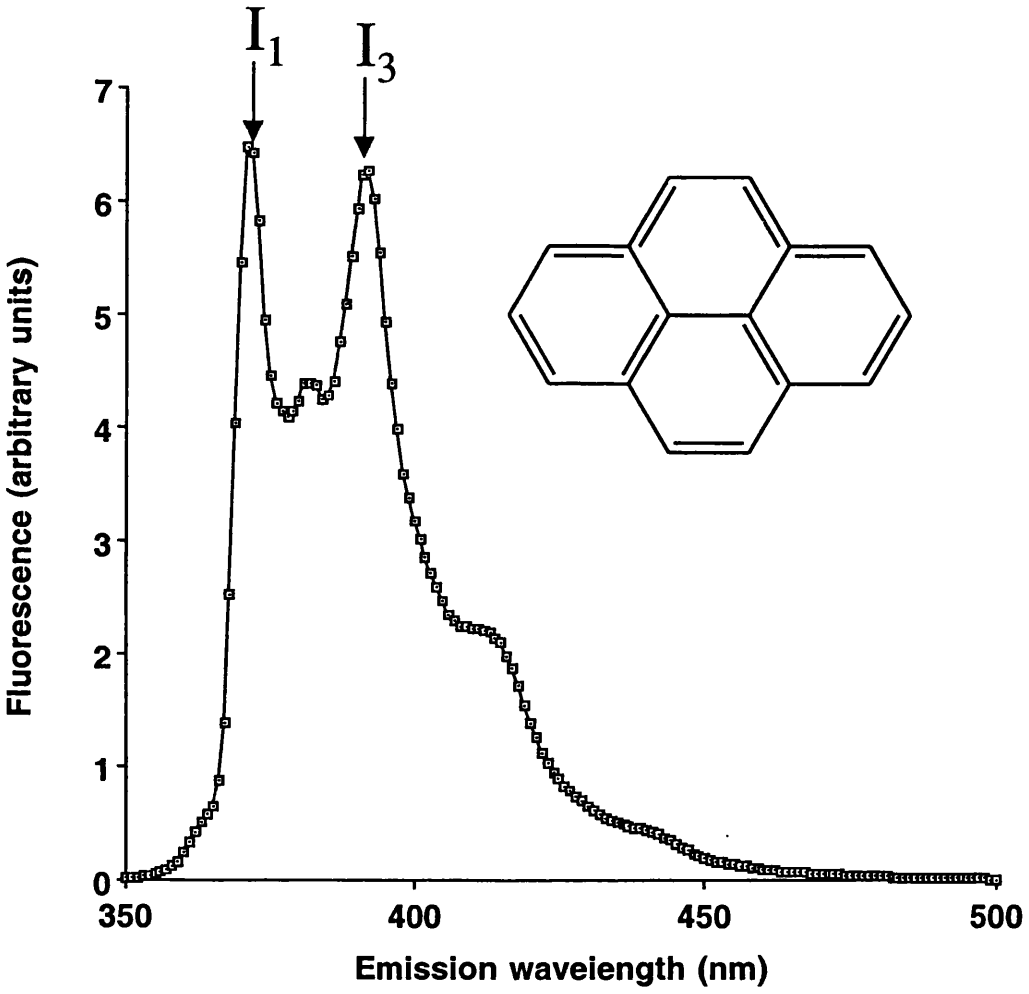



**Figure 4.1:** The structure of pyrene and its fluorescence spectrum (0.2mg/l in water) using an excitation wavelength of 330nm showing the intensity peaks $I_1$ (372nm) and $I_3$ (392nm).

microenvironment. The ratio ($I_1/I_3$) is therefore used to describe the polarity of a solution. An increase in this ratio is indicative of increased polarity and therefore a more hydrophilic environment for pyrene, the opposite also being the case (Winnik et al, 1991 and Gautier, 1995). The strong fluorescent signal of pyrene also allows the detection of small changes in intensity of the signal. A decrease in intensity indicates increased pyrene localisation in the solution as pyrene molecules will become self-quenching when closely associated.

*4.1.2* Drug release from HPMA copolymer-APE conjugates

Intracellular release of APE from HPMA copolymer-APE conjugates is essential for anti-tumour activity. This relies on effective access of cathepsin B to the GFLG linker and would theoretically be affected by solution conformation. Thus it was essential to characterise APE release from conjugates with different drug loading. This can be done by firstly isolating rat liver lysosomes as described in Section 2.3.4. This method has been used previously to examine the release of doxorubicin from PK1 (Subr et al, 1992) and will be used here as the same linker (GFLG) is used in the HPMA copolymer-APE conjugates

*4.1.3* Haemolytic activity of HPMA copolymer-APE conjugates

As mentioned previously ellipticines have been shown to cause severe haematotoxicity and minor immunogenicity upon i.v. administration (reviewed by Ohashi & Oki, 1996). Therefore it would be desirable for an HPMA copolymer-APE conjugate to be non-haematotoxic and non-immunogenic in order to facilitate parenteral administration. There has been extensive testing of the biocompatibility, immunogenic potential and toxicology of PK1 (Rihova et al, 1983; Simeckova et al, 1986; Rihova et al, 1989 and Duncan et al, 1998). These studies have shown that PK1 was not immunogenic (Rihova et al, 1989) and indeed other studies have shown that HPMA copolymers can reduce the immunogenicity of conjugated immunogenic proteins such as transferrin (Flanagan, 1987). Blood is the first biological environment encountered following parenteral administration and therefore any administered drug needs to be stable and non-toxic in this environment. As conjugation to HPMA copolymers has been shown to reduce immunogenicity of bound proteins and drugs and as ellipticines have only been shown to be mildly immunogenic (Alberici et al, 1986) screening for immunogenicity of the HPMA copolymer-APE conjugates was not considered necessary in the first instance. However it was felt to be important to determine the

haematotoxicity of the conjugates because of the known haemolytic potential of ellipticines (Section 1.5.2).

A simple method of determining any haematotoxicity was to incubate conjugates and free APE with a suspension of rat red blood cells and monitor release of haemoglobin spectrophotometrically (Section 4.2.3) as this method has been used previously to examine a conjugate of triton X-100 (Duncan et al, 1994) and a series of poly(amidoamines) (Richardson et al, 1999).

### *4.1.4* Cytotoxicity of HPMA copolymer-APE conjugates

Before HPMA copolymer-APE conjugates could be tested *in vivo* it was necessary to determine whether they might show anticancer activity. *In vitro* cytotoxicity of anticancer agents is traditionally assessed using a number of methods. These include assessment of cellular uptake of the macromolecular dye, trypan blue, to measure membrane integrity (Schlager & Adams, 1983); incorporation of a radiolabelled substrate such as [$^3$H]thymidine to measure DNA synthesis (Twentyman et al, 1984); enzyme release such as lactate dehydrogenase (LDH), another measure of membrane integrity (Choksakulnimitr et al, 1995) and metabolism of synthetic dyes such as MTT (Sgouras & Duncan, 1990).

Each of these methods has its own advantages and disadvantages. Macromolecular dyes can, over time, enter live as well as dead cells and so dye exclusion is therefore usually used as a crude method of excluding dead cells when making routine cell counts (as used in this study; Section 2.3.1.3 (Cook & Mitchell, 1989)). LDH release is a useful method particularly for detecting plasma membrane perturbation. LDH is found in the soluble cell fraction and therefore will be released into the extracellular fluid upon damage to the cell membrane. For testing non-membrane associated toxicity, however, this method would not be ideal as the processes of apoptosis and necrosis would be expected to damage cellular LDH leading to difficulties in properly analysing results (Choksakulnimitr et al, 1995). Sgouras & Duncan (1990) found that incorporation of [$^3$H]leucine and [$^3$H]thymidine can be inhibited by polymers binding to the cell membrane and preventing or limiting uptake of these substrates without necessarily causing a decrease in cell viability. This suggests that this method is not ideal for a study on polymer-drug conjugates.

Mosmann (1983) first described the use of MTT as a method of assessing cell viability. This method determines the metabolism of MTT to its formazan crystal

(reaction described in Section 2.3.1.6) which is carried out in the mitochondria. The formazan crystals are then determined using a colourimetric determination at 550nm. However there have been some reported difficulties associated with this assay. Some cell lines do not generate sufficient formazan for detection. There are also issues relating to the stability and solubility of the formazan metabolite in organic solvent systems (Sgouras & Duncan, 1990). Coloured drugs, for example doxorubicin, can interfere with the absorbance readout from an MTT assay (Wedge, 1991). However these problems can be overcome by using appropriate controls and suitable solvents (Sgouras & Duncan, 1990) and therefore this assay was to evaluate the *in vitro* cytotoxicity of the HPMA copolymer-APE conjugates containing different drug loadings (Section 2.3.1.6).

The aims of the studies described in this Chapter were to select suitable HPMA copolymer-APE conjugates for progression to anti-tumour studies in mouse models and to understand the effect of APE loading on solution conformation, tritosome-mediated APE release, haematocompatibility and *in vitro* cytotoxicity.

## 4.2 Methods

### *4.2.1* Determination of conjugate conformation using pyrene

Pyrene was dissolved to its maximum solubility in distilled water (0.2mg/l; $6x10^{-7}molL^{-1}$) and the solution filtered (using a syringe and 0.22μm filter) to remove any microcrystals. This stock solution was then used to prepare HPMA copolymer-APE and HPMA copolymer-aminopropanol conjugate solutions (3ml; 0-1mg/ml) in triplicate in cuvettes. The fluorescence spectrum of each sample was then recorded (excitation = 330nm and an emission scan of 350 to 450nm using a band pass of 4nm on both excitation and emission slits). The solution containing only pyrene was used to set the fluorescence maximum at 75% of the maximum detectable using this machine. This maximum was at 372nm in the spectrum taken within the 350 to 450nm range.

### *4.2.2* Tritosome-mediated release of APE from HPMA copolymer-APE conjugates

Tritosomes were isolated and assayed for protein content and enzyme activity using a standard substrate as described in Section 2.3.4. To determine thiol-protease degradation of HPMA copolymer-APE conjugates incubations were set up as described

in Section 2.3.4.4. A sample from any batch of tritosomes was retested the day before using that batch in a release study. Liberated APE (and in the case of control studies using PK1, doxorubicin) were quantified using the organic extraction and HPLC methods described in Sections 2.3.3.1 and 2.3.3.2 respectively.

***4.2.3*** Rat red blood cell lysis assay

Blood was obtained from male Wistar strain rats (250-350g body weight) by immediate cardiac puncture following asphyxiation with carbon dioxide and transferred into a heparin/lithium tube and kept on ice to minimise any damage to the erythrocytes. The blood was centrifuged at 1000g for 10min at 4°C, the supernatant discarded and some of the heparin beads carefully removed using a plastic pipette and then fresh pre-chilled (4°C) PBS (2ml) added. The red blood cell suspension was then centrifuged again at 1000g for 10min at 4°C and the process repeated twice until all the heparin beads had been removed. After the final centrifugation step the supernatant was removed and the pellet used to make a 2%w/v suspension of red blood cells, again using pre-chilled PBS.

Red blood cells (100µl of this 2%w/v suspension) were added to each well of a 96 well plate which already contained 100µl solutions of substances to be tested dissolved in PBS, or PBS alone as a control. To induce 100% lysis a 1%v/v solution of triton X-100 in PBS was used as a positive control (Duncan et al, 1994). These plates were then incubated for 1h at 37°C, and then they were centrifuged (1500g; 10min). Intact red blood cells pellet while any haemoglobin released remains in the supernatant. The supernatant was then pipetted into a fresh 96 well plate and assayed for haemoglobin at 550nm using a micro-titre platereader. The average absorbance of the wells containing triton X-100 was taken as 100% lysis and the absorbance of all other wells related to this value as a 100% haemoglobin release. Dextran was used as a negative control (0-5mg/ml) and poly(ethylene)imine (0-5mg/ml; $M_w$ approximately 750kDa) was used as a positive control (Duncan et al, 1991). An example of the haemolysis profiles of these reference compounds is shown in Figure 4.2 and both were used in each experiment to ensure reproducibility of the method.

***4.2.4*** *In vitro* cytotoxicity of HPMA copolymer-APE conjugates

First a growth curve was constructed for B16F10 cells (Figure 4.3) (methods as described in Section 2.3.1.5) and the doubling time of these cells was approximately 48h. Further experiments used cells in the exponential growth phase

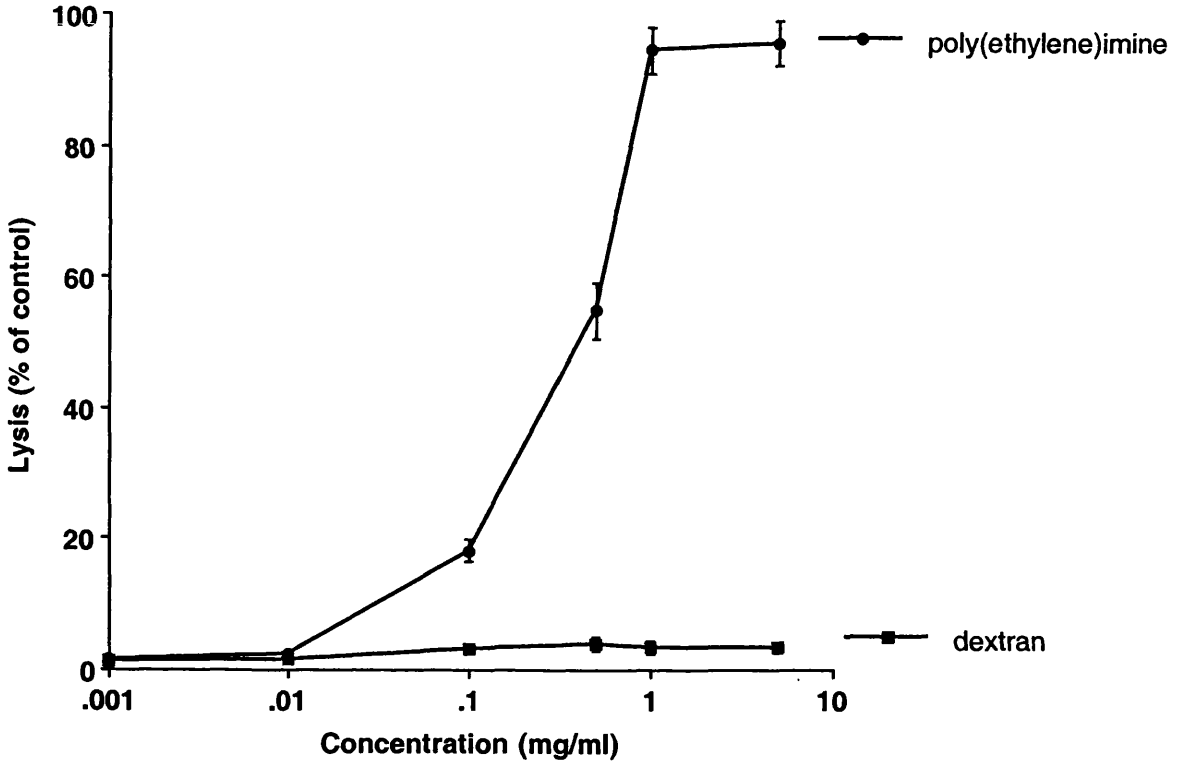



**Figure 4.2:** Haemolytic activity of dextran and poly(ethylene)imine.

**Note:** Data represent mean ± SD (n=6).

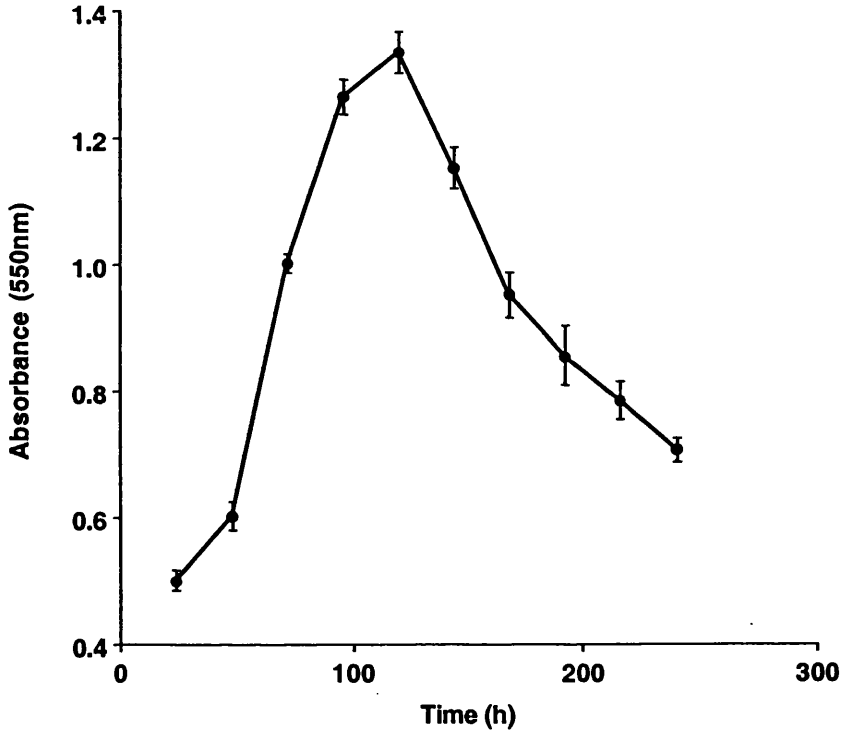



**Figure 4.3:** B16F10 growth curve.

**Note:** Data represent mean ± SD (n=6).

(i.e. the second or third day following sub-culture). Cytotoxicity of APE and HPMA copolymer-APE conjugates was determined using the method described in Section 2.3.1.6. Dextran (0-5mg/ml; Mw approximately 41kDa) was used as a negative control as it has been shown to be non-toxic and poly(L-lysine) (0-5mg/ml; Mw approximately 57kDa) was used as a positive control as it has been shown to be cytotoxic previously (Sgouras & Duncan, 1990). An example of a typical cytotoxicity profile for these control compounds is shown in Figure 4.4 and these reference controls incorporated into every experiment to ensure reproducibility.

## 4.3 Results

### *4.3.1* HPMA copolymer-APE conformation in solution

The $I_1$ (372nm) and $I_3$ (392nm) values for each conjugate were used to calculate the overall reduction in signal intensity, as a measurement of the localisation of pyrene in the solution. The $I_1/I_3$ ratio was also calculated for each concentration of conjugate to examine the local solution microenvironment characteristics.

Both HPMA copolymer aminopropanol (Figure 4.5) and HPMA copolymer-APE conjugates (Figure 4.6) caused a decrease in the overall fluorescence intensity of the spectrum. This can be clearly seen by plotting the pyrene $I_1$ value against conjugate concentration (Figure 4.7). The HPMA copolymer GFLG-aminopropanol conjugate showed little decrease in fluorescence over this concentration range (Figures 4.5 and 4.7). The HPMA copolymer GFLG-APE conjugate with the lowest loading (RK9) produces little decrease in pyrene $I_1$ over the concentration range used (Figures 4.6 and 4.7). However the HPMA copolymer GG-APE (RK4) and the other HPMA copolymer GFLG-APE conjugates (RK3 and RK10) all cause a considerable decrease in the pyrene fluorescence intensity (Figures 4.6 and 4.7). The pyrene $I_1/I_3$ ratio seen on addition of increasing concentrations of HPMA copolymer conjugates is shown in Figure 4.8. Figure 4.5 demonstrates also that APE (60μg/ml) does not itself cause any significant quenching of the pyrene solution.

### *4.3.2* Tritosome-mediated release of APE

Six batches of tritosomes were prepared for use in these studies and their protein content and thiol protease activity is summarised in Table 4.1. Batches RKT1, 2 and 4 were not used for tritosome release studies because of their low activity and protein content.

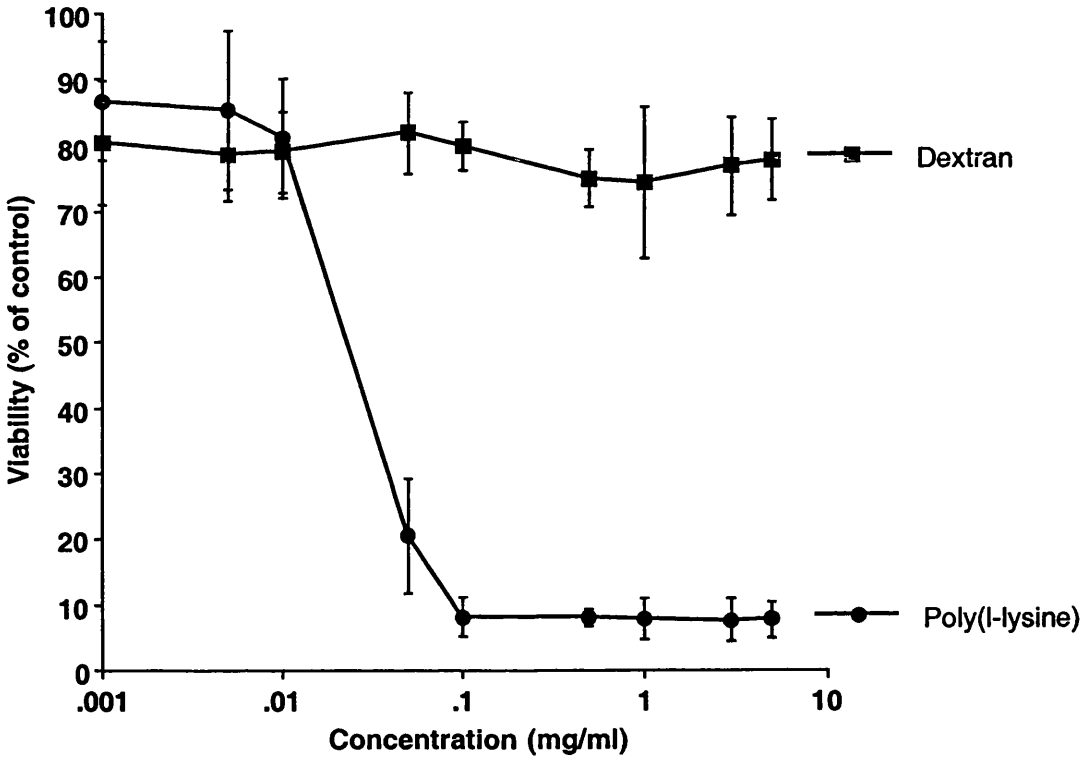



**Figure 4.4:** Cytotoxicity of dextran and poly(l-lysine) against B16F10 cells.

**Note:** Data represent mean ± SD (n=6).

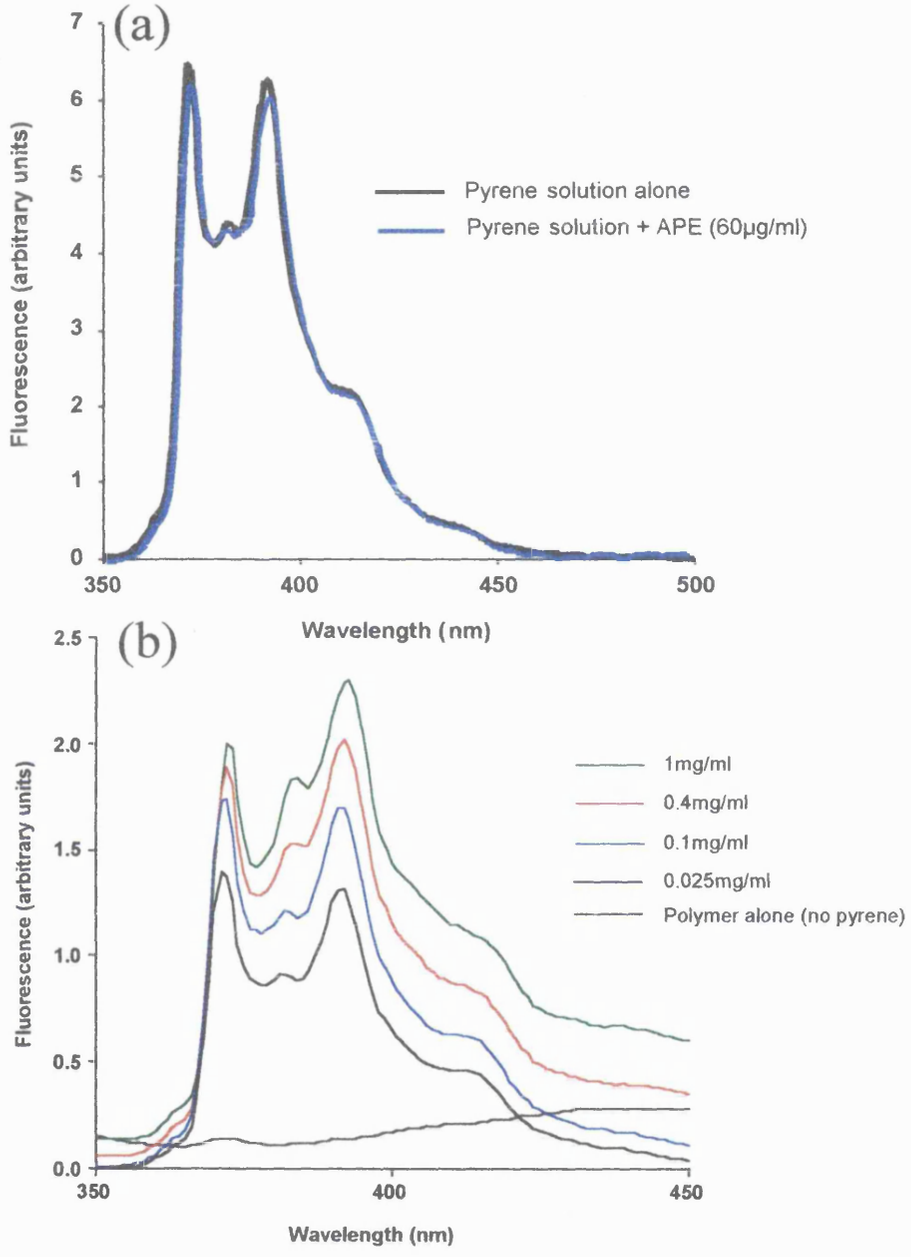



**Figure 4.5:** Fluorescence spectra of pyrene in the presence of APE and increasing concentrations of HPMA copolymer GFLG-aminopropanol.

**Note:** Panel a shows pyrene in the absence and presence of APE(60µg/ml) and panel b shows HPMA copolymer GFLG-aminopropanol.

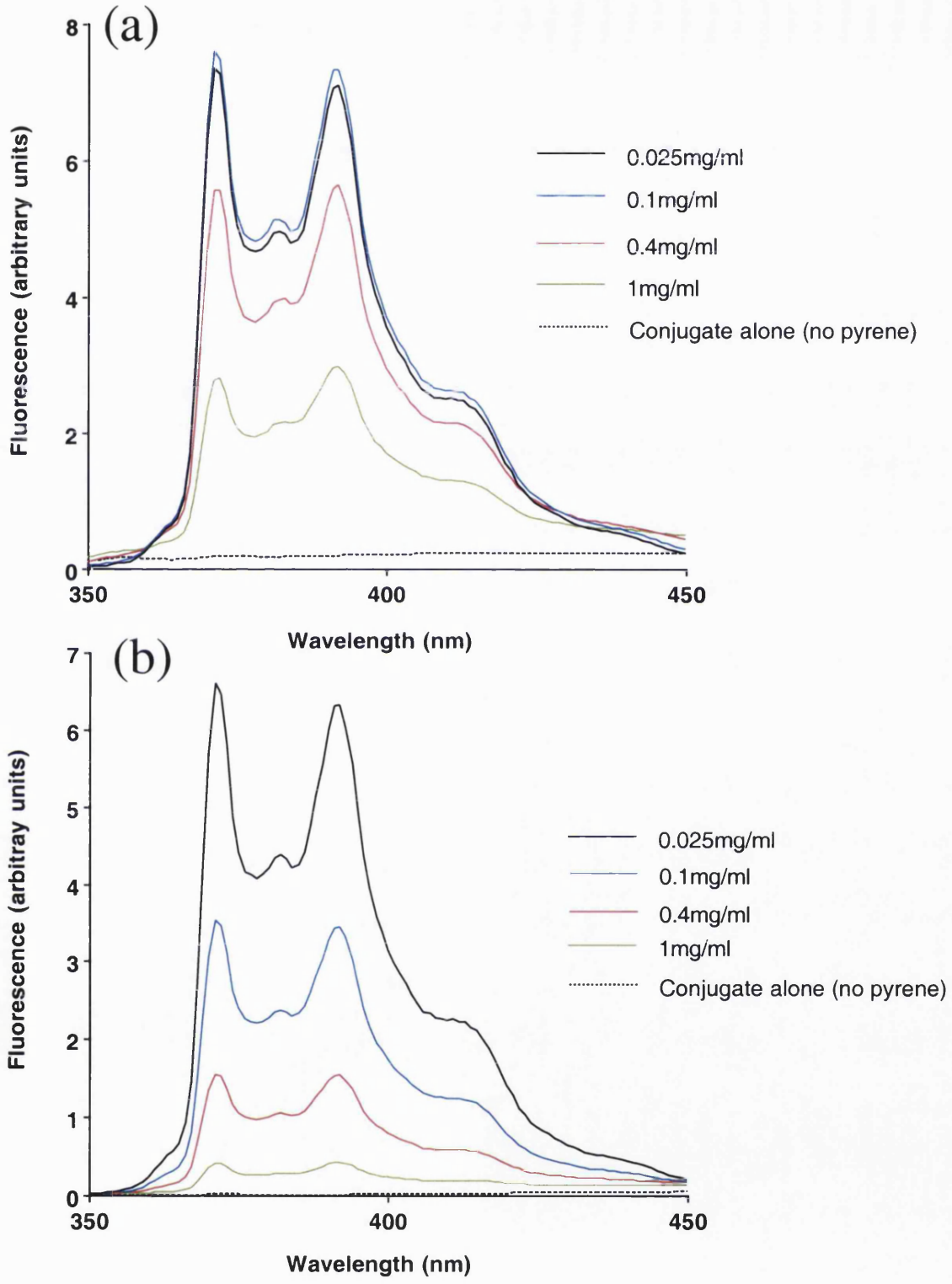



**Figure 4.6:** Fluorescence spectra of pyrene in the presence of increasing concentrations of the HPMA copolymer-APE conjugates.

**Note:** Panel a shows RK9 (HPMA copolymer GFLG-APE; 1.21%w/w APE) and Panel b shows RK1 (HPMA copolymer GFLG-APE; 3.07%w/w APE).

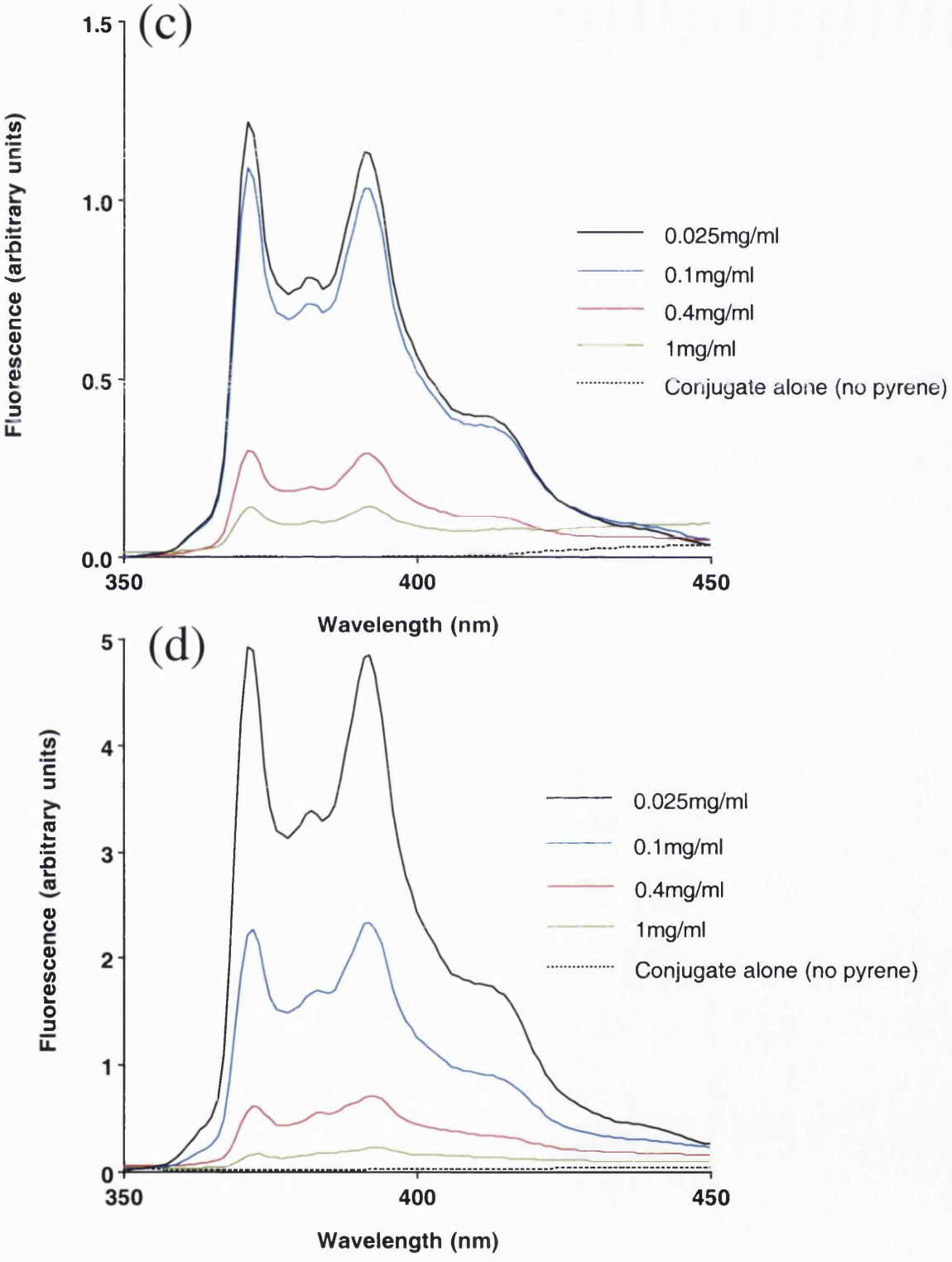



**Figure 4.6:** Fluorescence spectra of pyrene in the presence of increasing concentrations of the HPMA copolymer-APE conjugates.

**Note:** Panel c shows RK10 (HPMA copolymer GFLG-APE; 6.10%w/w APE) and Panel d shows RK4 (HPMA copolymer GG-APE; 4.27%w/w APE).

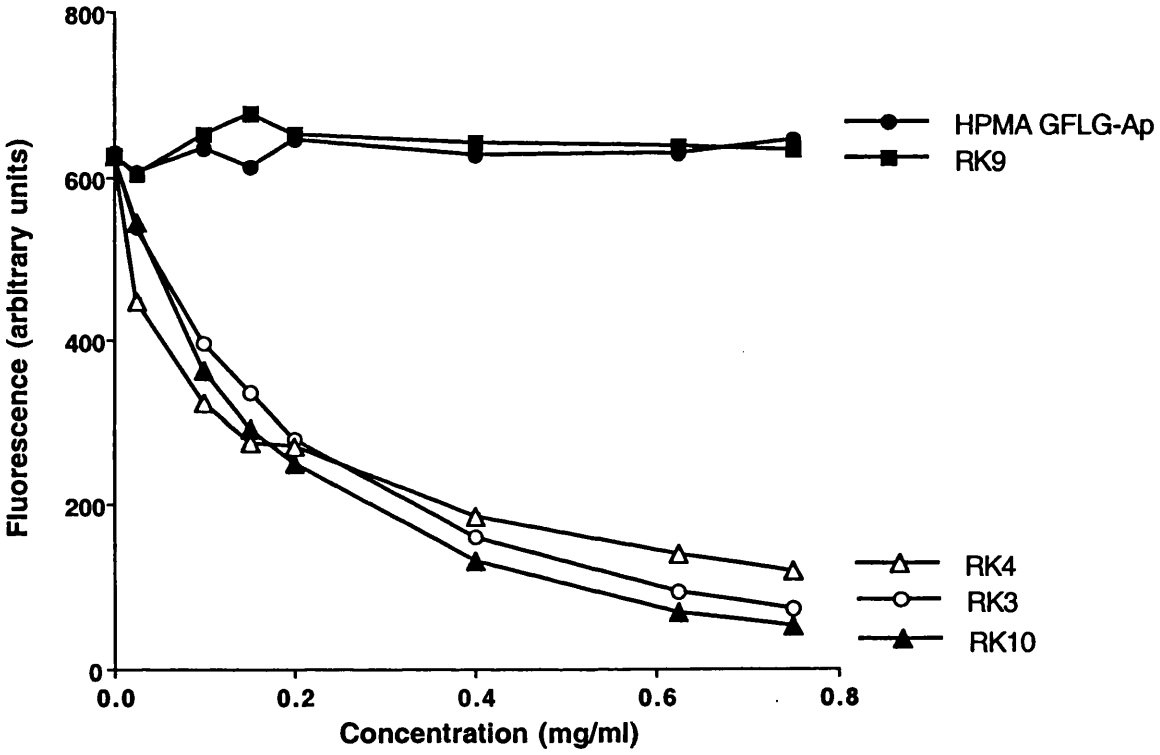



**Figure 4.7:** Relationship between pyrene $I_1$ and HPMA copolymer conjugate concentration.

**Note**: Data represent mean ±SD (n=3).

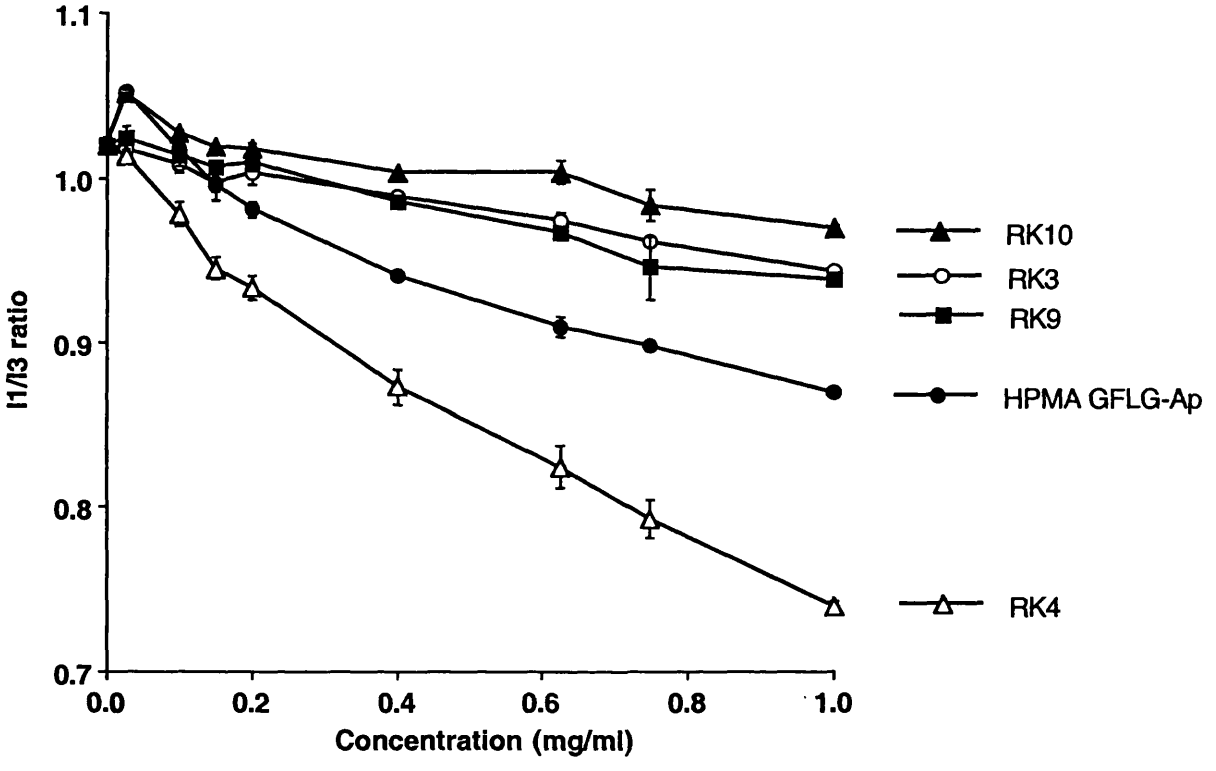



**Figure 4.8:** Relationship between the $I_1/I_3$ ratio of pyrene and HPMA copolymer conjugate concentration.

**Note:** Data represent mean ±SD (n=3).

**Table 4.1**: Characteristics of the tritosome batches.

| Code | Protein content (mg/ml) | Activity (nM/min/ml)* |
|---|---|---|
| RKT1† | 0.3 | 10.5 |
| RKT2† | 0.4 | 4.9 |
| RKT3 | 0.8 | 29.9 |
| RKT4† | 0.5 | 17.7 |
| RKT7 | 1.0 | 114.7 |
| RKT8 | 1.5 | 62.9 |

* Activity is expressed as nanomoles of NAp liberated/min/ml (see Section 2.3.4)

† Not used in degradation studies

A number of studies have been carried out using similar tritosome preparations to examine release of drug from PK1, HPMA copolymer-daunomycin and HPMA copolymer-melphalan conjugates (Subr et al, 1992 and Duncan et al, 1991) as all these conjugates utilise a GFLG linker. In these studies the GG linker was shown to be non-degradable and so an HPMA copolymer GG-APE conjugate was used in this study also as a comparator. All reaction solutions contained 1ml volume consisting of freshly prepared EDTA (1mM) and GSH (5mM) the remainder being conjugate, tritosomes and variable volumes of citrate phosphate buffer (pH5.5; prepared as described in Section 2.3.4.3). The first study undertaken (Figure 4.9) shows that APE is only released from the HPMA copolymer GFLG-APE conjugates in the presence of tritosomes while the HPMA copolymer GG-APE conjugate does not release drug even when incubated with tritosomes. APE is not released from either conjugate in the absence of tritosomes demonstrating the lack of non-specific degradability of these linkers. The second study (Figure 4.10) sought to examine the effect of substrate concentration on tritosome-mediated drug release using both HPMA copolymer GFLG-APE and PK1 as a comparison. The substrate concentration can be defined for these purposes as the quantity of bound drug present in the reaction. Three concentrations were chosen arbitrarily (25, 50 and 100μg/ml of reaction solution) and each were tested against the same activity of tritosomes.

Using an APE concentration of 50μg/ml in the reaction the effect of drug loading was examined using conjugates of low (RK9; 1.21%w/w APE), medium (RK3; 3.79%w/w APE) and high (RK10; 6.10%w/w APE) drug loading (see Table 3.2). As can be observed (Figure 4.11) the low and medium loaded conjugates released approximately 55% of bound drug within 5h while the high loaded conjugate released approximately 25%. Doxorubicin was cleaved from PK1 in this study (15%/5h) thus validating the experimental conditions.

### *4.3.3* Haematocompatibility of HPMA copolymer-APE conjugates

As the ellipticines have been shown previously to be haemolytic (Section 4.1.3) firstly the haemolytic potential of APE was examined. This study showed that APE (50% haemolysis at approximately 20μg/ml) is more haemolytic than ellipticine (50% haemolysis at approximately 800μg/ml) itself (Figure 4.12a). In a second series of experiments the haemolytic activity of a representative conjugate from each of the low, medium and high APE loadings of HPMA copolymer GFLG-APE conjugates and an HPMA copolymer GG-APE conjugate were compared to that of APE alone (Figure

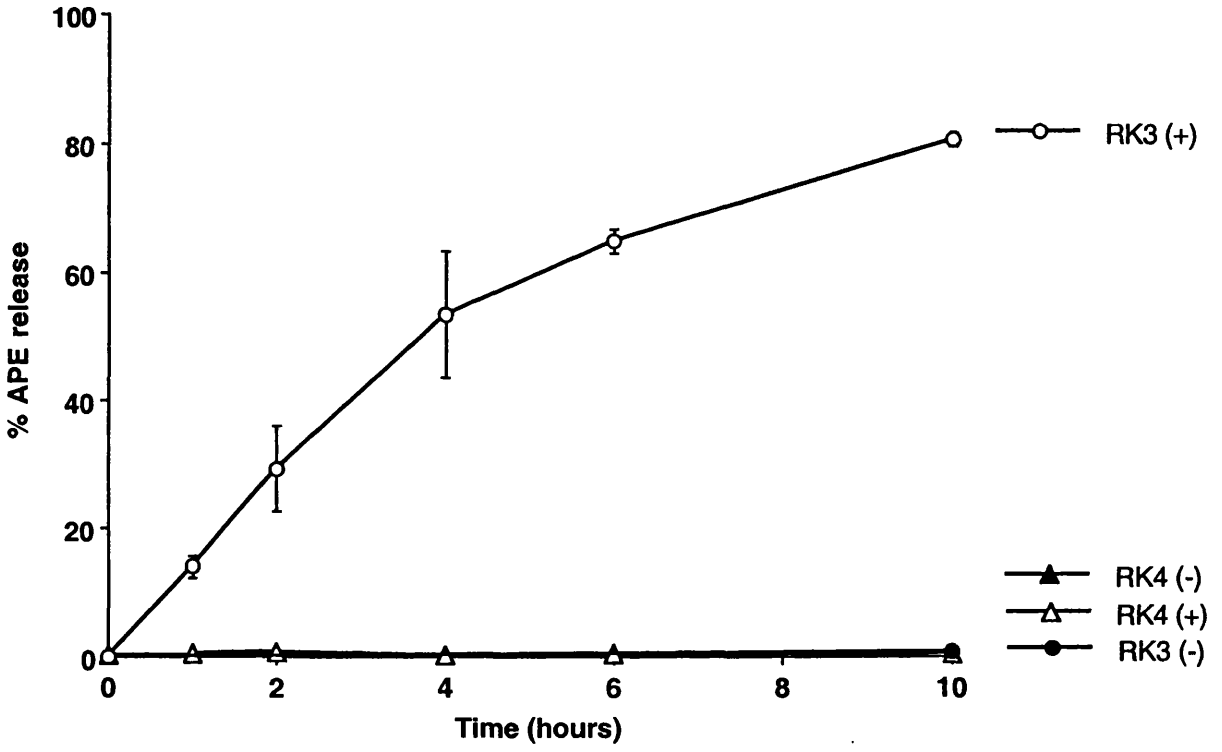



**Figure 4.9:** Release of APE from HPMA copolymer-APE conjugates in the presence (+) or absence (-) of tritosomes.

**Note:** RK3 is HPMA copolymer GFLG-APE (3.79%w/w APE). RK4 is HPMA copolymer GG-APE (4.27%w/w APE). Tritosome-containing tubes had tritosome batch RKT3 at an activity of 20ng/min/tube. Data represent mean ±SD (n=3)

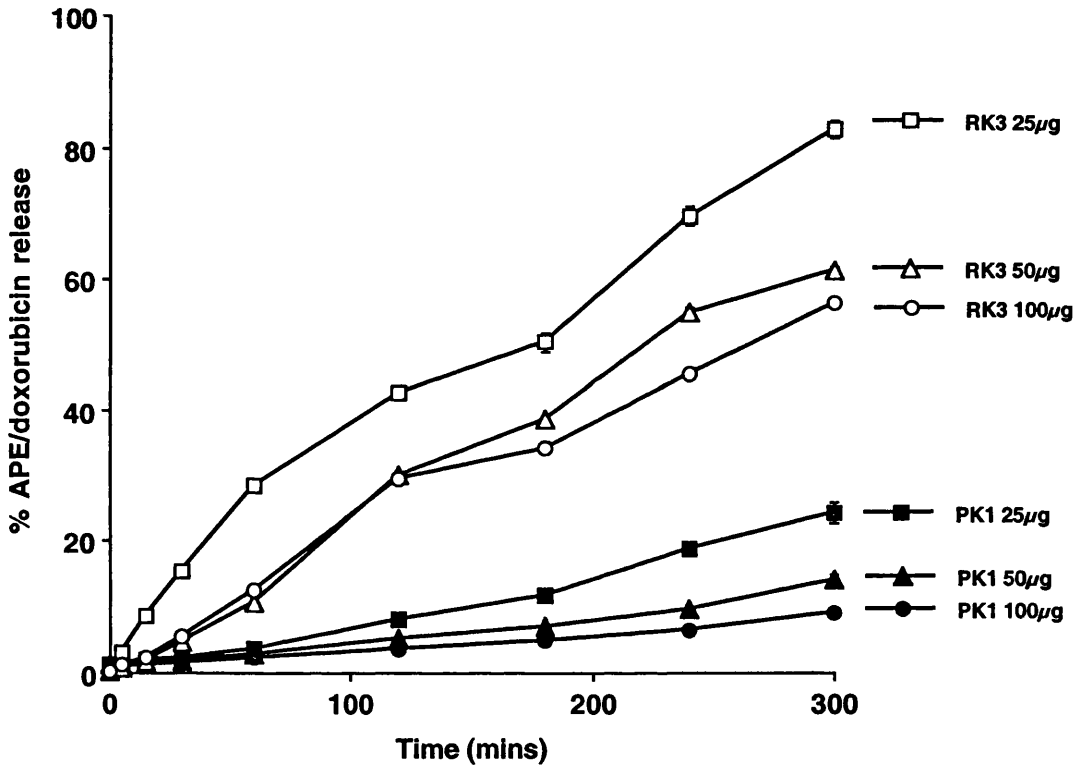



**Figure 4.10:** The effect of substrate concentration on tritosome-mediated release of APE from HPMA copolymer-APE and doxorubicin from PK1.

**Note:** RK3 is HPMA copolymer GFLG-APE (3.79%w/w APE). Tritosome-containing tubes had tritosome batch RKT7 at an activity of 20ng/min/tube. Data represent mean ±SD (n=3).

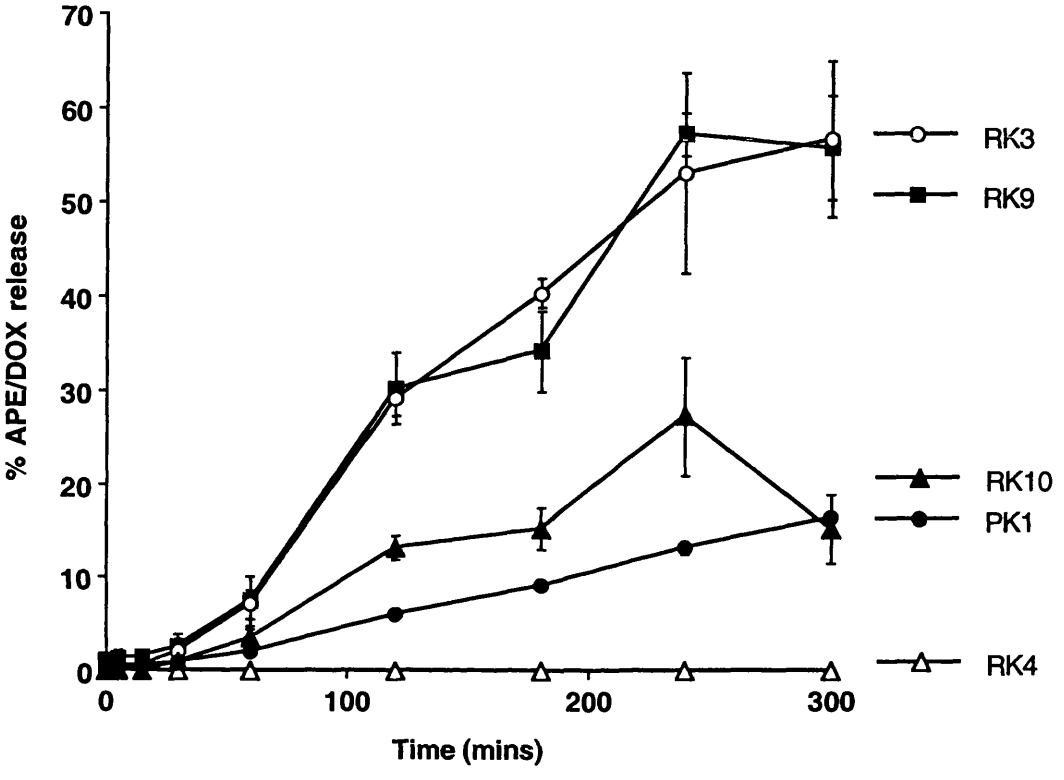



**Figure 4.11:** The effect of APE loading on tritosome-mediated release of APE from HPMA copolymer-APE and doxorubicin from PK1.

**Note:** RK3 is HPMA copolymer GFLG-APE (3.79%w/w APE). RK4 is HPMA copolymer GG-APE (4.27%w/w APE). RK9 is HPMA coploymer GFLG-APE (1.21%w/w APE). RK10 is HPMA copolymer GFLG-APE (6.10%w/w APE). Tritosome-containing tubes had tritosome batch RKT7 at an activity of 20ng/min/tube. Data represent mean ±SD (n=3)

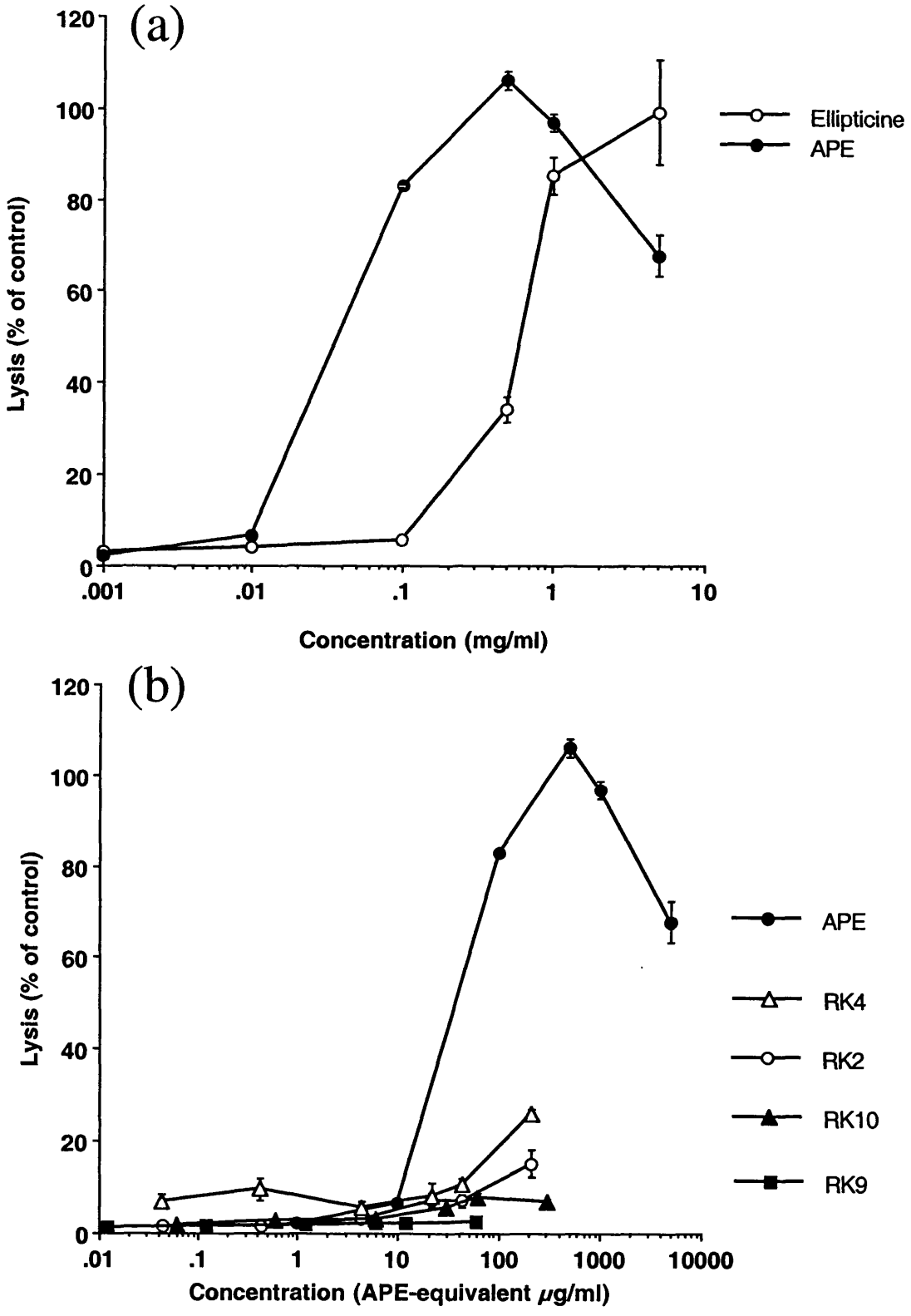



**Figure 4.12:** Haemolytic activity of APE, ellipticine and HPMA copolymer-APE conjugates.

**Note:** Panel a shows the comparative haemolysis caused by APE and ellipticine. Panel b shows the effect of drug loading on haemolysis. Data represent mean ±SE (n=6)

4.12b). All conjugates of different drug loading showed less than 20% haemolysis in this assay up to 100μg/ml of APE equivalence while APE alone gave over 80% lysis at this concentration.

### *4.3.4 In vitro* cytotoxicity

The *in vitro* cytotoxicity data can be expressed in either of two ways, either in terms of concentration of conjugates or as APE-equivalent concentration. The graph showing the concentration of conjugates versus APE alone shows that all the HPMA copolymer-APE conjugates are at least several hundred-fold less cytotoxic than free APE (Figure 4.13a). However, when this data is expressed in terms of APE-equivalence this gap narrows, particularly for the HPMA copolymer GFLG-APE conjugate of low APE loading and the HPMA copolymer GG-APE conjugate (Figure 4.13b). The remaining conjugates are still at least 100-fold less toxic than free APE.

## 4.4 Discussion

Pyrene has been used to give a myriad information on the hydrophobic/hydrophilic characteristics of a local solution environment. As described earlier the pyrene molecules in a solution containing areas of hydrophobicity would be distributed in high local concentrations in these areas. This would lead to self-quenching of the fluorescent signal which is easily detected. The intensity ratio ($I_1/I_3$) has been shown to decrease in more hydrophobic environments (e.g. 1.4 in water; 0.5 in hexane) and is therefore a sensitive measure of local solution hydrophobicity (Gautier, 1995). By dissolving a range of concentrations of HPMA copolymer-APE conjugates in a saturated solution of pyrene it was possible to examine the presence of areas of hydrophobicity in the HPMA copolymer-APE conjugates in solution by examining the fluorescence intensity of pyrene.

The coupling of a hydrophobic anticancer drug to the hydrophilic HPMA copolymer precursor was thought to affect the solution characteristics of the resultant conjugate but this has never been examined previously. This study sought to examine this effect and showed some unexpected results. The FL component of the GFLG linker has been shown to influence the solution characteristics of conjugates significantly. The conjugates seem to form micellar structures at low concentrations which is not unexpected considering the hydrophobicity of APE. A previous study

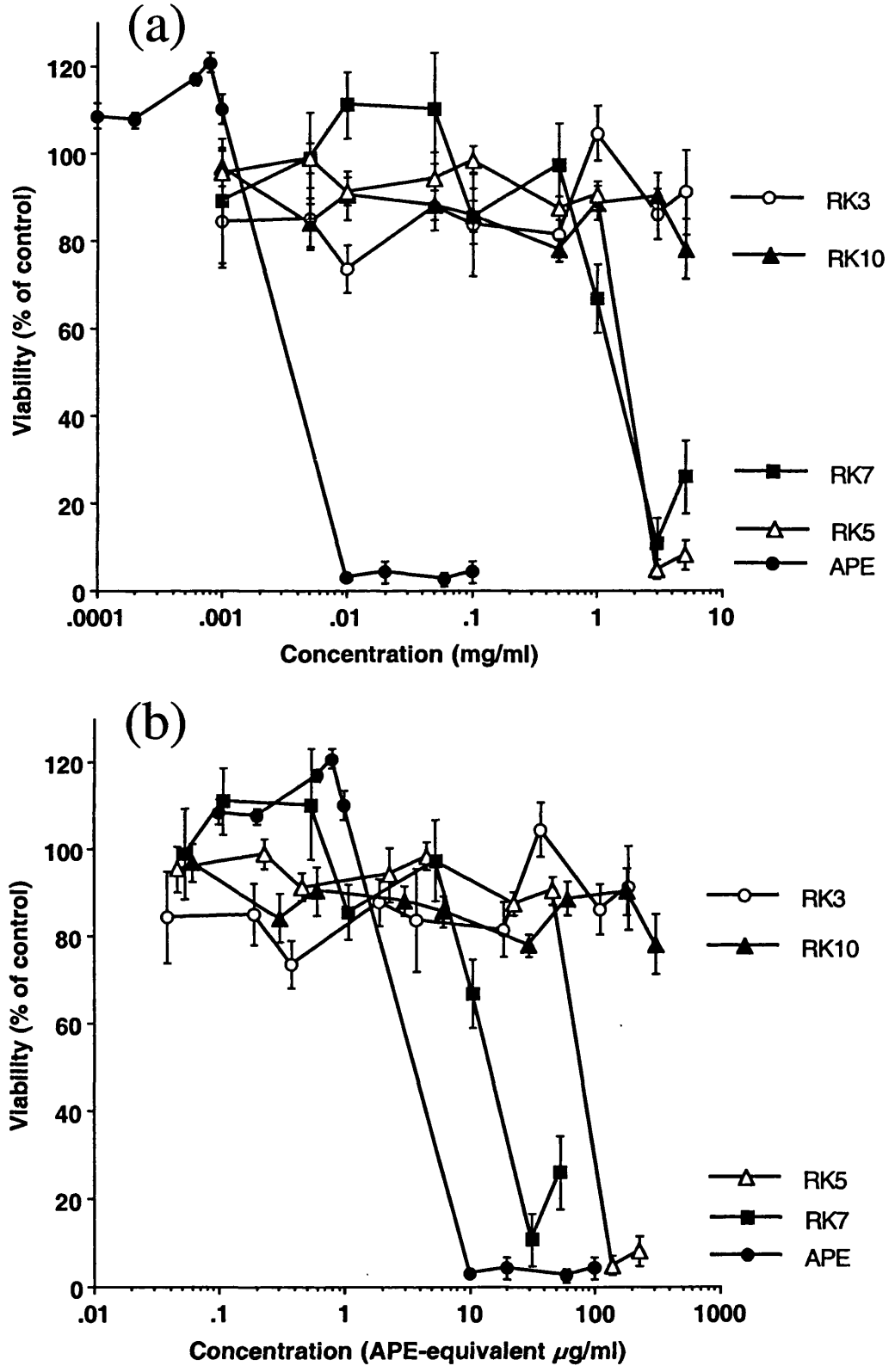



**Figure 4.13:** *In vitro* cytotoxicity of APE and HPMA copolymer-APE conjugates against B16F10 cells.

**Note:** Panel a shows the data in terms of concentration of APE and conjugates. Panel b shows the data in terms of APE-equivalent concentration. Data represent mean ±SE (n=6)

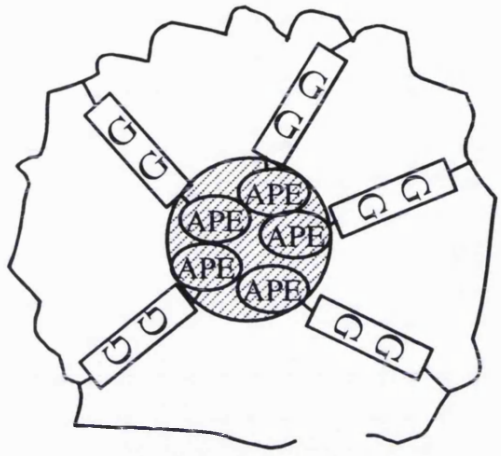
showed that pyrenyl molecules conjugated to poly(*N*-isopropylacrylamides) did indeed form such micelles (Ringsdorf et al, 1991). Also any micellar effect would be expected to be exacerbated by π-π stacking which is a common phenomenon between planar aromatic structures such as doxorubicin (Menozzi et al, 1984) and would be expected to occur with APE because of its similar planar aromatic structure. If the polymeric backbone is sufficiently flexible and hydrophilic (such as HPMA) then the hydrophobic drug could form these stacked conformations even after conjugation (Ilhan et al, 1999).

There is no evidence of drug loading affecting the pyrene $I_1/I_3$ ratio in the presence of the HPMA copolymer GFLG-APE conjugates but there may be differences in the size of the hydrophobic 'pockets' formed which were not detected using this approach. The clinical relevance of such a micellar structure is yet to be examined, if indeed these micellar structures reflect the situation following i.v. administration. To definitively determine the conformation of these conjugates it would be necessary to do structure property relationships of micelle stability. The stability of polymeric micelles is very strongly structure-dependent (Ringsdorf et al, 1992) and the equilibrium between an open chain coil and micelle forming units within the aqueous phase is very sensitive to the composition of the entire polymer (Ringsdorf et al, 1991).

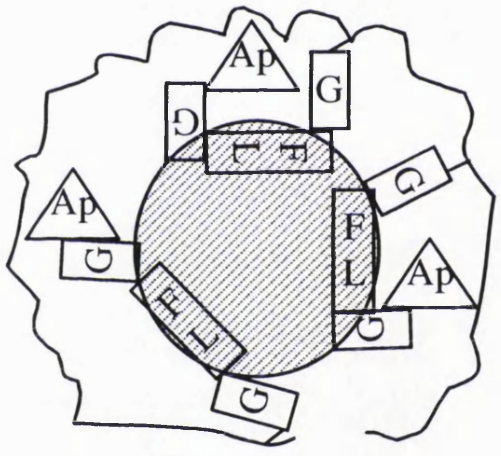
Examining Figure 4.8 it is interesting to observe that the HPMA copolymer GG-APE conjugate (RK4) seems to provide the most hydrophobic environment for the pyrene molecules. This is understandable though as the polymer has a clear distinction between its hydrophilic component (HPMA copolymer GG) and its only undisturbed hydrophobic part (APE) (represented in Figure 4.14). What is interesting is that the HPMA copolymer GFLG-APE conjugate of medium APE loading (RK3) seems to offer a less hydrophobic surrounding for pyrene than the comparable HPMA copolymer GFLG-aminopropanol. Again, a possible explanation might be that the APE containing conjugate, although more hydrophobic, cannot form a stable micelle due to the two glycyl units disturbing a defined separation of hydrophobic and hydrophilic domains. This could be explained by the formation of FL loops in both cases, the hydrophilic aminopropanol and HPMA copolymer GG elements stacking on the outside for the aminopropanol conjugate while the conformation of RK3 is more disturbed by the hydrophobic APE (represented in Figure 4.14). This is a speculative explanation but considering the sensitivity of micelle stability it may not be completely outlandish.

When considering the $I_1/I_3$ ratio data the three HPMA copolymer GFLG-APE conjugates of variable drug loading (RK3, RK9 and RK10) do not differ much because

**HPMA copolymer GG-APE**
**Most stable micelle**




**HPMA copolymer GFLG-Ap**
**Less stable micelle**




**HPMA copolymer GFLG-APE**
**Least stable micelle**

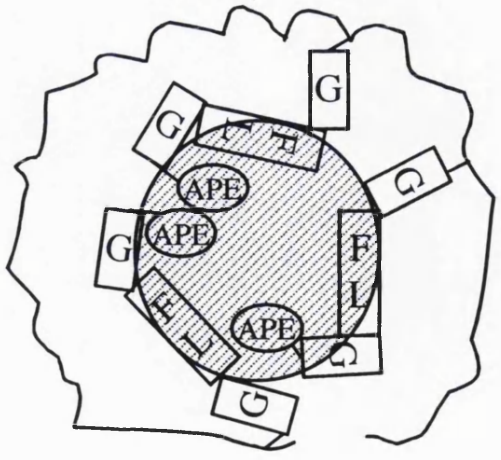



**Figure 4.14:** Proposed conformation of HPMA copolymer-APE and HPMA copolymer-aminopropanol conjugates.

**Note:** The shaded area represents the area of hydrophobicity central to each proposed micelle.

pyrene will sense the presence of hydrophobicity but not the size of these hydrophobic pockets (Figure 4.8). This may be the only difference between these conjugates.

The stability of the GFLG and GG linkers used in this study have been examined in rat plasma and serum (Rejmanova et al, 1985). A variety of different tri- and tetra-peptide linkers as well as one each of a penta- and a hexa-peptide linker were tested by detecting, using UV spectroscopy, the release of NAp from the terminal group of the linkers. The GFLG linker was chosen for the present study to synthesise HPMA copolymer-APE conjugates for the reasons outlined in Section 1.3.4.2. This linker released 1.5 and 1.7% of total NAp in plasma and serum respectively in a 5h time period. No significant release in the circulation has been reported for those polymer-drug conjugates which utilise this linker and PK1, for example, has been shown to release only small amounts of doxorubicin in the circulation of patients in clinical trial (Thomson et al, 1999).

Release of drug from the HPMA copolymer conjugates must occur for these conjugates to have anti-tumour activity. The tritosome-mediated APE release studies reported here have demonstrated the stability of the GFLG linked APE in the absence of tritosomes. This lack of non-thiol protease release of APE is also confirmed by the observed lack of haematotoxicity and *in vitro* cytotoxicity of the HPMA copolymer-APE conjugates. There seemed to be a limit of cathepsin B access in the case of the HPMA copolymer GFLG-APE conjugate of highest loading which showed approximately half the release of that seen from the low and medium loaded conjugates. Interestingly APE was released more rapidly from the low and medium loaded conjugate than doxorubicin was cleaved from PK1 under the same conditions at the same substrate concentration perhaps indicating that APE allows cathepsin B easier access to the terminal bond than doxorubicin. However as the ideal profile of drug exposure is likely to vary depending on the drug it is not known whether quicker release is a benefit. This has been demonstrated with the toxicity reported in animal studies of an HPMA copolymer GLFG-doxorubicin conjugate which released doxorubicin more rapidly than the GFLG linker in tritosome release studies (Subr et al, 1992).

This present study also demonstrated that at these concentrations of substrate and enzyme activity of tritosomes the enzymes were not saturated. This was not unexpected as previous studies on PK1 cited substrate concentrations of doxorubicin-equivalence of approximately 600μg/ml (Subr et al, 1992).

For a polymer-drug conjugate to be administered by the parenteral route it must be shown to not cause toxicity in the bloodstream either as the intact conjugate or by inappropriate release of active drug. Many anticancer drugs have been shown to extravasate at the site of administration into subcutaneous or subdermal tissue causing severe tissue necrosis because of the high local concentration of a toxic agent. For this reason potent anticancer agents are usually administered as slow infusions of low concentrations (British National Formulary, 2001) and those polymer-drug conjugates in clinical trial have also been administered in this way (Vasey et al, 1999 and Meerum Terwogt et al, 2001). It has been known for some time that macromolecules such as polymer-drug conjugates have longer half-lifes in the circulation than the equivalent low molecular weight compounds (reviewed by Duncan & Spreafico, 1994). Therefore a polymer-drug conjugate will be exposed to the blood and surrounding tissue for a longer period of time relative to administered free drug.

Toxicity could potentially occur by a number of mechanisms following administration. The polymer-drug conjugate could itself be toxic if the drug is still active when conjugated. The linker may be unstable and result in inappropriate drug release into the circulation hence causing toxicity.

As described in Section 4.1.3 it is well recognised that one of the major difficulties associated with the clinical use of ellipticines has been their tendency to be haematotoxic. HPMA copolymer conjugates of anticancer drugs have been shown to abrogate many of the usual toxicities of the administered drug in previous studies. The HPMA copolymer-APE conjugates were all significantly less toxic than APE alone in this haemolysis assay. This demonstrates the ability of HPMA copolymers to protect the drug from causing such toxicities while APE was shown to be haemolytic as expected from reports on other ellipticines (Lee, 1976). This study showed that APE is more haemolytic than ellipticine itself and therefore parenteral administration of APE could lead to haematotoxicity. The observed lack of haemolysis of the HPMA copolymer GFLG-APE conjugates bodes well for future administration of these conjugates in *in vivo* anti-tumour studies.

In the *in vitro* cytotoxicity assays there was some evidence of cytotoxicity for the two APE-containing conjugates which had been shown to adopt an open solution conformation in the pyrene studies. Some, but not all, of this toxicity could be explained by the presence of free drug in the HPMA copolymer-APE conjugate but

other mechanisms seem to play a role. The classical method of testing anticancer agents is to investigate their *in vitro* cell cytotoxicity against a range of cell lines and hence determine specificity towards certain cell and hence tumour types. This method is still widely used and indeed is the basis for testing compounds in the NCI screening process (Cragg et al, 1999). This simple test is suitable only for molecules which are active in their own right but is limited in the case of those molecules and systems requiring activation in some manner (e.g. prodrugs or drug delivery systems).

Polymer-drug conjugates rely on uptake by fluid-phase pinocytosis, in the absence of other carrier mechanisms (e.g. receptor-mediated uptake) as described in Section 1.4.4. The rate of uptake by this type of endocytosis is approximately 2μl of the surrounding medium being taken up per mg of protein per hour incubation (Duncan & Kopecek, 1984). Therefore only a small amount of conjugate will be taken up over the time course of a classical 72h cytotoxicity assay. As well as uptake the conjugate needs to be trafficked to the lysosome and the drug released before cytotoxic activity can occur. Therefore polymer-drug conjugates invariably show no or minimal toxicity in these assays although one must be careful to examine the role of free drug in any observed conjugate toxicity. It was still considered useful to evaluate the cytotoxicity of these conjugates as any unexpected toxicity could be determined.

Toxicity could be indicative of the presence of a significant quantity of free drug, instability of the linker, another biological process being capable of liberating free drug or perhaps an indication of activity of the drug even while conjugated. If one examines the cytotoxicity profile in terms of equivalent concentration of APE then all the HPMA copolymer-APE conjugates were less toxic than the parent APE as would be expected from their differing modes of cellular uptake. The HPMA copolymer GFLG-APE conjugate with the lowest loading (RK7) had a higher concentration of free APE than the other conjugates and a portion of its apparent toxicity can be explained by the presence of this free drug. However this does not completely explain the cytotoxicity of this conjugate. One possible explanation may be that the conjugate retains some activity towards the cell membrane and combined with the more open structure of this conjugate as determined in the conformation study the APE may be more exposed. Another possible explanation would be that because it forms a more open structure in solution this conjugate is interacting more closely with the cellular membrane and being internalised and therefore cleaved more quickly within the time scale of the experiment. This could also explain the cytotoxicity seen for the HPMA copolymer GG-APE

conjugate (RK4) as it also has been shown to adopt a more open structure in the solution conformation studies (Section 4.3.1).

The only conjugate which showed haemolytic activity was the HPMA copolymer GG-APE conjugate, RK4. This, in combination with the cytotoxicity of this conjugate, could suggest there being some element of surface activity mediated by the more open solution structure of this conjugate. It is important to note that while this haemolysis data seems to contradict the cytotoxicity of the HPMA copolymer GFLG-APE conjugates of low drug loading (RK7/RK9) cytotoxicity is a 72h assay while this red blood cell lysis assay was over 1h. In addition to this red blood cells do not have a process of endocytosis and their membranes have been shown to be more stable than other cellular membranes because of their different lipid composition and the presence of elevated amounts of cholesterol (reviewed by Weinstein, 1969). This would not therefore preclude increased uptake by endocytosis or some element of surface activity playing some role in the cytotoxicity of these two conjugates (namely an HPMA copolymer GFLG-APE conjugate of low drug loading or an HPMA copolymer GG-APE conjugate).

In summary HPMA copolymer-APE conjugates form complex micellar structures in solution. APE is only liberated by thiol proteases and therefore no *in vitro* cytotoxicity or haematotoxicity was observed. This reduction in haematotoxicity is important because of the clinical difficulties with haematotoxicity observed with the use of ellipticine derivatives (Section 1.5.2). There was no effect of drug loading on haematotoxicity but there seemed to be some effect on the *in vitro* cytotoxicity of the conjugates. These studies do not preclude investigation of any of the HPMA copolymer GFLG-APE conjugates in anti-tumour studies *in vivo*. Therefore the next study tested these HPMA copolymer GFLG-APE conjugates *in vivo*.

# Chapter Five

## *Effect of APE loading on anti-tumour activity of HPMA copolymer GFLG-APE conjugates*

## 5.1 Introduction

Conjugation of APE to HPMA copolymers reduced APE haematotoxicity and APE was shown to be released from the conjugates *in vitro* using thiol proteases isolated as lysosomes (Chapter 4). These observations justified testing HPMA copolymer-APE conjugates *in vivo* against murine tumour models. As described in Section 4.4 many of the anticancer agents in development are first tested against a panel of tumour cell lines *in vitro* to determine firstly whether they are active and secondly the spectrum of their anticancer activity. This approach is not suitable as a screen for drugs requiring activation such as prodrugs or polymer-drug conjugates due to the inherent limitations of these assays discussed in Section 4.4. It has been shown that HPMA copolymer conjugates are preferentially retained in solid tumours by the EPR effect (Section 1.4.3) and that HPMA copolymer paclitaxel, camptothecin and platinate have increased activity against a variety of solid tumours. PK1 has also shown activity in a number of murine and human xenograft tumour models *in vivo* which were not sensitive to doxorubicin alone (Duncan et al, 1992; Table 5.1).

The models used for pre-clinical evaluation of PK1 were mainly mouse models (Table 5.1) and were subsequently shown to be good predictors of the activity and reduction in toxicity observed in the Phase I human clinical trial (Vasey et al, 1999). In these mouse studies PK1 showed increased activity over doxorubicin alone and a lower incidence of toxicity allowing higher doses to be administered (Duncan et al, 1992). Therefore mouse tumour models were chosen as suitable for examining anti-tumour activity of HPMA copolymer GFLG-APE conjugates. In the first instance a s.c. B16F10 murine melanoma model in C57Bl male mice was chosen as this model has been widely used to study other HPMA copolymer conjugates (Duncan et al, 1992 and Gianasi et al, 1999). These s.c. B16F10 tumours take approximately 10 days to develop to a size of 25-50mm$^2$. When the tumour is not treated animals usually progress to the maximum allowable tumour area within a further 7 days. Therefore this is a particularly aggressive model and any anti-tumour activity in this model is promising. The B16F10 melanoma has shown significant and sustained accumulation of PK1 and HPMA copolymer-platinates by the EPR effect (Seymour et al, 1994 and Gianasi et al, 1999). Indeed Seymour et al (1994) showed that PK1 administered i.v. at a doxorubicin equivalent dose of 5mg/kg produced a greater than 10-fold increase in total drug levels in the tumour after 1h compared to that seen following administration of free doxorubicin given at the same dose. Also, following a lag phase during which PK1 is

**Table 5.1:** PK1 activity in a panel of *in vivo* tumour models (Duncan et al, 1992).

| Host animal | Cell line and origin | Site | Route of administration (dosing schedule) | Doxorubicin†† | | PK1†† | |
|---|---|---|---|---|---|---|---|
| | | | | Dose (mg/kg) | T/C (%) | Dose (mg/kg equiv.) | T/C (%) |
| CDF1 mice | L1210 murine leukaemia | i.p. | i.p. (Days 1, 2, 3) | 5 | 214 | 30 | 430 |
| CDF1 mice | L1210 murine leukaemia | i.p. | i.v. (Days 1, 2, 3) | 22 | 183 | 100 | 233 |
| CDF1 mice | M5076 murine reticulosarcoma | s.c. | i.v. (Days 5, 9, 15) | 10 | 125 | 20 | 319 |
| CDF1 mice | M5076 murine reticulosarcoma | s.c. | i.p. (Days 5, 9, 15) | 10 | 102 | 20 | >264 |
| CDF1 mice | M5076 murine reticulosarcoma | i.v. | i.v. (Days 3, 7, 11) | 10 | 148 | 30 | >244 |
| Wistar rats | †Walker sarcoma | s.c. | i.p. (Day 0) | 10 | 88 | 10 | >196 |
| DBA2 mice | †P388 murine leukaemia | s.c. | i.p. (Days 0, 1, 2) | 3 | 117 | 18 | >312 |
| C57BL/6J mice | †B16F10 murine melanoma | s.c. | i.p. (Days 0, 1, 2) | 5 | 133 | 36 | >320 |
| *nu/nu* mice | †LS174T human colorectal carcinoma xenograft | s.c. | i.p. (Days 0, 1, 2) | 5 | 70 | 18 | 200 |

†tumours established before treatment commenced

††Doses and T/C(%) shown represent the maximum anti-tumour activity seen in each experiment where a range of doses were typically administered

trafficked to the lysosome and doxorubicin is released, higher levels of free doxorubicin were maintained in the tumour over the subsequent 24h at this equal dose and higher doses of PK1 gave further improvements in tumour levels of doxorubicin. Therefore this tumour model displays the typical characteristics of the EPR effect (Section 1.4.3). It was hoped that HPMA copolymer-APE conjugates could benefit from this change in pharmacokinetic profile mediated by the nature of the HPMA copolymer conjugate.

The LS174T tumour model is a human colorectal xenograft model and was used in the evaluation of PK1 (Table 5.1). Such xenograft models have become vital to our understanding of human tumours since the development of mice lacking in T lymphocytes to eradicate immune reaction to the implantation or administration of human cancer cells (Van Putten, 1987). For that reason it was decided that a pilot study would be carried out on an HPMA copolymer GFLG-APE conjugate using a human non-small cell lung carcinoma model (CORL23) in *nu/nu* mice. This model has previously been used to evaluate PK1 activity and the polymer-directed enzyme prodrug therapy (PDEPT) concept. Satchi (1999) showed that PK1 had reduced activity compared to doxorubicin alone (T/C% 115 vs 130) when administered i.v. at an equal doxorubicin-equivalent dose of 10mg/kg. However there was no PK1-associated toxicity at this low dose and therefore dose escalation would be expected to lead to increased activity. CORL23 was therefore selected as a suitable xenograft model for testing HPMA copolymer-APE conjugates. These tumours, when injected s.c., are allowed to grow to a size of 25-50mm$^2$ (within 12 to 15 days) before administration of treatment. Untreated animals usually progress to the maximum allowable tumour area within approximately 30 days.

The extravasation of macromolecules into a variety of tumours in mice has been characterised based on the tumour size for a number of these model systems (Sat, 1999). Both PK1 and the macromolecular dye, Evans Blue, were injected as probes into tumour-bearing mice and the accumulation in each of the tumours measured. For many of the tumours examined smaller tumours took up more of the probes as these have been shown to be more angiogenic in nature and therefore to have a more leaky vasculature (Carmeliet & Jain, 2000). This was the case with the B16F10 model which showed uptake of approximately 14% dose/g of tumour after 1h for tumours weighing approximately 50mg and approximately 1% dose/g of tumour after 1h for tumours weighing approximately 1000mg. Therefore the B16F10 model is an example of a

tumour which shows tumour size-dependent uptake. The CORL23 model was also examined in a similar study and showed non tumour size-dependent uptake with approximately 8% dose/g of tumour across the weight range studied (10-500mg).

Therefore these two tumour models were selected to examine the anti-tumour activity of the HPMA copolymer GFLG-APE conjugates of different APE loading.

## 5.2 Methods

### *5.2.1* Cell preparation

The growth curve for B16F10 cells has been described in Section 4.2.4. A growth curve was also constructed for the CORL23 cells using the method described in Section 2.3.1.5 (Figure 5.1). These cells therefore have a doubling time of approximately 48h and all cell suspensions were thus prepared on day 2. Cell suspensions were prepared in saline using the method described in Section 2.3.1.7 when the cells were in the exponential growth phase.

### *5.2.2* Establishment of s.c. tumours

Tumours were established using cell suspensions in saline of $1x10^6$cells/ml (B16F10) or $1x10^7$cells/ml (CORL23) as described in Section 2.3.1.7. Of these suspensions 100µl was injected s.c. into the nape of the neck of the lightly anaesthetised C57Bl male mice (6-8 weeks old; approximately 25g body weight) or *nu/nu* male mice (6-8 weeks old; approximately 25g body weight) respectively. Tumours were allowed to establish to an area of 25-50mm$^2$ and animals were then randomised and APE, conjugate or saline (control) injected i.p. under light anaesthesia as described in Section 2.3.2.1.

### *5.2.3* Determination of APE MTD in tumour-bearing mice

The MTD can be defined as the highest dose which can be administered without significant toxicity to animals being observed. B16F10 melanoma cells were injected s.c. as for anti-tumour studies and tumours were allowed to establish to a diameter of 25-50mm$^2$ as described in Section 2.3.2.1. APE was then administered i.p. at a dose of 25mg/kg which was the reported MTD i.p. of APE for mice (NCI, personal communication). The animals were then monitored for tumour size and weight every

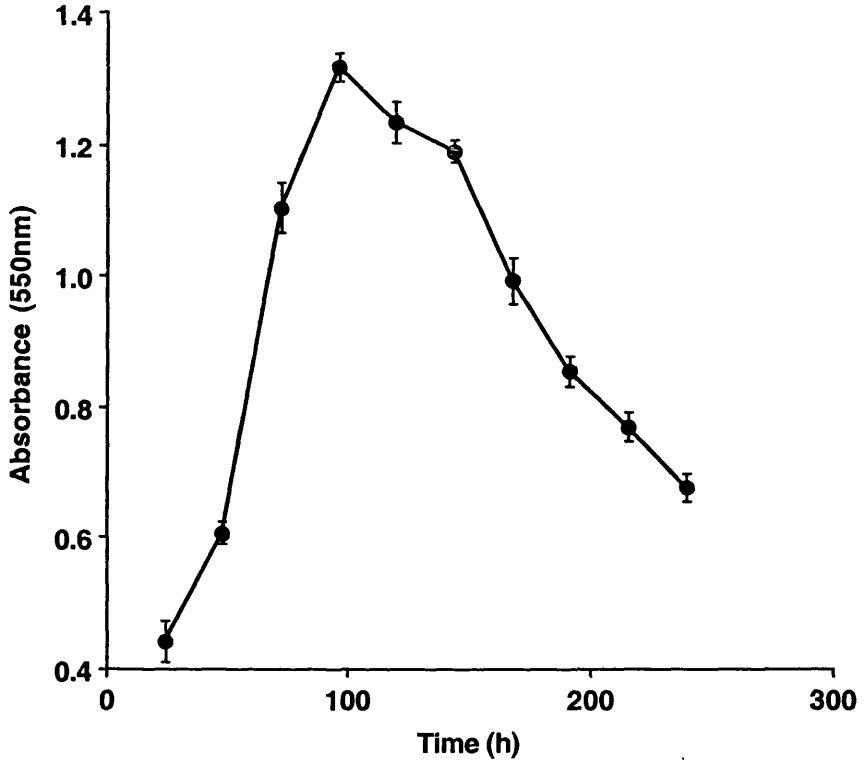



**Figure 5.1:** Growth curve of CORL23 cells grown from a seeding density of $1x10^4$ cells per well.

**Note:** Data represent mean ±SD (n=6).

day and animals were killed when tumours reached a size of 289mm$^2$ or when the weight of the animal dropped below 80% of initial body weight. The dose was then adjusted if toxicity was observed to find a dose which did not cause significant toxicity.

*5.2.4* Anti-tumour data expression

The measure of anti-tumour activity determined here was the average survival time of the treated group (T) divided by the average survival time of the control group (C) expressed as a percentage. This method has been used by the NCI and a substance is usually considered active if the T/C% is greater than 125. Toxic deaths are quoted as a combination of animals who were too sick to carry on the experiment and those animals found dead in cages during the experiment. These animals are not included in T/C% determinations.

## 5.3 Results

*5.3.1* Determination of APE MTD in tumour-bearing mice

The initial dose of 25mg/kg APE i.p. gave one toxic death observed as a 20% loss in body weight from the two animals and therefore it was decided to reduce the dose to 20mg/kg. This dose also gave one toxic death and so it was decided to further reduce the dose to 10mg/kg which gave no toxic deaths. Therefore, for the purposes of these studies, the MTD of APE was taken to be 10mg/kg. This is shown in Figure 5.2 as toxicity was observed as greater than 20% body weight loss in at least one animal of the two tested and this was the case for the groups dosed at 20 and 25mg/kg.

*5.3.2* Anti-tumour activity of HPMA copolymer-APE conjugates against B16F10 melanoma

Following this study to determine the MTD dose an anti-tumour experiment was carried out in the B16F10 model testing three doses of APE and HPMA copolymer GFLG-APE using a single dose i.p. on the day tumours reached an area of 25-50mm$^2$. This study utilised an HPMA copolymer GFLG-APE conjugate made from CPT-03 (Table 3.1) and containing 5.5%w/w APE (less than 0.04% of which was free) and is reported in Searle et al (2001). The results of this study are reported in Table 5.2 as this gave the information on which further studies were carried out using the HPMA copolymer GFLG-APE conjugates containing different loading of APE, described in

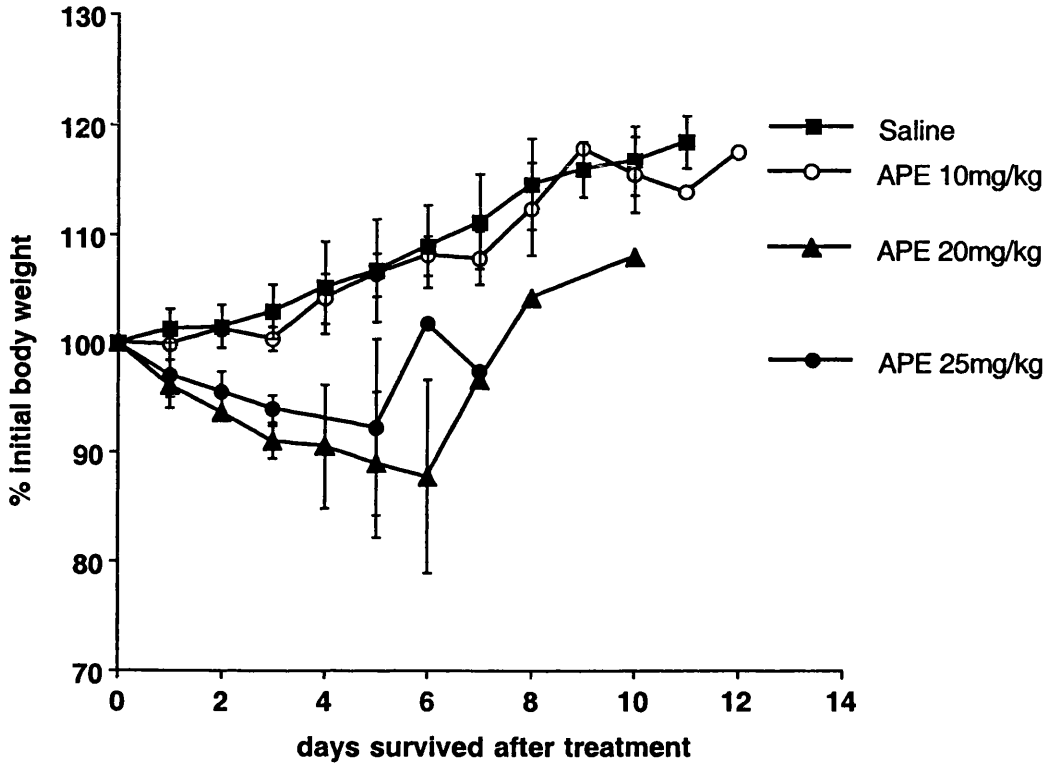



**Figure 5.2:** Toxicity of 20 and 25mg/kg doses observed as 20% body weight loss in the MTD determination of APE in mice bearing s.c. B16F10.

**Note:** Saline is shown as a control which shows no toxicity. Symbols represent mean ±SD (n=2).

**Table 5.2:** Anti-tumour activity of APE and HPMA copolymer GFLG-APE conjugates against s.c. B16F10 murine melanoma.

| Treatment | Dose (mg/kg) | Mean time to tumour progression ± SE | T/C (%) ± SE | Toxic deaths |
|---|---|---|---|---|
| saline | - | 7.7 ± 0.4 | 100 | 0/14 |
| APE | 1 | 9.8 ± 0.6 | 126.7 ± 8.0[b] | 0/9 |
| APE | 5 | 9.0 ± 0.8 | 116.7 ±10.9[NS] | 0/10 |
| APE | 7.5 | 7.2 ± 0.9 | 93.3 ±11.1[NS] | 1/5 |
| HPMA copolymer –APE* | 1 | 11.0 ± 0.7 | 142.6 ±9.6[c] | 1/14 |
| HPMA copolymer –APE* | 5 | 9.9 ± 1.4 | 128.0 ±17.6[NS] | 0/8 |
| HPMA copolymer –APE* | 10 | 8.8 ± 1.1 | 110.0 ±13.9[NS] | 1/5 |

* doses are indicated in APE-equivalent. [NS] not significant, [b] $p<0.01$, [c] $p<0.001$ (Student's t test vs saline controls).

this study. On increasing the dose of APE and HPMA copolymer GFLG-APE an unexpected result was encountered in that the T/C% was less than for the 1mg/kg dose (Table 5.2).

From the study described previously it was decided to examine the anti-tumour activity of the HPMA copolymer GFLG-APE conjugates containing different loading using a single i.p. dose of 5mg/kg of APE equivalence. The results of this study are shown in Table 5.3. No significant anti-tumour activity was noted (using Student's t test) although the T/C% value for RK2 was 135. This shows that while there is some activity for RK2 and RK8 (119%) there is none observed for the highest drug loading conjugate RK10 or for APE alone (in accordance with Searle et al, 2001) at this dose. This could potentially be due to the reduced release of APE from the RK10 conjugate observed in the tritosome release studies (Chapter 4). None of the treatments showed toxicity observed as weight loss of greater than 20% (Figure 5.3) and no gross organ toxicity was noted on examination of liver, heart, lungs, kidneys and spleen in any animals in this study (Figure 5.4). This opens the possibility of using higher doses of conjugates provided the problem of dose escalation can be elucidated.

The conjugate which gave the best activity here was the conjugate containing a medium loading of APE (RK2; 4.34%w/w APE) and so this would seem to be the best drug loading for further anti-tumour studies. For this reason a pilot study was conducted against the CORL23 human tumour xenograft in *nu/nu* mice using the same conjugate of medium drug loading (RK2). This was a simple study comparing the HPMA copolymer GFLG-APE conjugate to saline treatment to elucidate any activity of the conjugate in this tumour model. The results of the study are shown in Table 5.4. As can be observed RK2 is significantly more active than saline treatment. This tumour is slower growing than the B16F10 melanoma and so any differences in tumour growth can be observed more easily. The tumour growth for the mice treated with RK2 is very much slower than the control group (Figure 5.5). The difficulties associated with toxic deaths are clearly demonstrated here with one toxic death in both the control (saline) and treated (RK2) groups. Neither group showed significant weight loss during the study (Figure 5.5) and neither group showed any gross abnormalities to liver, lung, heart, kidneys or spleen upon examination.

**Table 5.3:** The effect of APE loading on the anti-tumour activity of HPMA copolymer GFLG-APE conjugates against B16F10 melanoma.

**Note:** These conjugates contained 1.20%w/w RK8, 4.34%w/w RK2 and 6.10%w/w RK10 of APE and each had less than 0.1%w/w free APE (Table 3.2).

| Treatment | Dose (mg/kg) | Initial tumour area $mm^2$ (mean ± SD) | Days survived after treatment (mean±SD) | T/C % (± SD) | Toxic deaths |
|---|---|---|---|---|---|
| Saline | - | 27.5, 30, 27.5, 36, 25.5 (29.3 ± 4.1) | 6, 7, 9, 6, 8 (7.2 ± 1.3) | 100 (± 18.1) | 0/5 |
| APE | 5 | 52.5, 25, 25, 27.56, 23.4 (30.7 ± 12.3) | 4, 10, 4, 6, 6 (6 ± 2.4) | 101 (± 32.1)$^{NS}$ | 2/5 |
| RK8* | 5 | 30.25, 25, 42, 36, 27.5 (32.2 ± 6.9) | 6, 10, 11, 6, 10 (8.6 ± 2.4) | 119 (± 33.4)$^{NS}$ | 0/5 |
| RK2* | 5 | 25, 27.5, 25, 27.5, 26.95 (26.4 ± 1.3) | 6, 10, 12, 7, 10 (9 ± 2.4) | 135 (± 28.6)$^{NS}$ | 1/5 |
| RK10* | 5 | 24.75, 31.2, 30, 31.35, 26.52 (28.8 ± 3) | 8, 6, 7, 4, 8 (6.6 ± 1.7) | 100 (± 13.3)$^{NS}$ | 1/5 |

* doses are indicated in APE-equivalent. $^{NS}$ not significant (Student's t test vs saline controls).

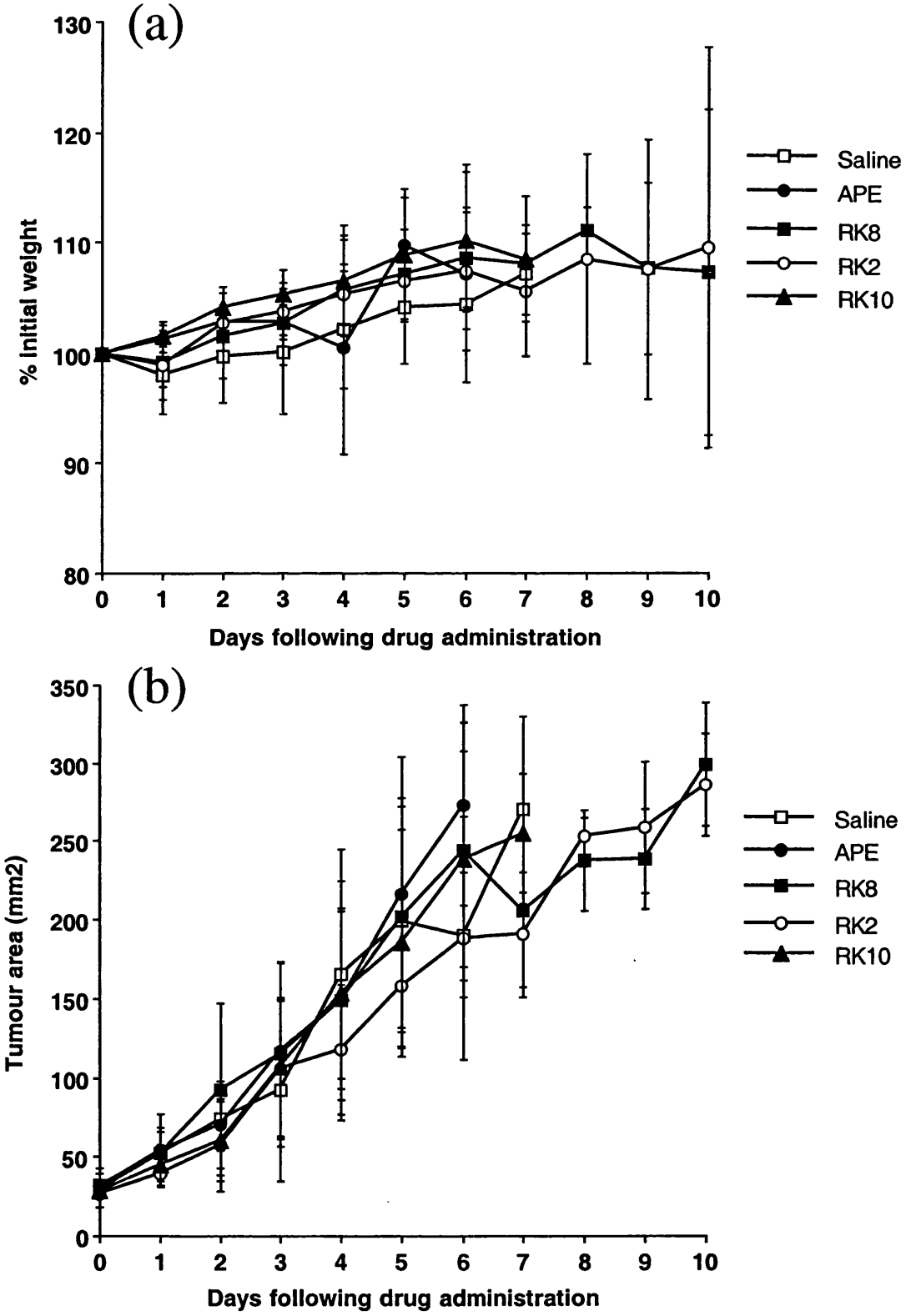



**Figure 5.3:** Body weight and antitumour activity of saline, APE and HPMA copolymer GFLG-APE conjugates

Note: Panel a shows mouse weight following drug administration and panel b shows tumour are over the course of the antitumour study. Data represent mean ±SD in both cases (n=3 at least).

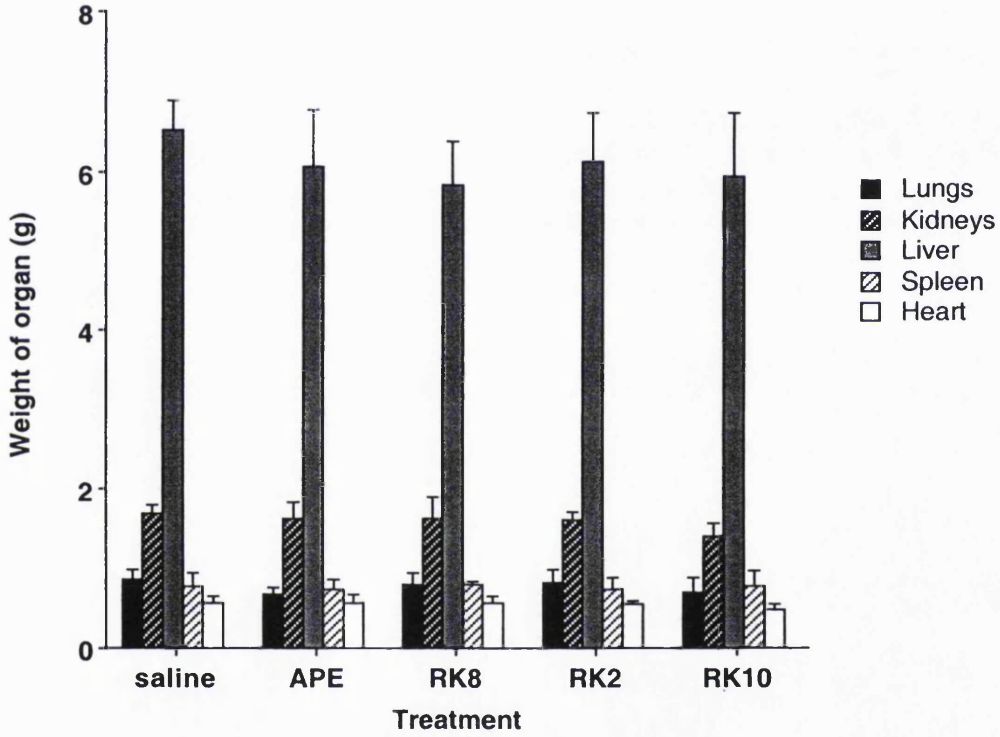



**Figure 5.4:** Effect of saline (control), APE and HPMA copolymer GFLG-APE conjugates of different loading on organ weight of organs removed at the end of an antitumour study.

**Note:** Data represent mean ±SD (n=3 at least).

**Table 5.4**: Activity of RK2 (4.34%w/w APE) against the CORL23 human tumour xenograft in *nu/nu* mice.

| **Treatment** | **Dose (mg/kg)** | **Initial tumour area $mm^2$ (mean ± SD)** | **Days survived after treatment (mean±SD)** | **T/C % (± SD)** | **Toxic deaths** |
|---|---|---|---|---|---|
| Saline | - | 27, 30, 27.5, 25, 27.5, 30 (27.8 ± 1.9) | 35, 7, 40, 32, 28, 28 (28.3 ± 11.4) | 100 (± 15.6) | 1/6 |
| RK2* | 1 | 40, 25, 36, 25, 25, 25 (29.3 ± 6.8) | 47, 46, 32, 51, 75, 45 (49.3 ± 14.1) | 162 (± 38.7)[b] | 1/6 |

* doses are indicated in APE-equivalent. [b] $p<0.05$ (Student's t test vs saline controls).

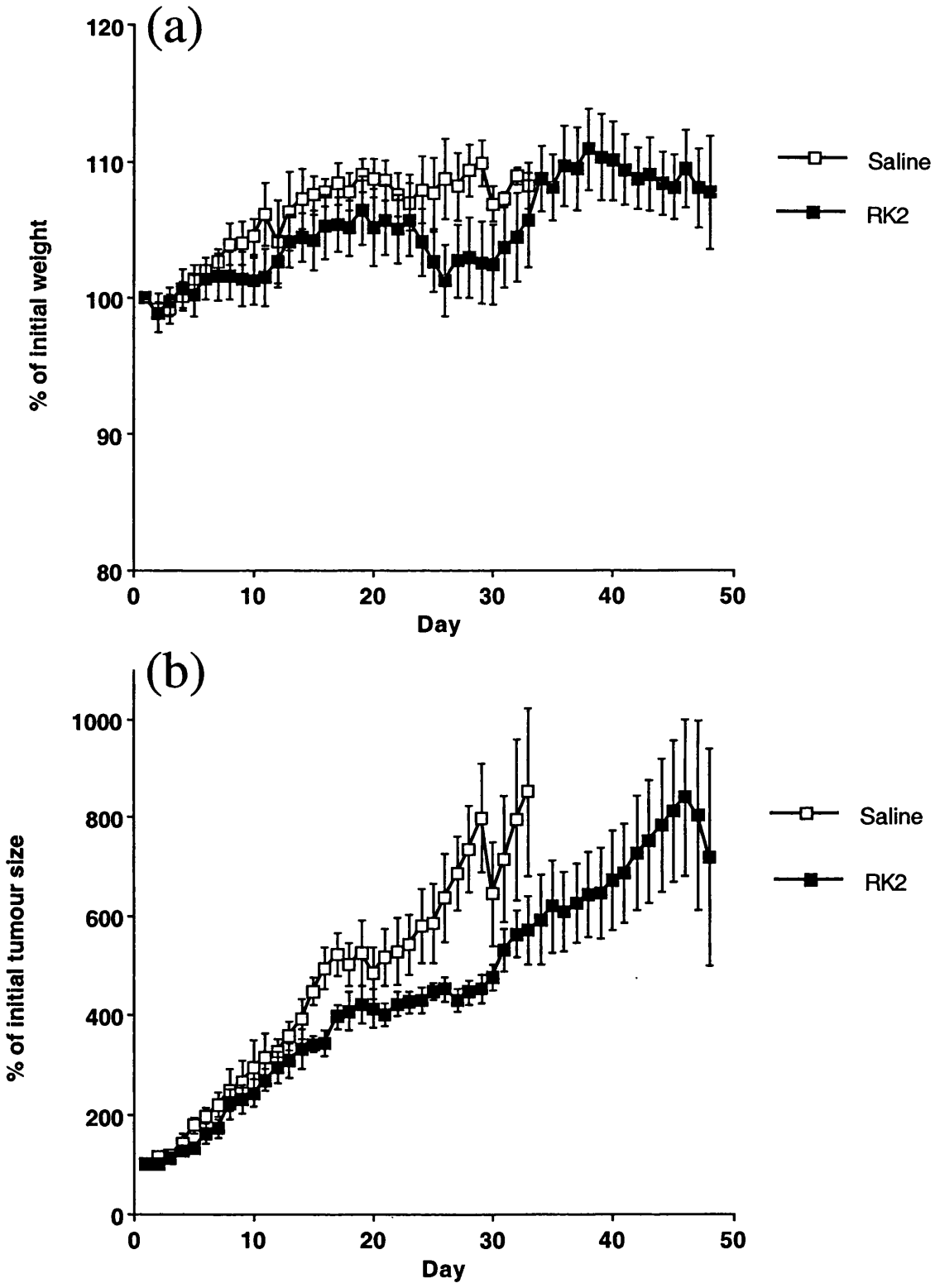



**Figure 5.5:** Effect of saline and HPMA copolymer GFLG-APE (RK2) on mouse weight and tumour area.

**Note:** Panel a shows the effect of drug administration on mouse body weight and b shows the effect on tumour area over the course of the antitumour study. Data represent mean ±SD in both cases (n=3 at least).

## 5.4 Discussion

The early study of HPMA copolymer GFLG-APE reported in Searle et al (2001) and described here in Table 5.1 was encouraging as the T/C% for HPMA copolymer GFLG-APE at a dose of 1mg/kg was significant against the control treatment, saline ($p<0.001$; Student's t test). The later study, described in Table 5.3 showed some activity for the conjugates containing low and medium drug loadings but it was not statistically significant when using the 5mg/kg dose of APE equivalence. It is important to note, however, that a single dose i.p. study of PK1 showed low activity in this tumour model (O'Hare et al, 1993). Increased activity of PK1 in the B16F10 model was only evident upon increasing dose and frequency of dosing (Duncan et al, 1992 and Seymour et al, 1994). The observed sustained reduction in tumour growth over time for such a low dose of APE equivalence of the conjugates described here was promising This is particularly true when considering the high repeated doses necessary for activity of the newly developed ellipticine analogues (S16020-2 and S30972-1) in the B16 model (Malonne et al, 2000).

The data obtained in this thesis using single doses of HPMA copolymer GFLG-APE are compared with the later studies using PK1 and the reported activity of the ellipticine analogues which are undergoing clinical testing in Table 5.5. It is interesting to note that an altered dosing regimen of PK1 administration showed significant activity in the B16F10 model and therefore dosing regimen optimisation is a vital aspect of the development of polymer-drug conjugates. It is necessary to optimise such regimens if the potential of polymer-anticancer drug conjugates is going to be fully realised. The anti-tumour activity of the ellipticine analogues are obtained at cumulative doses of up to 360mg/kg administered over three weeks. Therefore the anti-tumour activity observed in this model for the HPMA copolymer GFLG-APE conjugates at 1mg/kg of drug equivalence is promising. These results also show how difficult it is to treat the B16F10 model as even high doses of the active ellipticine analogues, which are in clinical trial, gave no long term survivors in this model. Long term survivors were only observed in the highest dose of PK1 in the small number of animals tested.

The results of the pilot study in the CORL23 human tumour xenograft model are also interesting for many of the same reasons. The low dose of 1mg/kg of APE equivalence gave a sustained decrease in the rate of tumour growth during this study. A full study is warranted in this model based on this pilot study and the activity observed.

**Table 5.5:** Comparison between HPMA copolymer GFLG-APE, S16020-2, S30972-1 and PK1 administered i.p. in the B16F10 model.

**Note:** The HPMA copolymer GFLG-APE conjugates contained 1.20%w/w RK8, 4.34%w/w RK2 and 6.10%w/w RK10

| Drug | Dose* | T/C (%) | Long-term survivors | Reference |
|---|---|---|---|---|
| RK8[a] | 5 | 119 | 0/5 | |
| RK2[a] | 5 | 135 | 0/5 | |
| RK10[a] | 5 | 100 | 0/5 | |
| S16020-2[b] | 10 | 139 | 0/9 | |
| | 20 | 161 | 0/9 | |
| | 40 | 53 | 0/9 | |
| S30972-1[b] | 10 | 137 | 0/9 | Malonne et al, 2000 |
| | 20 | 184 | 0/9 | |
| | 40 | 148 | 0/9 | |
| PK1[c] | 18 | 209 | 0/10 | |
| | 27 | 293 | 0/4 | Duncan et al, 1992 |
| | 36 | 320 | 3/3 | |

* doses of conjugates are in drug-equivalence

[a] single dose administered on day of tumour establishment

[b] Nine doses administered in the three weeks following tumour establishment

[c] Three doses administered on days 0, 1 and 2 of tumour establishment

In summary HPMA copolymer GFLG-APE conjugates have shown activity in two tumour models *in vivo*. The conjugate of medium drug loading showed the best activity in the B16F10 study. No toxicity of the HPMA copolymer GFLG-APE conjugates was evident in any of these studies so there is the possibility of dose escalation. However attempts to give increased doses seemed to give lower activity than the 1mg/kg dose. This phenomenon needs to be examined to determine what is giving this inexplicable result. All the studies reported here have used the i.p. route of drug administration and therefore the comparison between i.p. dosing and i.v. dosing needs to be examined to optimise this feature of conjugate testing.

There were unexpected toxic deaths in the study of Searle et al (2001) with one toxic death reported at a dose of 1mg/kg APE equivalence of the HPMA copolymer GFLG-APE conjugate. It is difficult sometimes to attribute early deaths in these studies to toxicity as there are compounding factors. The B16F10 tumour has been shown to be a good tumour model of metastasis (reviewed by Glinsky & Glinsky, 1996) and therefore unexpected metastases could be fatal. Another difficulty is that the C57Bl strain of male mice are particularly aggressive and fighting is not uncommon and it is therefore sometimes difficult to distinguish toxic deaths from those caused by fighting.

The possible explanations for the decreased activity of HPMA copolymer GFLG-APE conjugates with increased doses are that the compounds may have precipitated in the peritoneal cavity; there was increased liver clearance; there was a preferred accumulation site other than the tumour or that there was dramatic decrease of the release of APE from the conjugate when the concentration of polymer increases (Searle et al, 2001).

HPMA copolymer GFLG-APE conjugates have therefore demonstrated anti-tumour activity in these early studies but further optimisation of dosing schedules and routes of administration are necessary before these conjugates can be considered for further development. The lead candidates for such studies would seem to be the conjugates containing a medium APE loading (3.07-4.34%w/w).

# Chapter Six

## *The window chamber model: visualisation of the intratumoural distribution of PK1*

## 6.1 Introduction

It has been shown that polymer-drug conjugates benefit from the EPR effect resulting in increased tumour accumulation compared to low molecular weight anticancer agents (Section 1.4.2). This has been proven for a number of polymer-drug conjugates using a variety of murine tumour models using both tumour excision and drug content determination by various methods (e.g. PK1; Duncan et al, 1992) as well as various imaging studies (e.g. HPMA copolymer-camptothecin; Caiolfa et al, 2000). In the case of PK1 imaging trials have also been carried out as a side-arm to the Phase I clinical study using $^{131}$I-labelled PK1 (Vasey et al, 1999). While this particular isotope was found to be not optimal for imaging, tumour accumulation was noted and importantly no significant accumulation was observed at unexpected sites. This reduction in non-tumour deposition was also shown by the decrease in cardiotoxicity of PK1 compared to doxorubicin alone, suggesting decreased PK1 accumulation in the heart. Future studies using methods such as gamma camera and positron emission tomography have been suggested as means of better characterising the EPR effect. One method which has been reported has been the use of an $^{123}$I-labelled PK2 conjugate in a clinical imaging study using single photon emission computed tomography (Julyan et al, 1999). This shows that there are many promising methods of imaging polymer-drug conjugates in future studies and helping our understanding of the EPR effect in humans.

However the pattern and extent of intratumoural distribution of such polymer-drug conjugates has not been reported. It has been shown previously that antibodies (reviewed by Jain & Baxter, 1988) and liposomes (Yuan et al, 1995) do extravasate well into tumour tissue but penetrate poorly beyond the immediate vicinity of the blood vessels. This has been attributed to factors such as the elevated interstitial pressure of tumours as well as slow interstitial diffusion of these systems. This is a vital aspect of drug delivery as lack of penetration of anticancer agents into solid tumours has been shown to result in low exposure of certain cell populations to the cytotoxic agent and to lead to resistance to therapy (reviewed by Tungaal et al, 1999). One method that has been most widely used to examine the structure of tumours has been the use of excised tumour sections from solid tumours. This has been used as a means of examining the vasculature of tumours (Konerding et al, 1999) as well as detecting tumour markers (reviewed by Fenton et al, 1999). However to use this method to examine intratumoural drug distribution would require the use of many animals to determine the time course and extent of extravasation and, due to practical limitations, the earliest

time points immediately following parenteral administration, particularly in the first minute, cannot be studied.

One method which has gained widespread use recently, particularly to examine tumour blood flow characteristics, has been the window chamber model. This method was first developed in 1982 (reviewed in Li et al, 2000) and has been used in a number of studies on blood flow characteristics of tumours since then (reviewed by Jain, 1998). A number of variations exist of the window chamber (described in Jain, 2001; Figure 6.1), the rat and mouse window chambers being most widely used to examine tumour characteristics. Perhaps one of the most powerful aspects of this technique is that the tumour can be visualised in real time and video captured and re-examined to, for example, study blood flow characteristics. In addition one animal can be used to examine the complete time course of intratumoural distribution of a drug or marker therefore reducing the effect of inter-animal variation as long as a suitable number of animals are used to allow for any gross variations. This technique has recently been used to examine the migration of implanted tumour cells towards the pre-existing blood supply in the surrounding tissue (Li et al, 2000) and also to examine the antivascular activity of combretastatin A4-phosphate (Tozer et al, 2001). In the first study the tumour cells used expressed green fluorescent protein and therefore tumours were examined using fluorescence microscopy while in the second study simple light microscopy was used. This underlines the versatility of this model as almost any imaging method can potentially be utilised for visualisation.

PK1 is the most widely studied of the polymer-drug conjugates and its EPR characteristics have been described earlier (Section 1.3). PK1 was therefore selected as a better probe than using an HPMA copolymer-APE conjugate to establish this method of visualising polymer-drug conjugates and study their intratumoural distribution using the window chamber model. The inherent fluorescence properties of doxorubicin provide an ideal probe for visualising PK1 distribution. A P22 carcinosarcoma tumour in BD9 rats was selected as the tumour to use for these studies. This model has been used previously to study the mechanism of action of vasoactive compounds such as combretastatin A4 phosphate (Tozer et al, 2001). These window chambers can be monitored for up to 3h to examine any change in distribution pattern.

After this time period tumours can be re-examined under light microscopy to determine whether there were any gross changes in blood flow. This would help

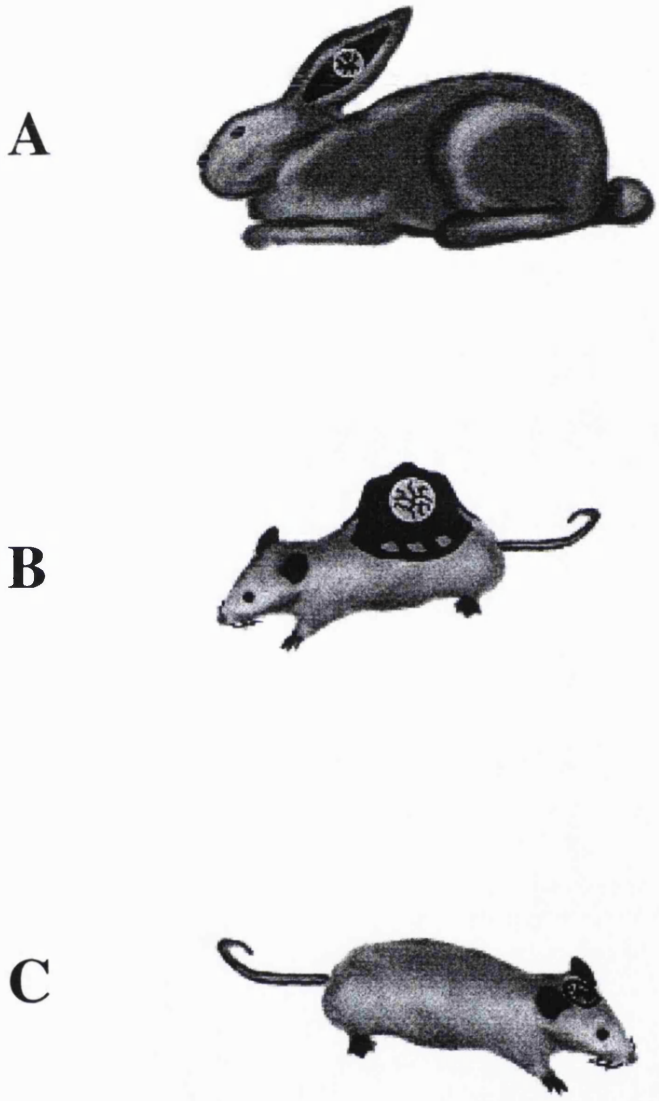



**Figure 6.1:** The type of window chambers available (from Jain, 2001).

**Note:** The rabbit ear window chamber (A) has mostly been used for physiological measurements of blood flow while the rat and mouse skinfold (B) and cranial (C) models have been used more for tumour studies.

determine whether doxorubicin was being released near the vasculature, due to poor penetration of the conjugate, and then causing a local cytotoxic effect.

## 6.2 Methods

### *6.2.1* Window chamber surgery

Male BD9 rats (weighing 150-200g) were prepared for surgery by first being anaesthetised using 10mg/kg fluanisone containing 0.32mg/kg fentanyl citrate and 5mg/kg midazolam administered i.p. They were then kept under anaesthesia for the duration of the surgery. All hair on the back of each rat was then removed using hair removal cream and standard clippers. The rat was then transferred to the surgical area, wrapped in sterile drapes leaving the back exposed, onto a heated pad and full aseptic technique was used from this point onwards. The skin of the back was then tented upwards and sutured into a C clamp using 4/0 Ethilon sutures to stretch the skin slightly and then keep the skin stretched. The two ends of each suture, on one side of the C clamp, were then clamped using artery forceps to prevent them from slipping and the suture tightened to tent the skin (Figure 6.2). One side of the aluminium frame of the window chamber was then used to mark where the window would be on each side. A fibre optic light is then used to trans-illuminate the skin. The bottom edge of the circle where the window will be was then sliced using a scalpel. A fine toothed forceps was used to lift up the thick tissue layer and a scissors was used to make a hole in the centre of this incision. The scissors were then opened and closed carefully to free the outer layers of skin, leaving the deepest fascial layer. The remainder of the circle was then cut using the scissors. The animal was turned around and the other side was prepared in the same manner, while protecting the exposed tissue using a sterile gauze soaked in warm saline. When this was completed the window chamber frame was used to line up and punch holes through the skin to allow its attachment on both sides. Both sides of the window chamber were then lined up and stainless steel screws used to join the frames together with the skin between. Before fully tightening the screws the areas of exposed fascia were adjusted, if necessary, so that they were in the correct position in the centre of the window. The window chamber frames were then further re-enforced by suturing them together.

To implant tumours firstly the areas of fascia were dried using sterile swabs. The tumour fragments were then implanted on or near a blood vessel, usually near the

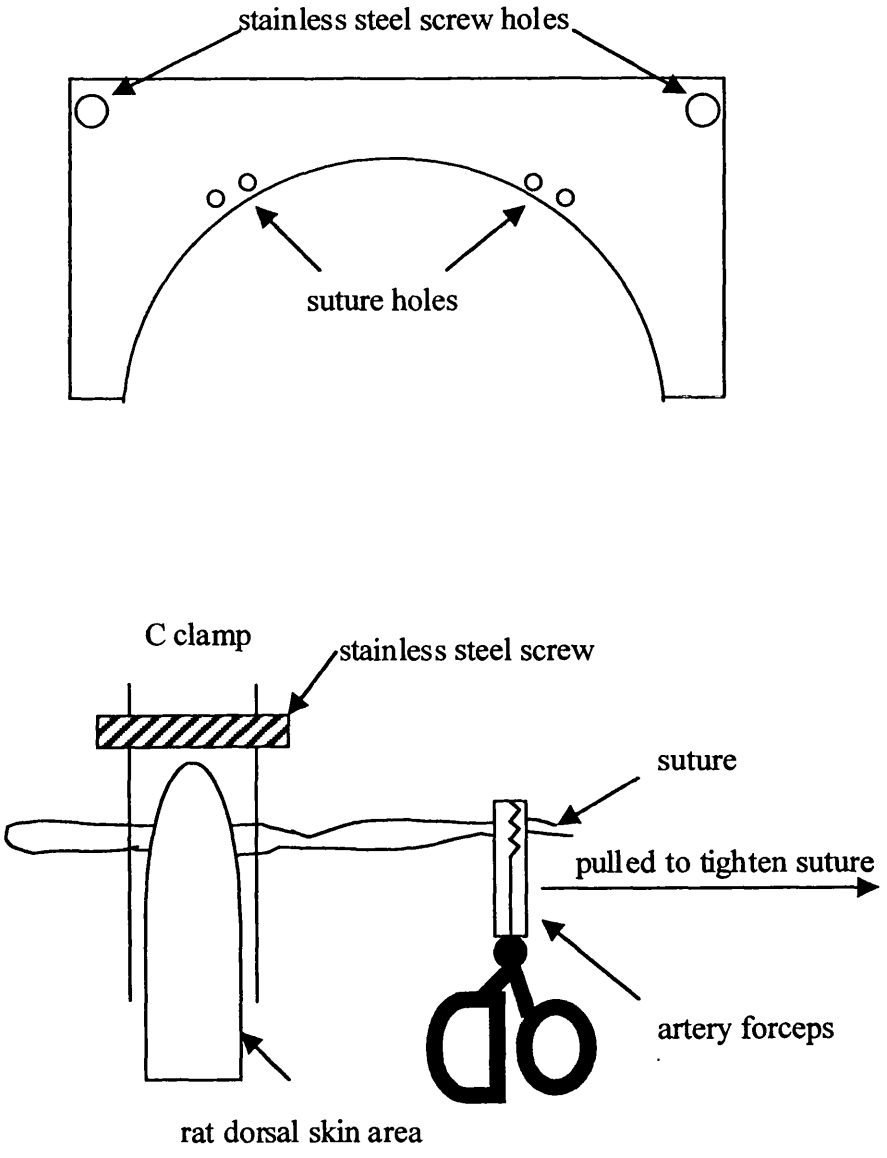



**Figure 6.2:** Schematic of C clamp from a front and side view.

top of the window to allow for any movement of the fragment which tends to fall downwards. The tumour fragments were early generation (generation 0-10) P22 carcinosarcoma fragments removed from a donor rat and sliced, in media, into pieces with a diameter of approximately 0.5mm using a scalpel and kept in warm media until use. Saline was slowly added to the window, so as not to disturb the fragment, and then a glass window was carefully placed on top of the saline, so as not to introduce air bubbles. This window was then secured using a C-shaped spring. The same procedure was carried out on the other side of the window, except no tumour was implanted. The C clamp was removed and antibiotic cream was applied to the edges of the window chamber frame. Dextrose saline solution (3ml) was administered to the rat i.p. while the animals were allowed to recover on a heated pad. Subsequently animals were kept in a warm room (32-34°C) until the day of the experiment.

### *6.2.2* Tumour examination and PK1 administration

Following surgery tumours took approximately 7-14 days to develop to a diameter of approximately 4mm, when they would be used in an experiment. For the window chamber visualisation studies the rats were again anaesthetised using the same regimen as before and kept under anaesthesia for the duration of the study. The window chamber frame was attached to a modified microscope stand to allow visualisation. Rats were monitored for rectal temperature, which was maintained at 35-37°C using a thermostatically controlled heating pad beneath the rat and an overhead infrared lamp.

Before PK1 was administered the tumour was examined using a light microscope first under low and then high power to find an area within the tumour containing typical vasculature. For fluorescence visualisation of PK1 the excitation source was of 546 +/- 5nm wavelength, passed through a 580nm dichroic cube on an inverted Nikon Diaphot 200 fluorescence microscope. The microscope setup and a picture of a window chamber are shown in Figure 6.3. Emission was visualised through a 580nm long pass filter. The processes of extravasation and distribution were viewed and recorded using a video system (Sony DSR-30P digital videocassette recorder) coupled to a 3-CCD colour camera (JVC KYF-55) or to a monochrome intensified CCD camera (Phillips FM800 fibre optic coupled to an XX1666/CJ10 image intensifier). The sensitivity was set to minimise inherent fluorescence prior to injection.

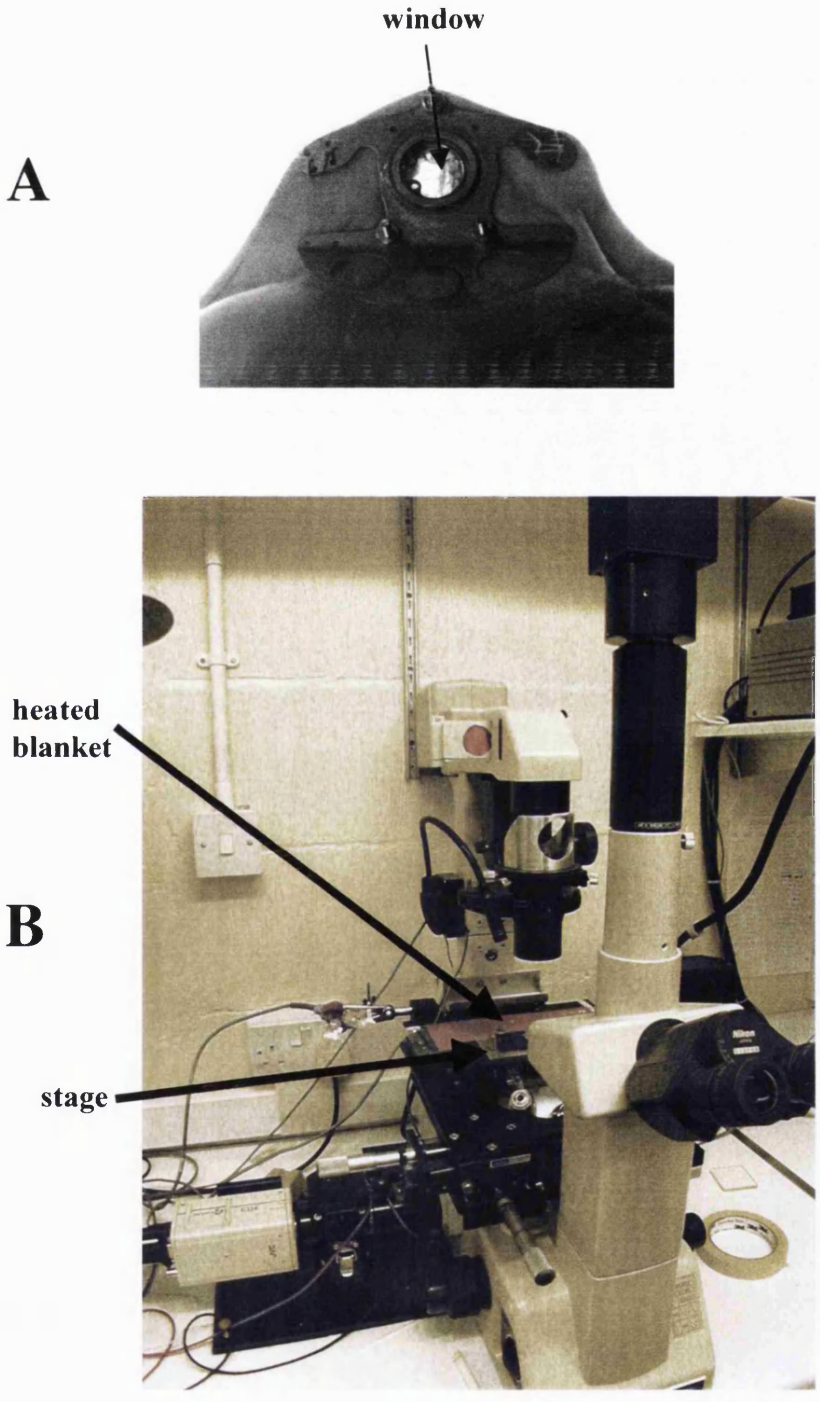



**Figure 6.3:** Picture of one side of a window chamber frame (A) and of the microscope set-up for imaging (B).

To begin the experiment PK1 in PBS was injected via an intravenous cannula in a tail vein.

*6.2.3* Determination of doxorubicin content of tumours

At the end of the 3h experiments blood was removed from the animals using a cannulated tail artery or using cardiac puncture following anaesthetic overdose. Tumours were excised from the window using a scalpel. Samples were stored in a freezer until analysis. Total and free doxorubicin levels in the blood and tumour samples were determined by extraction and HPLC as described previously (Section 2.3.3.2)

**6.3 Results**

Before any further experiments could be started it was necessary to deduce a suitable dose of PK1 which could be administered. The first dose attempted was 10mg/kg of doxorubicin equivalence. However as PK1 was being injected the fluorescence was already being visualised in the tumour and the injection stopped. It was calculated that 4mg/kg doxorubicin had been administered and therefore this dose was used for further studies.

Figure 6.4 shows an example of what the window looks like under light microscopy at the lowest magnification (x1.6). Figures 6.5 and 6.6 show a series of still images taken from each of four studies (all at magnification x20) all utilising 4mg/kg of doxorubicin equivalence of PK1. The first image in each series shows the area of tumour selected, under light microscopy, as being suitable for further study viewed from the surface adjacent to the fascial layer. These areas contain a number of blood vessels which are believed to be mainly venules but it is difficult to fully characterise a tumour blood vessel. These areas are towards the centre of the tumour in all cases and contain vessels which are easily distinguishable using light microscopy.

At the time of injection the fluorescence image of all tumours examined was dark in the field studied due to adjustment of the gain. Immediately after PK1 was injected (10sec) fluorescence can be seen localised in nearly all the blood vessels within the same field (second image in each series). After 1min PK1 was seen to extravasate

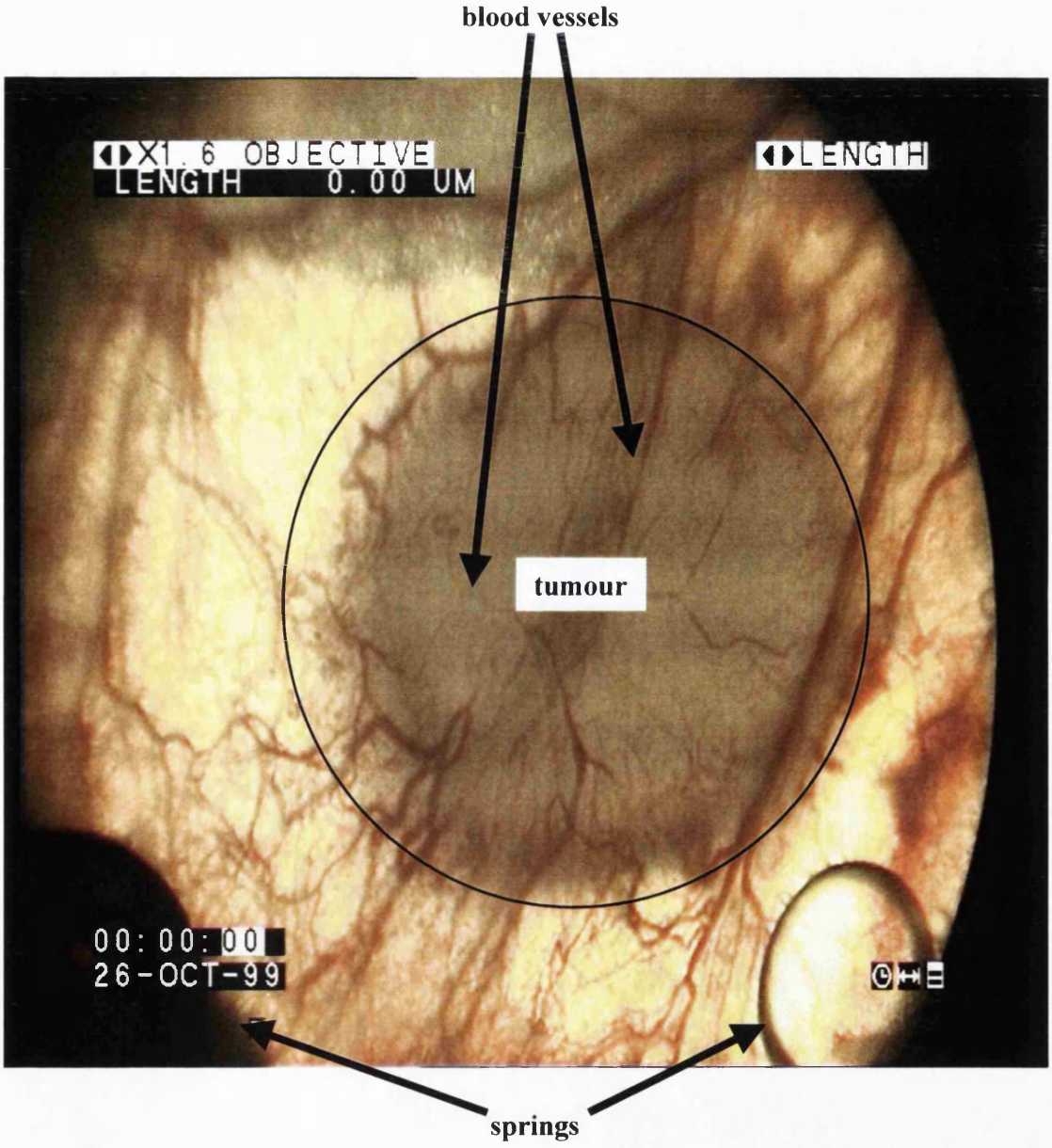



**Figure 6.4:** Trans-illuminated picture of a window chamber preparation under the lowest magnification (x1.6).

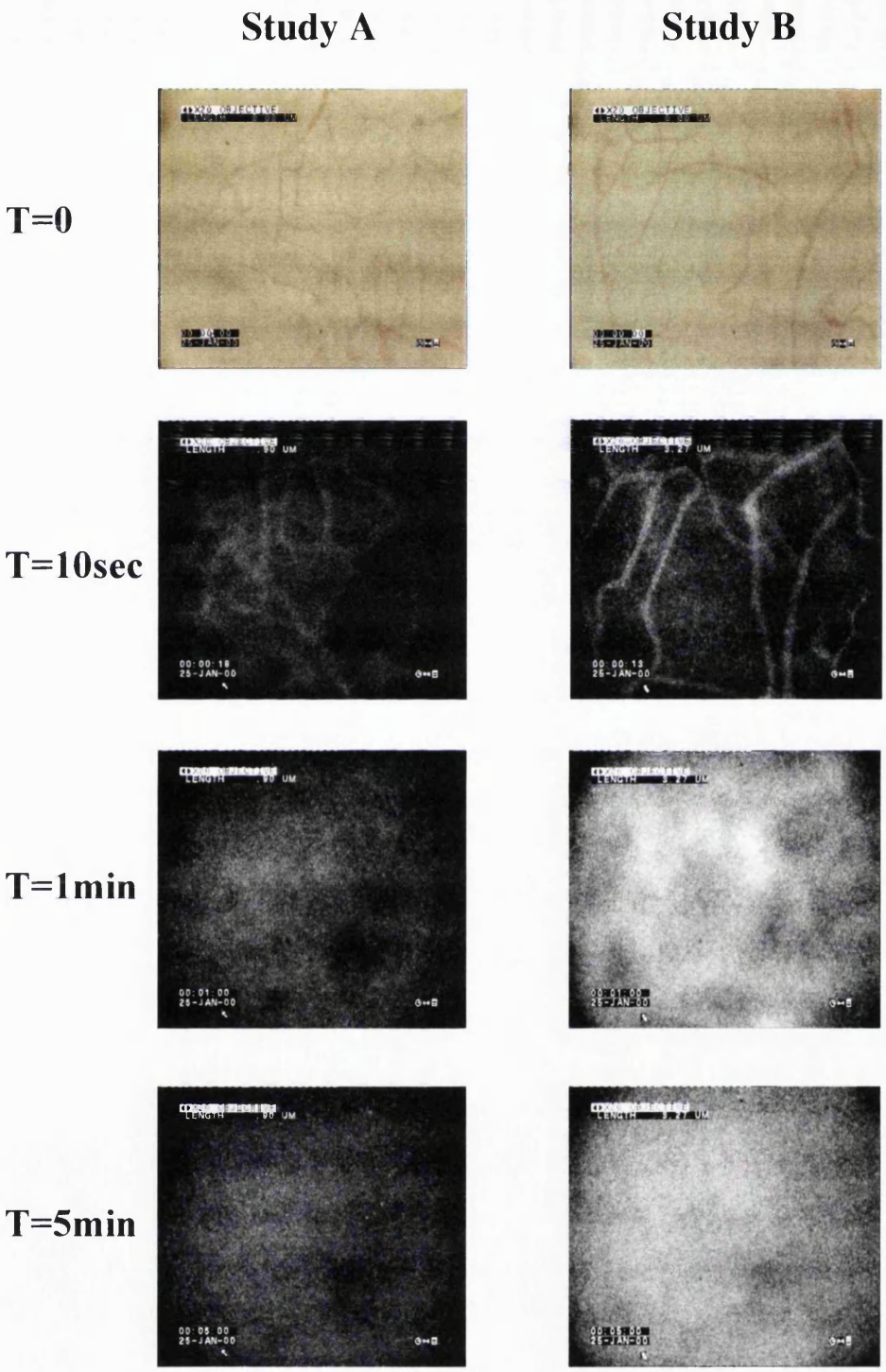



**Figure 6.5:** A series of still images taken from two studies showing time-dependent distribution of PK1 following injection of 4mg/kg (doxorubicin equivalence).

**Note:** The darker area in the bottom right of the fluorescence images is a camera artefact. Differences in intensity are due to differing levels of autofluorescence prior to PK1 administration.

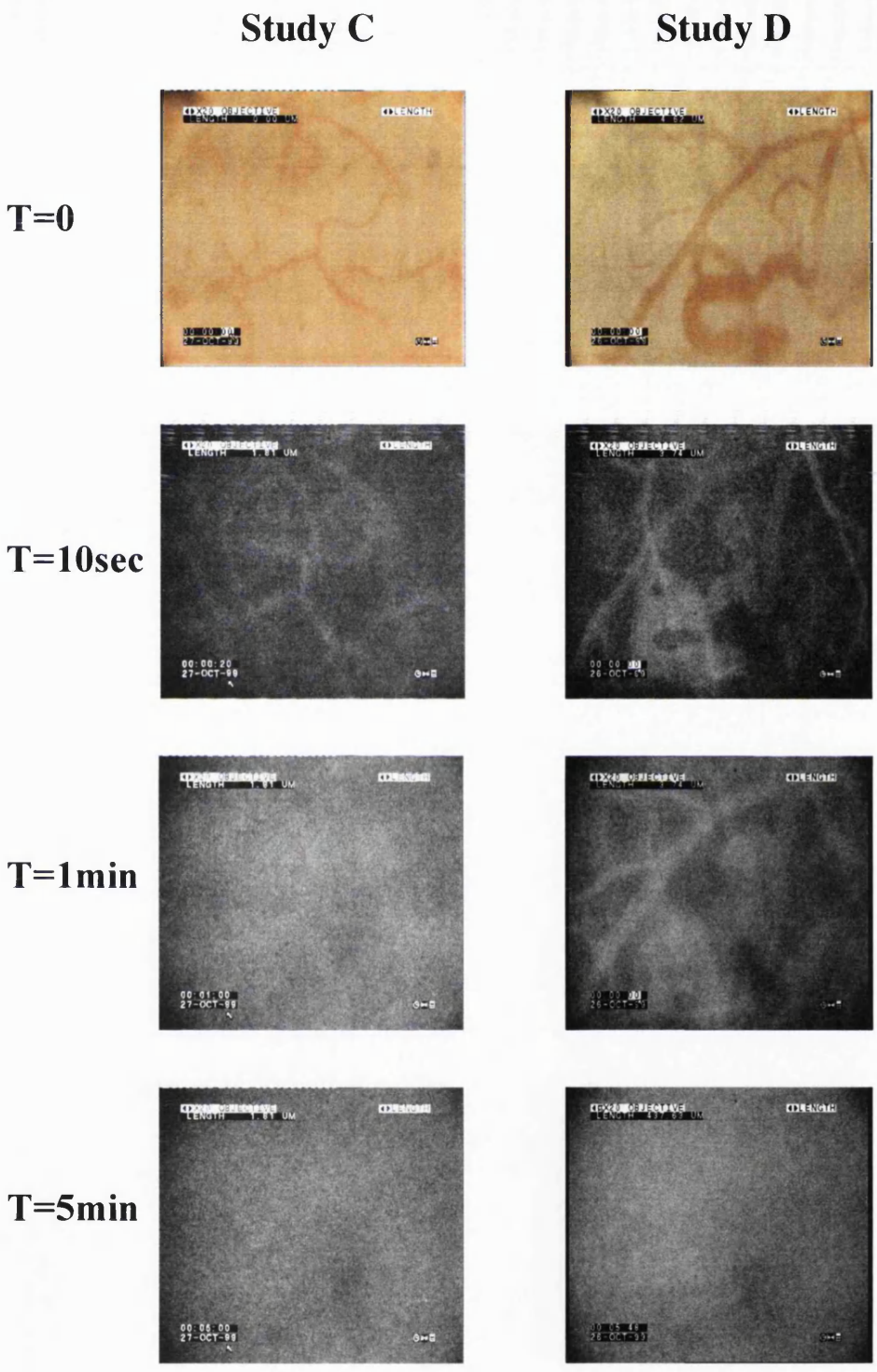



**Figure 6.6:** A series of still images taken from two further studies showing time-dependent distribution of PK1 following injection of 4mg/kg (doxorubicin equivalence).

**Note:** The darker area in the bottom right of the fluorescence images is a camera artefact. Differences in intensity are due to differing levels of autofluorescence prior to PK1 administration.

with fluorescence beginning to distribute evenly throughout the tumour (third image in each series). After 10min there was an almost homogenous distribution of fluorescence throughout the tumour with no evidence of areas of non-accessibility (fourth image in each series). The dark area in the bottom right area of all fluorescent images can be attributed to a camera artefact. This profile of uniform distribution remained throughout the duration of the experiment, up to 3h which is the longest time permitted under the animal project licence.

At the end of the 3h studies no gross changes in tumour blood flow were discernible. Analysis of blood and P22 carcinosarcoma tumour samples following PK1 administration (3h) showed total conjugate levels of 3.5 and 1.1% dose/ml or g respectively. There was no evidence of doxorubicin being released from PK1 within this time period, as no free doxorubicin was detected in the tumour or blood samples by this HPLC analysis method.

**6.4 Discussion**

This study clearly demonstrates that PK1 extravasates very rapidly and is quickly and homogeneously distributed intratumourally. This is in contrast to studies using rhodamine labelled liposomes (containing egg phosphatidylcholine: cholesterol: polyethylene glycol: distearoylphosphatidylethanolamine / rhodamine labelled distearoylphosphatidylethanolamine in a molar ratio of 10: 5: 0.8: 0.1; approximately 90nm diameter). This study utilised a human colon adenocarcinoma (LS174T) xenograft implanted into window chambers on severe combined immunodeficient (SCID) mice and showed perivascular deposits of fluorescent spots (<20μm in diameter) (Figure 6.7; Yuan et al, 1994).

The lack of obvious vascular effects of PK1 contrasts with combretastatin A4 phosphate which causes vascular shutdown in this same model after 20min which is clearly visible using light microscopy (Tozer et al, 2001). These observations support the distribution profile observed in these studies showing widespread intratumoural distribution of PK1. The principle site of PK1 activity would therefore seem to be intratumoural rather than at the level of the tumour endothelium.

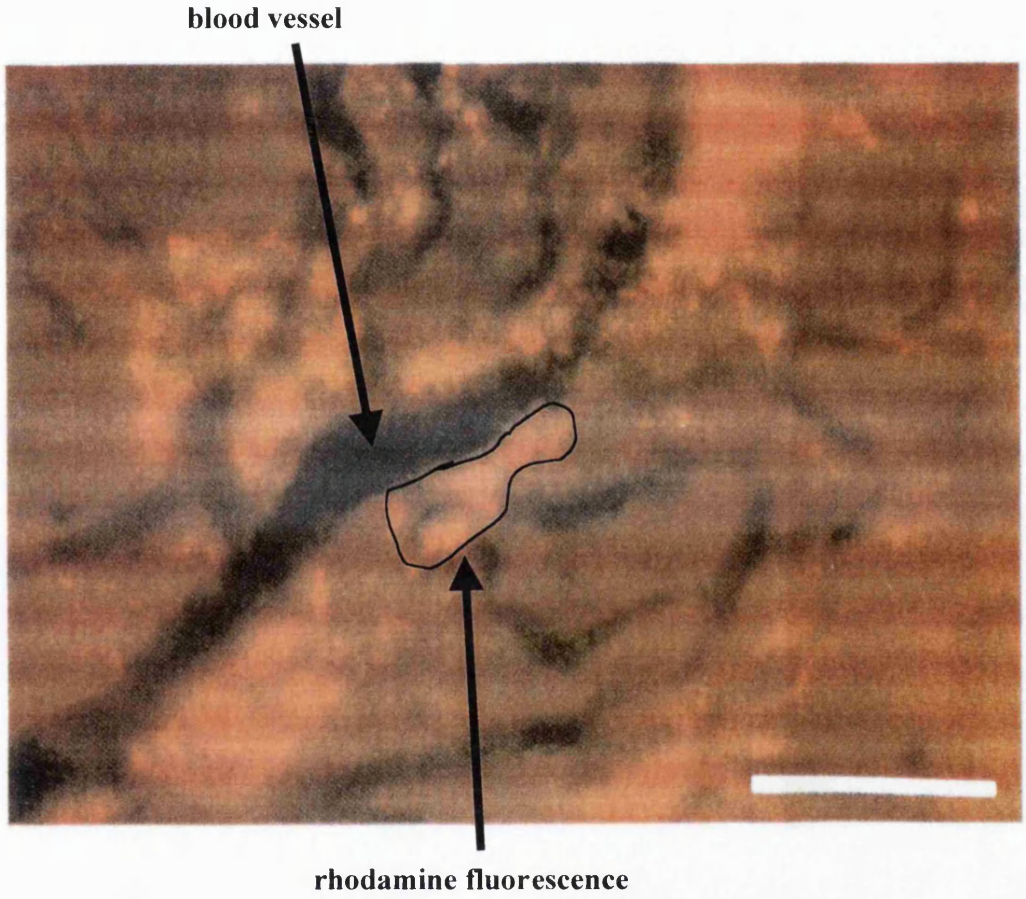



**Figure 6.7:** Intratumoural distribution of liposomes of approximately 90nm in LS174T tumours implanted in dorsal window chambers on SCID mice showing characteristic perivascular deposits (from Yuan et al, 1994)

It is known that to exert its anti-tumour effects doxorubicin must be released from the PK1 conjugate and the GFLG linker was designed specifically to be cleaved by intralysosomal thiol-dependent proteinases (Duncan et al, 1983). The time dependence of doxorubicin release from PK1 in s.c. B16F10 tumours previously suggested that lysosomotropic delivery is an essential step for PK1 activation (Seymour et al, 1994). The results of this study are not inconsistent with those of published studies in mice (Table 6.1) taking into account that these earlier studies used a 1h time point and blood clearance of PK1 has been shown to follow an exponential profile (Sat, 1999).

Future studies aim to investigate the extravasation characteristics in larger tumours (here the P22 carcinosarcoma used had a size range of 1.8 to 5.4mm diameter) and other tumour types in order to confirm the results seen here. If this is the case then other HPMA copolymer conjugates (such as HPMA copolymer GFLG-APE) could be tested to see if this intratumoural distribution profile is a general effect of HPMA copolymer-conjugates. The effect of polymer conformation on these characteristics will need to be examined also using novel polymeric architectures, such as dendrimers, as a means of optimising these structures and polymer-drug conjugates generally for tumour extravasation and intratumoural distribution. The next stage of development would be to integrate these studies with clinical imaging studies in humans to determine the clinical relevance of these observations.

If clinical imaging studies do confirm this distribution profile one clinical implication of this study would be in the treatment of metastases using HPMA copolymer-anticancer conjugates. Metastases are difficult to treat using conventional surgery and radiotherapy methods and are often the cause of fatalities in patients with solid tumours. These are small tumours and therefore would be expected to have an irregular vasculature which would be similar in size to the tumours visualised here. HPMA copolymer-anticancer conjugates could therefore be employed as an effective treatment for metastases, perhaps in combination with conventional chemotherapy.

**Table 6.1:** Comparison of present study with previous accounts for the total amount of doxorubicin (dox) present in blood and tumour samples.

| Species | Strain-Tumour type | Time post injection | Total dox %dose/ml blood | Total dox % dose/g tumour | Reference |
|---|---|---|---|---|---|
| Rat* | BD9-P22 | 3 h | 3.5 | 1.07 | |
| Mouse | $DBA_2$-None | 60 min | 18 | ND | Seymour et al, 1990 |
| Mouse | Balb/c-Meta 7 | 60 min | ND | 2.5 | Sat, 1999 |
| Mouse | nu/nu-MAXF 449 | 60 min | ND | 1.0 | Sat, 1999 |
| Mouse | nu/nu-PAXF 546 | 60 min | ND | 2.3 | Sat, 1999 |
| Mouse | C57Bl/ B16F10 | 60 min | 20 | 5 | Sat, 1999 Seymour et al, 1994 |

* Present study described in Section 6.3

ND denotes not determined

# Chapter Seven

## *General discussion*

The continuing success of the progression of classical polymer-drug conjugates to clinical trial expounds the potential of this strategy to improve the pharmacokinetic, toxicological and ultimately clinical anticancer activity of the attached drugs (reviewed by Duncan et al, 2001). What is also interesting is that many of these conjugates have shown responses in Phase I clinical trials in chemotherapy refractory patients, which is something that is usually only observed in less than 5% of patients entering Phase I trials. It was hoped that the principles derived from studying those conjugates, which contain clinically used drugs, could be applied to other non-classical anticancer compounds. This thesis describes a polymer-drug conjugate synthesised using such a non-clinically studied anticancer drug, namely APE. The effect of drug loading of a polymer-drug conjugate has not been described before for the polymer-drug conjugates in clinical development and therefore this aspect of conjugate structure was also examined. This thesis has also, for the first time, visualised the tumoural extravasation of a polymer-drug conjugate (PK1), using the rat dorsal window chamber model.

The synthesis of a polymer-drug conjugate needs to be closely controlled to synthesise a conjugate with the lowest quantity of free drug possible in a reproducible manner. A series of HPMA copolymer-APE conjugates containing a range of drug loading have been synthesised in this study, all of which contain less than 0.1%w/w APE present as the free drug. These conjugates consist of three groups of HPMA copolymer GFLG-APE conjugates containing low (0-1.21%w/w APE), medium (3.07-4.34%w/w APE) and high (5.85-6.10%w/w APE) drug loadings. A series of HPMA copolymer GG-APE conjugates were also synthesised (4.27-4.90%w/w APE) for use as a comparator in drug release studies. This thesis demonstrates the importance of examining these HPMA copolymer conjugates, in this case HPMA copolymer-APE, as new chemical entities and not simply as a drug delivery system, as the physicochemical nature of the attached drug and the degree of drug loading can significantly influence *in vitro* and *in vivo* properties of the conjugate.

The anti-tumour activity of HPMA copolymer GFLG-APE observed in the B16F10 and CORL23 *in vivo* models in this thesis demonstrates the anticancer potential of these conjugates. The observed activity of HPMA copolymer GFLG-APE at low doses of APE equivalence in the s.c. B16F10 model is particularly relevant with the recent study of Malonne et al (2000) examining the activity of S16020-2 and S30972-1 (ellipticine derivatives) in this same model. These compounds showed anti-tumour activity in the B16F10 model but using considerably higher doses administered

repeatedly than those used in this thesis. The anti-tumour activity observed in the pilot study against the s.c. CORL23 xenograft model using the HPMA copolymer GFLG-APE conjugate compared to saline alone is particularly interesting with the recently described lack of activity of PK1 administered as a single dose in this model (Satchi et al, 2001). The reduced activity of HPMA copolymer GFLG-APE conjugates with increasing dose observed in this thesis in the B16F10 study, however, will need to be resolved before further studies using HPMA copolymer-APE conjugates could be attempted with an optimised dosing regimen. Selection of appropriate tumour models is also particularly important in the evaluation of polymer-drug conjugates as the EPR-mediated uptake and tumoural levels of cathepsin B are vital for the activity of these conjugates. A full anti-tumour study, in a panel of tumour models such as that used to evaluate PK1, will need to be carried out, using an optimised dosing regimen, to more fully examine the activity of HPMA copolymer GFLG-APE conjugates.

**Is APE the best drug for conjugation?**

APE was chosen as the natural anticancer drug to be used in this study for the reasons outlined in Section 3.1. In retrospect perhaps a more potent agent may have been chosen to better examine the benefits of conjugation to the HPMA copolymer conjugate. However APE was chemically suitable for the strategy of conjugation by aminolysis to the HPMA copolymer precursor and ellipticines have interesting anticancer activity but also marked toxicities, which conjugation was shown to largely overcome. In the tumour models examined HPMA copolymer-APE conjugates also had increased activity over free APE at low doses, demonstrating the importance of increased tumour targeting mediated by the HPMA copolymer.

**Is HPMA the best polymer to use?**

For many years the polymer of choice for the design of polymer-drug conjugates has been HPMA based on its proven lack of toxicity and excellent solubilisation properties. It was therefore the best polymer to use in this study to design HPMA copolymer conjugates of naturally-derived non-classical anticancer agents. Indeed conjugates of other non-classical anticancer agents have been recently described using HPMA copolymers. These include an HPMA copolymer GFLG-

aminopropylgeldanamycin conjugate, geldanamycin being a benzoquinone ansamycin anticancer antibiotic (Kasuya et al, 2001).

HPMA is not perhaps the ideal polymer, however, as its lack of degradability limits the molecular weight to below that of the renal threshold. Therefore the design of degradable polymers will be vital to the future use of these conjugates. In our group alone systematic studies have been ongoing to identify suitable polymers for this purpose. In the last few years, a potentially degradable carrier poly(L-glutamic acid) conjugated to paclitaxel (Li et al, 1998) and more recently camptothecin (Singer et al, 2001) have been described, with a molecular weight of approximately 50kDa. These conjugates have shown good pre-clinical activity and poly(L-glutamic) paclitaxel has shown good activity in a Phase I trial (Sludden et al, 2001).

**What have we learned about polymer-drug linkers?**

The importance of the choice of a suitable linker in a polymer-drug conjugate has recently been re-enforced by the toxicity reported at clinical trial of the 'MAG'-camptothecin conjugate (Twelves, 2002), described in Section 3.1. These conjugates contained camptothecin linked by a spacer with a terminal ester bond, which may be releasing the drug inappropriately. Therefore in this thesis HPMA copolymer-APE conjugates have used the GFLG linker, chosen on the basis of the widespread use and benefits of this linker, described in Section 1.3.4.2. However, this linker has been used for a long period of time and I believe that there is scope for the development of novel linkers, such as suitable acid-labile or slow hydrolytically-labile linkers. These can be designed to capitalise on some of the basic tumour characteristics which are constantly being discovered. This would help broaden the choice of anticancer agent which could be conjugated as its most potent form, rather than the use of derivatives which may not be as active which are currently conjugated.

**What is the influence of drug loading on polymer-drug conjugates?**

Only one range of drug loading has been reported for each of the polymer-drug conjugates in development. The data obtained in this thesis show that HPMA copolymer-APE conjugates form complex structures in solution. It may be expected that this will be the case for many of the other polymer-drug conjugates being examined due to the hydrophobicity, in the majority of cases, of the pendent drug. The

significance of conjugate conformation needs to be more closely examined for all the polymer-drug conjugates to optimise this feature of design. In this study there was a noticeable effect of conformation observed with the HPMA copolymer GFLG-APE conjugate of highest drug loading showing reduced APE release in tritosome release studies with subsequent reduced *in vivo* activity in the B16F10 tumour model.

**Understanding more about the EPR effect**

Many are still sceptical about the benefits of EPR-mediated targeting and intratumoural distribution of polymer-drug conjugates. Therefore the second aspect of this study has been to utilise the rat dorsal window chamber model to visualise, for the first time, the distribution profile of HPMA copolymer conjugates following extravasation into a solid tumour (using PK1 as a model compound with suitable properties for visualisation). This study showed that PK1 extravasated more rapidly than might have been expected from previous studies and then was distributed widely throughout the tumour. There was no evidence of limited distribution within the tumours examined. Studies on other tumour types will be important to characterise and understand this effect fully. The tumours examined are relatively small and therefore this favourable distribution may not exist for large solid tumours in man. Sat (1999) has shown that such small tumours show better EPR-mediated targeting. However, metastases are often the cause of cancer deaths and this increased accumulation and distribution of polymer-drug conjugates throughout the tumour mass may become important in the treatment of metastatic tumours. This could also be extrapolated to the treatment of small tumours which often develop and re-establish following the removal of a larger tumour. This would strengthen the case for the examination of combination chemotherapy using polymer-drug conjugates.

The work reported here is just the beginning and future studies must utilise the window chamber technology to examine the extravasation and intratumoural distribution of other polymer-drug conjugates. Another use of this model would be to examine the effect of polymeric architecture on extravasation as new polymer architectures are being developed for use as polymer-anticancer drug conjugates (such as dendrimers (Malik et al, 1999)).

The continuing development of novel methods of visualisation, such as the window chamber model, greatly aid our understanding of the development and potential treatment of tumours. This is particularly important with the development of

novel methods of examining human tumours which will be used to standardise the animal models and characterise the relevance of the EPR effect in humans.

**Will HPMA copolymer GFLG-APE become a clinical candidate?**

The potential of HPMA copolymer GFLG-APE conjugates is yet to be fully determined. The reasons for the reduction in anti-tumour activity with increasing dose has to be elucidated, using further anti-tumour studies. These studies are warranted based on the activity observed at very low doses of APE equivalence in the two tumour models studied. Examination of the body distribution and EPR-mediated localisation of HPMA copolymer-APE conjugates would need to be completed, especially to determine the effect of drug loading and dose on these characteristics. Combining these studies with window chamber examination of HPMA copolymer-APE would help give a clearer idea of how this particular conjugate is behaving *in vivo*.

Studies using the window chamber model and other visualisation techniques in the cancer field will, I believe, lead to a better understanding of the conjugate characteristics required to successfully target anticancer drugs to the tumour using the EPR effect. Combining this type of information with the large amount of studies and early clinical trials which have already been reported for polymer-drug conjugates will result in better rational design of the next generation of anticancer polymer-drug conjugates.

The synthesis of more closely defined polymeric structures, particularly in terms of polydispersity and reduction in free drug content will also aid the development of the next generation of polymer-drug conjugates. New treatment strategies, such as the development of the two step PDEPT approach (Satchi et al, 2001) and the development of novel polymeric architectures ensures an exciting future for polymer-drug conjugates as anti-tumour agents.

**Using polymer-drug conjugates to treat other diseases**

Finally as polymer-drug conjugates have been shown to be biocompatible their use need not be limited to cancer and this more widespread interest in the development of polymer-drug conjugates can only benefit the field of polymer therapeutics. Other

conjugates have recently been described for use in diseases other than cancer. Examples of this are an HPMA copolymer 8-aminoquinoline antileishmanial conjugate (Nan et al, 2001) and an HPMA copolymer lectin anti-ulcerative colitis / Barrett's oesophagus conjugate (Wroblewski et al, 2001) which have been recently described. This demonstrates the potential of polymer-drug conjugates in other diseases based on the clinical experience and considerable preclinical data with HPMA copolymers. Here the development of degradable polymers will help, particularly for those diseases where repeated dosing, over long periods, will be necessary and the use of non-degradable polymers would be undesirable such as in the treatment of inflammation, where there may also be hyperpermeability to macromolecules.

# References

Access Pharmaceuticals Ltd. website (2001) www.accesspharma.com

Alakhov VY, Moskaleva EY, Batrakova EV and Kabanov AV (1996) Hypersensitization of multidrug resistant human ovarian carcinoma cells by pluronic P85 block copolymer. *Bioconj. Chem.*, **7**: 209-216

Alberici GF, Fellous R, Bidart JM, Troalen F, Mondesir JM, Goodman A, Ho DH and Bohuon C (1986) Human antibodies to the antineoplastic drug elliptinium: characterization and structure-activity relationships. *J. Allergy Clin. Immunol.*, **77(4)**: 624-630

Allen TM and Moase EH (1996) Therapeutic opportunities for targeted liposomal drug delivery. *Adv. Drug Del. Rev.*, **21**: 117-133

Allen TM (2000) Solving drug delivery problems with liposomal carriers. In *Controlled Drug Delivery: designing technologies for the future ACS Symposium Series 752*, Park K and Mrsny R (eds) pp 100-109. American Chemical Society, Washington, U.S.A.

Bailon P and Berthold W (1998) Polyethylene glycol-conjugated pharmaceutical proteins. *Pharm. Sci. Technol. To.*, **1(8)**: 352-356

Balosso J, Giocanti N and Favaudon V (1991) Additive and supraadditive interaction between ionizing-radiation and pazelliptine, a DNA topoisomerase inhibitor, in chinese-hamster V-79 fibroblasts. *Cancer Res.*, **51(12)**: 3204-3211

Bangham AD, Standish HM and Watkins JC (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.*, **13**: 238-252

Baselga J (2001) Clinical trials of Herceptin® (trastuzumab). *Eur. J. Cancer*, **37**: S18-24

Batrakova EV, Dorodnych TY, Klinskii EY, Kliushnenkova EN, Shemchukova OB, Goncharova ON, Arjakov SA, Alakhov VY and Kabanov AV (1996) Anthracycline antibiotics non-covalently incorporated into the block copolymer micelles: In vivo evaluation of anti-cancer activity. *Br. J. Cancer*, **74(10)**: 1545-1552

Biltonen RL and Friere E (1978) Thermodynamic characterization of conformational states of biological macromolecules using differential scanning calorimetry. *CRC Crit. Rev. Biochem.*, **5(2)**: 85-124

Blumhardt L (2000) Phase III studies with the synthetic copolymer glatiramer acetate: a new treatment for multiple sclerosis. *Proc. 4th Intl. Symp. Polym. Ther.*: 24

Brem H, Piantadosi S, Burger PC, Walker M, Selker R, Vick NA, Black K, Sisti M, Brem S, Mohr G, Muller P, Morawetz R and Schold SC (1995) Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. *Lancet*, **345(8956)**: 1008-1012

British National Formulary (2001) Number 41. British Medical Association and Royal Pharmaceutical Society of Great Britain, London, U.K.

Brocchini S and Duncan R (1999) Pendent drugs, release from polymers, In *Encyclopedia of Controlled Drug Delivery*, Mathiowitz E (ed) pp 786-816. John Wiley & Sons, New York, U.S.A.

Brown KG, Erfurth SC, Small EW and Peticolas WL (1972) Conformationally dependent low-frequency motions of proteins by laser Raman spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.*, **69(6)**: 1467-1469

Buske C, Feuring-Buske M, Unterhalt M and Hiddemann W (1999) Monoclonal antibody therapy for B cell non-hodgkin's lymphomas: emerging concepts of a tumour-targeted strategy. *Eur. J. Cancer*, **35(4)**: 549-557

Caiolfa VR, Zamai M, Fiorino A, Frigerio E, Pellizzoni C, d'Argy R, Ghiglieri A, Castelli MG, Farao M, Pesenti E, Gigli M, Angelucci F and Suarato A (2000) Polymer-bound camptothecin: initial biodistribution and antitumour activity studies. *J. Control. Rel.*, **65**: 105-119

Cappelaere P, Demaille MC, Azab M and Degardin M (1993) Phase I study of retelliptine dihydrochloride (RTE) using daily i.v. infusion for 3 days. *Proc. Am. Soc. Clin. Oncol.*, **12**: 129

Carmeliet P and Jain RK (2000) Angiogenesis in cancer and other diseases. *Nature*, **407**: 249-257

Cassidy J, Duncan R, Morrison GJ, Strohalm J, Plocova D, Kopecek J and Kaye SB (1989) Activity of N-(2-hydroxypropyl)methacrylamide copolymers containing daunomycin against a rat tumor model. *Biochem. Pharmacol.*, **38(6)**: 875-879

Charcosett JY, Saucier JM and Jacquemin-Sablon A (1988) Reduced DNA topoisomerase II activity and drug-stimulated DNA cleavage in 9-hydroxyellipticine resistant cells. *Biochem. Pharmacol.*, **37(11)**: 2145-2149

Choi W-M, Kopeckova P, Minko T and Kopecek J (1999) Synthesis of HPMA copolymer containing adriamycin bound via an acid-labile spacer and its activity toward human ovarian carcinoma cells. *J. Bioact. Compat. Polym.*, **14**: 447-456

Choksakulnimitr S, Masuda S, Tokuda H, Takakura Y and Hashida M (1995) In vitro cytotoxicity of macromolecules in different cell culture systems. *J. Control. Rel.*, **34**: 233-241

Connors TA (1996) New anti-cancer agents. *Anti-Cancer Drug.*, **7(Suppl. 3)**: 179-185

Cook JA and Mitchell VB (1989) Viability measurements – cell systems. *Anal. Biochem*, **179(1)**: 1-7

Cragg GM, Boyd MR, Khanna R, Newman DJ and Sausville EA (1999) Natural product drug discovery and development: the United States National Cancer Institute role. *In Phytochemicals in Human Health Protection, Nutrition and Plant Defense*, Romeo (ed) pp 1-29. Plenum publishers: New York, U.S.A.

Cragg GM, Newman DJ and Snader KM (1997) Natural products in drug discovery and development. *J. Nat. Prod.*, **60**: 52-60

CRC Cancerstats (2000): Mortality-UK, The Cancer Research Campaign, U.K.

Dalton LK, Demerac S, Elmes BC, Lorder JW, Swan JM and Teitel T (1967) Synthesis of the tumor-inhibitory alkaloids ellipticine, 9-methoxyellipticine and related pyrido[4,3-b]carbazoles. *Aust. J. Chem.*, **20**: 2715-2727

Dang W, Daviau T, Ying P, Zhao Y, Nowotnik D, Clow CS, Tyler B and Brem H (1996) Effects of Gliadel® wafer initial molecular weight on the erosion of wafer and release of BCNU. *J. Control. Rel.*, **42**: 83-92

Danhauser-Reidl S, Hausmann E, Schick HD, Bender R, Dietzfelbinger H, Rastetter J and Hanauske AR (1993) Phase I clinical and pharmacokinetic trial of dextran conjugated doxorubicin (AD-70, DOX-OXD). *Invest. New Drugs*, **11(2-3)**: 187-195

Dean RT (1977) Lysosomes. In *The Institute of Biology's studies in Biology no.84* pp 1-54. Edward Arnold (Publishers) Ltd: London U.K.

Debruyne FMJ, Denis L, Lunglmayer G, Mahler C, Newling DWW, Richards B, Robinson MRG, Smith PH, Weil EHJ and Whelan P (1988) Long-term therapy with a depot luteinizing-hormone-releasing hormone analog (Zoladex) in patients with advanced prostatic-carcinoma. *J. Urol.*, **140(4)**: 775-777

De Duve C, De Barsy T, Poole B, Trouet A, Tulkens P and Van Hoof F (1974) Commentary. Lysosomotropic agents. *Biochem. Pharmacol.*, **23(18)**: 2495-2531

De Vita VT, Hellman S and Rosenberg SA (1993) In *Cancer: principles and practice of oncology* ($4^{th}$ ed), JB Lippincott Company, Philadelphia, U.S.A.

Devraj R, Jurayi J, Fernandez JA, Barrett JF and Cushman M (1996) Synthesis of a series of cytotoxic 2-acyl-1,2-dihydroellipticines which inhibit topoisomerase II. *Anticancer Drug Des.*, **11(4)**: 311-324

Dimitrijevic S and Duncan R (1998) Synthesis and characterization of *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer-emetine conjugates. *J. Bioact. Compat. Polym.*, **13**: 165-178

Djuric Z, Everett CK and Valeriote FA (1992) DNA damage and cytotoxicity in L1210 cells by ellipticine and a structural analogue, N-2-(diethylaminoethyl)-9-hydroxyellipticinium chloride. *Cancer Res.*, **52**: 1515-1519

Dodane V and Vilivalam VD (1998) Pharmaceutical applications of chitosan. *Pharm. Sci. Technol. To.*, **1(6)**: 246-253

Donato MT, Goethals F, Gomez-Lechon MJ, Deboyser D, DeCoster I, Roberfroid M and Castell JV (1992) Toxicity of the antitumoral drug datelliptium in hepatic cells: use of models *in vitro* for the prediction of toxicity *in vivo*. *Toxicol. In Vitro*, **6(4)**: 295-302

Duncan R, Cable HC, Lloyd JB, Rejmanova P and Kopecek J (1983) Polymers containing enzymatically degradable bonds, 7: design of oligopeptide side-chains in poly[*N*-(2-hydroxypropyl)methacrylamide] copolymers to promote efficient degradation by lysosomal enzymes. *Makromol. Chem.*, **184**: 1997-2008

Duncan R and Kopecek J (1984) Soluble synthetic polymers as potential drug carriers. *Adv. Polym. Sci.*, **57**: 52-101

Duncan R (1986) Lysosomal degradation of polymers used as drug carriers. *CRC Crit. Rev. Biocompat.*, **2(2)**: 127-145

Duncan R, Hume IC, Yardley HJ, Flanagan PA, Ulbrich K, Subr V and Strohalm J (1991) Macromolecular prodrugs for use in targeted cancer chemotherapy: melphalan covalently coupled to *N*-(2-hydroxypropyl)methacrylamide copolymers. *J. Control. Rel.*, **16**: 121-136

Duncan R (1992) Drug-polymer conjugates: potential for improved chemotherapy. *Anti-Cancer Drugs*, **3**: 175-210

Duncan R, Ferruti P, Sgouras D, Tuboku-Metzger A, Ranucci E and Bignotti F (1994) A polymer-triton X-100 conjugate capable of pH-dependent red blood cell lysis: a model system illustrating the possibility of drug delivery within acidic intracellular compartments. *J. Drug Targeting*, **2**: 341-347

Duncan R and Spreafico F (1994) Polymer conjugates – pharmacokinetic considerations for design and development. *Clin. Pharmacokinet.*, **27(4)**: 290-306

Duncan R, Dimitrijevic S and Evagorou EG (1996) The role of polymer conjugates in the diagnosis and treatment of cancer. *STP Pharm. Sci.*, **6(4)**: 237-263

Duncan R, Coatsworth JK and Burtles S (1998) Preclinical toxicology of a novel polymeric antitumour agent: HPMA copolymer-doxorubicin (PK1). *Human. Exp. Toxicol.*, **17**: 93-104

Duncan R (2000) Polymer therapeutics into the 21st century. In *Controlled Drug Delivery: designing technologies for the future ACS Symposium Series 752*, Park K and Mrsny R (eds) pp 350-363. American Chemical Society, Washington, U.S.A.

Duncan R, Gac-Breton S, Keane R, Musila R, Sat YN, Satchi R and Searle F (2001) Polymer-drug conjugates, PDEPT and PELT: basic principles for design and transfer from the laboratory to clinic. *J. Control. Rel.*, **74**: 135-146

Elcock AH, Rodger A and Richards WG (1996) Theoretical studies of the intercalation of 9-hydroxyellipticine in DNA. *Biopolymers*, **39(3)**: 309-326

Erukova VY, Krylova OO, Antonenko YN and Melik-Nubarov NS (2000) Effect of ethylene oxide and propylene oxide block copolymers on the permeability of bilayer lipid membranes to small solutes including doxorubicin. *Biochim. Biophys. Acta*, **1468**: 73-86

Etrych T, Jelinkova M, Rihova B and Ulbrich K (2001) New HPMA copolymers containing doxorubicin bound via pH-sensitive linkage: synthesis and preliminary in vitro and in vivo biological properties. *J. Control. Rel.*, **73**: 89-102

Fenton BM, Paoni SF, Lee J, Koch CJ, Lord EM (1999) Quantification of tumour vasculature and hypoxia by immunohistochemical staining and $HbO_2$ saturation measurements. *British J. Cancer*, **79(3/4)**: 464-471

Firestone RA, Willner D, Hofstead SJ, King HD, Kaneko T, Braslawsky GR, Greenfield RS, Trail PA, Lasch SJ, Henderson AJ, Casazza AM, Hellstrom I and Hellstrom KE (1996) Synthesis and antitumor activity of the immunoconjugate BR96-Dox. *J. Control. Rel.*, **39**: 251-259

Flanagan PA (1987) Evaluation of the pinocytic uptake and cellular processing of antibody-N-(2-hydroxypropyl)methacrylamide copolymer conjugates and estimation of their potential use in "targeted" drug delivery - PhD thesis. University of Keele U.K.

Forssen EA, Malé-Brune R, Adler-Moore JP, Lee MJA, Schmidt PG, Krasieva TB, Shimizu S and Tromberg BJ (1996) Fluorescence imaging studies for the disposition of daunorubicin liposomes (DaunoXome) within tumor tissue. *Cancer Res.*, **56**: 2066-2075

Forssen EA (1997) The design and development of DaunoXome® for solid tumor targeting in vivo. *Adv. Drug Del. Rev.*, **24**: 133-150

Fraier D, Frigerio E, Pianezzola E, Strolin Benedetti M, Cassidy J and Vasey P (1995) A sensitive procedure for the quantitation of free and N-(2-hydroxypropyl) methacrylamide polymer-bound doxorubicin (PK1) and some of its metabolites, 13-dihydrodoxorubicin, 13-dihydrodoxorubicinone and doxorubicinone, in human plasma and urine by reversed-phase HPLC with fluorimetric detection. *J. Pharm. Biomed. Anal.*, **13(4/5)**: 625-633

Frenoy JP (1986) Effect of physical environment on the conformation of ricin. Influence of low pH. *Biochem. J.*, **240(1)**: 221-226

Froelich-Ammon SJ, Patchan MW, Osheroff N and Thompson RB (1995) Topoisomerase II binds to ellipticine in the absence or presence of DNA. *J. Biol. Chem.*, **270(25)**: 14998-15004

Fung LK and Saltzman WM (1997) Polymeric implants for cancer chemotherapy. *Adv. Drug Del. Rev.*, **26**: 209-230

Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, Martin F, Huang A and Barenholz Y (1994) Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res.*, **54**: 987-992

Gautier S (1995) Hydrophobisation du vecteur poly(L-lysine citramide): comportement physico-chimique et aptitude a solubiliser des molecules lipophiles dans l'eau - PhD thesis. University of Montpellier France

Gewirtz DA (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem. Pharmacol.*, **57**: 727-741

Gianasi E, Wasil M, Evagorou EG, Keddle A, Wilson G and Duncan R (1999) HPMA copolymer platinates as novel antitumour agents: *in vitro* properties, pharmacokinetics and antitumour activity *in vivo*. *Eur. J. Cancer*, **35(6)**: 994-1002

Giavazzi R, Albini A, Bussolino F, DeBraud F, Presta M, Ziche M and Costa A (2000) The biological basis for antiangiogenic therapy. *Eur. J. Cancer*, **36**: 1913-1918

Gill PS, Wernz J, Scadden DT, Cohen P, Mukwaya GM, Von Roenn JH, Jacobs M, Kempin S, Silverberg I, Gonzales G, Rarick MU, Myers AM, Shepherd F, Sawka C, Pike MC and Ross ME (1996) Randomized Phase III trial of liposomal daunorubicin versus doxorubicin, bleomycin and vincristine in AIDS-related Kaposi's sarcoma. *J. Clin. Oncol.*, **14**: 2353-2364

Glinsky GV and Glinsky VV (1996) Apoptosis and metastasis: a superior resistance of metastatic cancer cells to programmed cell death. *Cancer Lett.*, **101**: 43-51

Godwin A, Hartenstein M, Muller AHE and Brocchini S (2001) Narrow molecular weight distribution precursors for polymer-drug conjugates. *Angew. Chem.*, **113(3)**: 614-617

Goodwin S, Smith AF and Horning EC (1959) Alkaloids of *Ochrosia elliptica Labill. J. Am. Chem. Soc.*, **81**: 1903-1908

Greenwald RB (1997) Drug delivery systems: anticancer prodrugs and their polymeric conjugates. *Exp. Opin. Ther. Patents*, **7(6)**: 601-609

Greenwald RB, Conover CD, Pendri A, Choe YH, Martinez A, Wu D, Guan S, Yao Z and Shum KL (1999) Drug delivery of anticancer agents: water soluble 4-poly(ethylene glycol) derivatives of the lignan, podophyllotoxin. *J. Control. Rel.*, **61**: 281-294

Gregoriadis G (1995) Engineering liposomes for drug delivery: progress and problems. *Trends Biotechnol.*, **13(12)**: 527-537

Hillery A (2000) Anti-vascular targeting: a novel approach to cancer treatment. *Pharm. Sci. Technol. To.*, **3(1)**: 4

Hirano T, Klesse W and Ringsdorf H (1979) Polymeric derivatives of activated cyclophosphamide as drug delivery systems in anti-tumor chemotherapy. *Makromol. Rev.*, **180**: 1125-1131

Hirano T, Ringsdorf H and Zaharko DS (1980) Antitumor activity of monomeric and polymeric cyclophosphamide derivatives compared with in vitro hydrolysis. *Cancer Res.*, **40(7)**: 2263-2267

Hoes CJT, Grootoonk J, Duncan R, Hume IC, Bhakoo M, Bouma M, Bouma JMW and Feijen J (1993) Biological properties of adriamycin bound to biodegradable polymeric carriers. *J. Control. Rel.*, **23(1)**: 37-54

Huinink WWT, Terwogt JM, Dubbelman R, Valkenet L, Zurlo MG, Schellens JHM and Beijnen JH (1998) Phase I and pharmacokinetics study of PNU166945, a polymer formulated paclitaxel. *Proc. 3rd Intl. Symp. Polym. Ther.*: 12

Ilhan F, Gray M, Blanchette K and Rotello VM (1999) Control of polymer solution structure via intra- and intermolecular aromatic stacking. *Macromolecules*, **32**: 6159-6162

Jain RK and Baxter LT (1988) Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: significance of elevated interstitial pressure. *Cancer Res.*, **48**: 7022-7032

Jain RK (1989) Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J. Natl. Cancer Inst.*, **81(8)**: 70-76

Jain RK (1998) The next frontier of molecular medicine: delivery of therapeutics. *Nat. Med.*, **4(6)**: 655-657

Jain RK (2001) Delivery of molecular medicine to solid tumors: lessons from in vivo imaging of gene expression and function. *J. Control. Rel.*, **74(1-3)**: 7-25

Jones M-C and Leroux J-C (1999) Polymeric micelles – a new generation of colloidal drug carriers. *Eur. J. Pharm. Biopharm.*, **48**: 101-111

Julyan PJ, Seymour LW, Ferry DR, Daryani S, Boivin CM, Doran J, David M, Anderson D, Christodoulou C, Young AM, Hesslewood S and Kerr DJ (1999) Preliminary clinical study of the distribution of HPMA copolymers bearing doxorubicin and galactosamine. *J. Control. Rel.*, **57(3)**: 281-290

Kaminski MS, Zasadny KR, Francis IR, Fenner MC, Ross CW, Milik AW, Estes J, Tuck M, Regan D, Fisher S, Glenn SD and Wahl RL (1996) Iodine-131-anti-B1 radioimmunotherapy for B-cell lymphoma. *J. Clin. Oncol.*, **14(7)**: 1974-1981

Kasuya Y, Lu Z-R, Kopeckova P, Minko T, Tabibi SE and Kopecek J (2001) Synthesis and characterization of HPMA copolymer-aminopropylgeldanamycin conjugates. *J. Control. Rel.*, **74**: 203-211

Kataoka K, Matsumoto T, Yokoyama M, Okano T, Sakurai, Fukushima S, Okamoto K and Kwon GS (2000) Doxorubicin-loaded poly(ethylene glycol)-poly(β-benzyl-L-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance. *J. Control. Rel.*, **64**: 143-153

Keating MJ, Holmes R, Lerner S and Ho DH (1993) L-asparaginase and PEG asparaginase – past, present and future. *Leuk. Lymphoma*, **10(Suppl)**: 153-157

Kerbel RS (1997) A cancer therapy resistant to resistance. *Nature*, **390**: 335-336

Khayat D, Borel C, Azab M, Paraisot D, Malaurie E, Bouloux C and Weil M (1992) Phase-I study of datelliptium chloride, hydrochloride given by 24-h continuous intravenous-infusion. *Cancer Chemo. Pharmacol.*, **30(3)**: 226-228

Kohler G and Milstein C (1975) Continuous culture of fused cells secreting antibody of predefined specificity. *Nature*, **256**: 495-497

Konerding MA, Malkusch W, Klapthor B, Van Ackern C, Fait E, Hill SA, Parkins C, Chaplin DJ, Presta M and Denekamp J (1999) Evidence for characteristic vascular patterns in solid tumours: quantitative studies using corrosion casts. *British J. Cancer*, **80(5/6)**: 724-732

Konno T, Maeda H, Iwai K, Tashiro S, Maki S, Morinaga T, Mochinaga M, Hiraoka T and Yokoyama I (1983) Effect of arterial administration of high-molecular-weight anticancer agent SMANCS with lipid lymphographic agent on hepatoma: a preliminary report. *Eur. J. Clin. Oncol.*, **19(8)**: 1053-1065

Kopecek J and Duncan R (1987) Targetable polymeric prodrugs. *J. Control. Rel.*, **6**: 315-327

Kopecek J, Kopeckova P, Minko T and Lu Z-R (2000) HPMA copolymer-anticancer drug conjugates: design, activity and mechanism of action. *Eur. J. Pharm. Biopharm.*, **50**: 61-81

Kranz DM, Manning TC, Rund LA, Cho BK, Gruber MM and Roy EJ (1998) Targeting tumor cells with bispecific antibodies and T cells. *J. Control. Rel.*, **53**: 77-84

Krishna R and Mayer LD (2000) Multidrug resistance (MDR) in cancer: mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur. J. Pharm. Sci.*, **11**: 265-283

Kwon G, Suwa S, Yokoyama M, Okano T, Sakurai Y and Kataoka K (1994) Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxide-aspartate) block copolymer-adriamycin conjugates. *J. Control. Rel.*, **29**: 17-23

Lee IP (1976) A possible mechanism of ellipticine-induced hemolysis. *J. Pharmacol. Exp. Ther.*, **196(3)**: 525-535

Lee MD, Dunne TS, Chang CC, Ellestad GA, Siegel MM, Morton GO, McGahren WJ and Borders DB (1987) Calichemicins, a novel family of antitumor antibiotics. 2. Chemistry and structure of calichemicin-gamma-1. *J. Am. Chem. Soc.*, **109(11)**: 3466-3468

Lee EC and Muller SJ (1999) Flow light scattering studies of polymer coil conformation in solutions under shear: effect of solvent quality. *Polymer*, **40**: 2501-2510

Le Mee S, Pierre A, Markovits J, Atassi G, Jacquemin-Sablon A and Saucier J-M (1998) S16020-2, a new highly cytotoxic antitumor olivacine derivative: DNA interaction and DNA topoisomerase II inhibition. *Mol. Pharmacol.*, **53**: 213-220

Li C, Yu D-F, Newman RA, Cabral F, Stephens LC, Hunter N, Milas L and Wallace S (1998) Complete regression of well-established tumors using a novel water-soluble poly(l-glutamic acid)-paclitaxel conjugate. *Cancer Res.*, **58**: 2404-2409

Li C, Price JE, Milas L, Hunter NR, Ke S, Yu D-F, Charnsangavej C and Wallace S (1999) Antitumour activity of poly(L-glutamic acid)-paclitaxel on syngeneic and xenografted tumors. *Clin. Cancer Res.*, **5**: 891-897

Li C-Y, Shan S, Huang Q, Braun RD, Lanzen J, Hu K, Lin P and Dewhirst MW (2000) Initial stages of tumor cell-induced angiogenesis: evaluation via skin window chambers in rodent models. *J. Natl. Cancer Inst.*, **92(2)**: 143-147

Lloyd JB (2000) Lysosome membrane permeability: implications for drug delivery. *Adv. Drug Del. Rev.*, **41**: 189-200

Lobert S, Vulevic B and Correia JJ (1996) Interaction of vinca alkaloids with tubulin: a comparison of vinblastine, vincristine and vinorelbine. *Biochemistry*, **35(21)**: 6806-6814

Madden T, Tran HT, Beck D, Huie R, Newman RA, Pusztai L, Wright JJ and Abbruzzese JL (2000) Novel marine-derived anticancer agents: a Phase I clinical, pharmacological and pharmacodynamic study of dolastatin 10 (NSC 376128) in patients with advanced solid tumors. *Clin. Cancer Res.*, **6**: 1293-1301

Maeda H, Seymour LW and Miyamoto Y (1992) Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconj. Chem.*, **3(5)**: 351-362

Maeda H and Matsumura Y (1989) Tumoritropic and lymphotropic principles of macromolecular drugs. *Crit. Rev. Ther. Drug Carrier Syst.*, **6(3)**: 193-210

Maeda H, Wu J, Sawa T, Matsumura Y and Hori K (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Control. Rel.*, **65**: 271-284

Malik N, Evagorou E and Duncan R (1999) Dendrimer-platinate: a novel approach to cancer chemotherapy. *Anti-Cancer Drugs*, **10**: 767-776

Malonne H, Farinelle S, Decaestecker C, Gordower L, Fontaine J, Chaminade F, Saucier J-M, Atassi G and Kiss R (2000) In vitro and in vivo pharmacological characterizations of the antitumor properties of two new olivacine derivatives, S16020-2 and S30972-1. *Clin. Cancer Res.*, **6**: 3774-3782

Martindale (1993) 30$^{th}$ edition, Reynolds JE (ed). The Pharmaceutical Press, London U.K.

Matsumura Y and Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.*, **46(12I)**: 6387-6392

McCafferty J and Glover DR (2000) Engineering therapeutic proteins. *Curr. Opin. Struct. Biol.*, **10**: 417-420

McCaffrey JA and Bajorin DF (1998) Therapy for good risk germ cell tumors. *Semin. Oncol.*, **25(2)**: 186-193

Meerum Terwogt JM, ten Bokkel Huinink WW, Schellens JHM, Schot M, Mandjes IAM, Zurlo MG, Rocchetti M, Rosing H, Koopman FJ and Bejnen JH (2001) Phase I clinical and pharmacokinetic study of PNU166945, a novel water-soluble polymer-conjugated prodrug of paclitaxel. *Anti-Cancer Drugs*, **12**: 315-323

Mehvar R (2000) Dextrans for targeted and sustained delivery of therapeutic and imaging agents. *J. Control. Rel.*, **69**: 1-25

Mellman I (1996) Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.*, **12**: 575-625

Menozzi M, Valentini L, Vannini E and Arcamone F (1984) Self-association of doxorubicin and related-compounds in aqueous-solution. *J. Pharm. Sci.*, **73(6)**: 766-770

Mezo G, Remenyi J, Kajtar J, Barna K, Gaal D and Hudecz F (2000) Synthesis and conformational studies of poly(L-lysine) based branched polypeptides with ser and glu/leu in the side chains. *J. Control. Rel.*, **63**: 81-95

Minko T, Kopeckova P, Pozharov and Kopecek J (1998) HPMA copolymer bound adriamycin overcomes *MDR1* gene encoded resistance in a human ovarian carcinoma cell line. *J. Control. Rel.*, **54**: 223-233

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.*, **65**: 55-63

Muggia FM, Hainsworth JD, Jeffers S, Miller P, Groshen S, Tan M, Roman L, Uziely B, Muderspach L, Garcia A, Burnett A, Greco FA, Morrow CP, Paradiso LJ and Liang LJ (1997) Phase II study of liposomal doxorubicin in refractory ovarian cancer: antitumor activity and toxicity modification by liposomal encapsulation. *J. Clin. Oncol.*, **15(3)**: 987-993

Nakanishi T, Fukushima S, Okamoto K, Suzuki M, Matsumura Y, Yokoyama M, Okano T, Sakurai Y and Kataoka K (2001) Development of the polymer micelle carrier system for doxorubicin. *J. Control. Rel.*, **74**: 295-302

Nan A, Dhammika Nanayakkara NP, Walker LA, Yardley V, Croft SL and Ghandehari H (2001) *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers for targeted delivery of 8-aminoquinoline antileshmanial drugs. *J. Control. Rel.*, **77**: 233-243

NCI (National Cancer Institute) website (2001) www.nci.nih.gov

Newman DJ, Cragg GM and Snader KM (2000) The influence of natural products upon drug discovery. *Nat. Prod. Rep.*, **17**: 215-234

Nomura T, Saikawa A, Morita S, Sakeda T, Yamashita F, Honda K, Takakura Y and Hashida M (1998) Pharmacokinetic characteristics and therapeutic effects of mitomycin C-dextran conjugates after intratumoural injection. *J. Control. Rel.*, **52**: 239-252

Nucci ML, Shorr R and Abuchowski A (1991) The therapeutic value of poly(ethylene glycol)-modified proteins. *Adv. Drug Del. Rev.*, **6**: 133-151

O'Hare KB, Duncan R, Strohalm J, Kopeckova P, Kopecek J and Ulbrich K (1993) Macromolecular prodrugs containing doxorubicin and melanocyte stimulating hormone in vitro and in vivo evaluation against murine melanoma. *J. Drug Target.*, **1**: 217-230

Ohashi M and Oki T (1996) Ellipticine and related anticancer agents. *Exp. Opin. Ther. Patents*, **6(12)**: 1285-1294

Ohkuma S and Poole B (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. U.S.A.*, **75(7)**: 3327-3331

Ohnama T, Holland JF and Meyer P (1972) *Erwinia carotovora* asparaginase in patients with prior anaphylaxis to asparaginase from *E. coli*. *Cancer*, **30**: 376-381

Ohya Y, Huang TZ, Ouchi T, Hasegawa K, Tamura J, Kadowaki K, Matsumoto T, Suzuki S and Suzuki M (1991) Synthesis and antitumor activity of α-1, 4-polygalactosamine and N-acetyl-α-1, 4-polygalactosamine immobilized 5-fluorouracils through hexamethylene spacer groups via urea, urea bonds. *J. Control. Rel.*, **17**: 259-266

Okuno S, Harada M, Yano T, Yano S, Kiuchi S, Tsuda N, Sakamura Y, Imai J, Kawaguchi T and Tsujihara K (2000) Complete regression of xenografted human carcinomas by camptothecin analogue-carboxymethyl dextran conjugate (T-0128). *Cancer Res.*, **60(11)**: 2988-2995

Ottenbrite RM, Kuus K and Kaplan AM (1983) Characteristic biological effects of anionic polymers. In *Polymers in Medicine: biomedical and pharmacological applications*, Chiellini E and Giusti P (eds) pp 3-22. Plenum Press, New York, U.S.A.

Palmer DH, Seymour LW, Ferry DR, Hussain SA, Hesslewood S, Julyan PJ, Poyner R, Doran J, Young AM, Burtles S and Kerr DJ (2001) Targeting drugs to the liver: clinical evaluation of polymer-bound doxorubicin for the treatment of liver cancer. *Br. J. Cancer*, **85(Suppl 1)**: #7.7

Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C and Martin FJ (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 11460-11464

Parnaud G, Tache S, Peiffer G and Corpet DE (2001) Pluronic F68 block polymer, a very potent suppressor of carcinogenesis in the colon of rats and mice. *Br. J. Cancer*, **84(1)**: 90-93

Pechar M, Ulbrich K, Subr V, Seymour LW and Schacht EH (2000) Poly(ethylene glycol) multiblock copolymer as a carrier of anti-cancer drug doxorubicin. *Bioconj. Chem.*, **11(2)**: 131-139

Pederson D, Gabriel D and Hermans J (1971) Potentiometric titration of poly-L-lysine: the coil to beta transition. *Biopolymers*, **10(11)**: 2133-2145

Pendri A, Conover CD and Greenwald RB (1998) Antitumor activity of paclitaxel-2'-glycinate conjugated to poly(ethylene glycol): a water-soluble prodrug. *Anti-Cancer Drug Design*, **13(5)**: 387-395

Polymers: Biomaterials and Medical Applications (1989) Kroschwitz JI (ed). John Wiley & Sons, New York, U.S.A.

Potmesil M (1994) Camptothecins: from bench research to hospital wards. *Cancer Res.*, **54**: 1431-1439

Przybylski M, Zaharko DS, Chirigos MA, Adamson RH, Schultz RM and Ringsdorf H (1978) DIVEMA-methotrexate: immune-adjuvant role of polymeric carriers linked to antitumor agents. *Cancer Treat. Rep.*, **62(11)**: 1837-1843

Putnam D and Kopecek J (1995) Enantioselective release of 5-fluorouracil from N-(2-hydroxypropyl)methacrylamide-based copolymers via lysosomal-enzymes. *Bioconj. Chem.*, **6(4)**: 483-492

Raguenez-Viotte G, Dadoun C, Buchet P, Ducas-Telle T and Fillastre JP (1988) Renal toxicity of the antitumour drug N-methyl-9-hydroxyellipticinium acetate in the Wistar rat. *Arch. Toxicol.*, **61**: 292-297

Ranucci E, Spagnoli G, Ferruti P, Sgouras D and Duncan R (1991) Poly(amidoamine)s with potential as drug carriers: degradation and cellular toxicity. *J. Biomat. Sci., Polym. Ed.*, **2**: 303-315

Rejmanova P, Labsky J and Kopecek J (1977) Aminolyses of monomeric and polymeric *p*-nitrophenyl esters of methacryloylated amino acids. *Makromol. Chem.*, **178**: 2159-2168

Rejmanova P, Kopecek J, Duncan R and Lloyd JB (1985) Stability in rat plasma and serum of lysosomally degradable oligopeptide sequences in *N*-(2-hydroxypropyl)methacrylamide copolymers. *Biomaterials*, **6**: 45-48

Richardson S, Ferruti P and Duncan R (1999) Poly(amidoamine)s as potential endosomolytic polymers: evaluation in vitro and body distribution in normal and tumour-bearing animals. *J. Drug Target.*, **6(6)**: 391-404

Rihova B, Ulbrich K, Kopecek J and Mancal P (1983) Immunogenicity of N-(2-hydroxypropyl)-methacrylamide copolymers – potential hapten or drug carriers. *Folia. Microbiol.*, **28**: 217-227

Rihova B, Bilej M, Vetvicka V, Ulbrich K, Strohalm J, Kopecek J and Duncan R (1989) Biocompatibility of *N*-(2-hydroxypropyl) methacrylamide copolymers containing adriamycin. *Biomaterials*, **10**: 335-342

Rihova B (1998) Receptor-mediated targeted drug or toxin delivery. *Adv. Drug Del. Rev.*, **29**: 273-289

Ringsdorf H (1975) Structure and properties of pharmacologically active polymers. *J. Polym. Sci. Polymer Symp.*, **51**: 135-153

Ringsdorf H, Venzmer J and Winnik FM (1991) Fluorescence studies of hydrophobically modified poly(N-isopropylacrylamides). *Macromolecules*, **24**: 1678-1686

Ringsdorf H, Simon J and Winnik FM (1992) Hydrophobically-modified poly(*N*-isopropylacrylamides) in water: a look by fluorescence techniques at the heat-induced phase transition. *Macromolecules*, **25**: 7306-7312

Ringsdorf H, Sackmann E, Simon J and Winnik FM (1993) Interactions of liposomes and hydrophobically-modified poly-(N-isopropylacrylamides): an attempt to model the cytoskeleton. *Biochim. Biophys. Acta*, **1153**: 335-344

Rizzo V (2000) Validated characterization of polymer-drug conjugates. *Proc. 4th Intl. Symp. Polym. Ther.*, 17

Rouesse J, LeChevalier T, Caille P, Mondesir J, Sancho-Garnier H, May-Levin F, Spielmann M, Dejager R and Amiel J (1985) Phase II study of ellipticinium in advanced breast cancer. *Cancer Treat. Rep.*, **69**: 707-708

Rouesse J, Spielmann M, Turpin F, Le Chevalier T, Azab M and Mondesir JM (1993) Phase II study of elliptinium acetate salvage treatment of advanced breast cancer. *Eur. J. Cancer*, **29A(6)**: 856-859

Rubin P and Casarett G (1966) Microcirculation of tumors. I. Anatomy, function and necrosis. *Clin. Radiol.*, **17**: 220-229

Ruckdeschel JC and Archer S (1989) Rational design of a novel ellipticine derivative with significant activity against human lung cancer cell lines. *Proc. Am. Assoc. Cancer Res.*, **30**: A2420

Ryan DP, Supko JG, Eder JP, Seiden MV, Demetri G, Lynch TJ, Fischman AJ, Davis J, Jimeno J and Clark JW (2001) Phase I and pharmacokinetic study of ecteinascidin 743 administered as a 72-hour continuous intravenous infusion in patients with solid malignancies. *Clin. Cancer Res.*, **7(2)**: 231-242

Ryser HJ and Shen WC (1978) Conjugation of methotrexate to poly(l-lysine) increases drug transport and overcomes drug resistance in cultured cells. *Proc. Natl. Acad. Sci. U.S.A.*, **75(8)**: 3867-3870

Sainsbury M (1990) Ellipticines. In *The chemistry of antitumour agents*, Wilman DEV (ed) pp 410-435. Blackie & Son Ltd., London, U.K.

Saleh MN, Sugarman S, Murray J, Ostroff JB, Healey D, Jones D, Daniel CR, LeBherz D, Brewer H, Onetto N and LoBuglio AF (2000) Phase I trial of the anti-Lewis Y drug immunoconjugate BR96-doxorubicin in patients with Lewis Y-expressing epithelial tumors. *J. Clin. Oncol.*, **18(11)**: 2282-2292

Sat YN (1999) Factors that influence tumour targeting by the enhanced permeability and retention (EPR) effect – PhD thesis. University of London U.K.

Satchi R (1999) PDEPT: polymer directed enzyme prodrug therapy - PhD thesis. University of London U.K.

Satchi R, Connors TA and Duncan R (2001) PDEPT: polymer-directed enzyme prodrug therapy I. HPMA copolymer-cathepsin B and PK1 as a model combination. *Br. J. Cancer*, **85(7)**: 1070-1076

Schlager SI and Adams AC (1983) Use of dyes and radioisotopic markers in cyto-toxicity tests. *Meth. Enzymol.*, **93**: 233-245

Schwartz MK (1995) Tissue cathepsins as tumor markers. *Clin. Chim. Acta*, **237**: 67-78

Searle F, Gac-Breton S, Keane R, Dimitrijevic S, Brocchini S, Sausville EA and Duncan R (2001) *N*-(2-Hydroxypropyl)methacrylamide copolymer-6-(3-aminopropyl)-ellipticine conjugates. Synthesis, in vitro and preliminary in vivo evaluation. *Bioconj. Chem.*, **12(5)**: 711-718

Senter PD, Svensson HP, Schreiber GJ, Rodriguez JL and Vrudhula VM (1995) Poly(ethylene glycol)-doxorubicin conjugates containing beta-lactamase-sensitive linkers. *Bioconj. Chem.*, **6(4)**: 389-394

Seymour LW, Duncan R, Strohalm J and Kopecek J (1987) Effect of molecular weight (Mw) of *N*-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal and intravenous administration to rats. *J. Biomed. Mat. Res.*, **21**: 1341-1358

Seymour LW, Ulbrich K, Steyger PS, Brereton M, Subr V, Strohalm J and Duncan R (1994) Tumour tropism and anti-cancer efficacy of polymer-based doxorubicin prodrugs in the treatment of subcutaneous murine B16F10 melanoma. *British J. Cancer*, **70**: 636-641

Seymour LW, Miyamato Y, Maeda H, Brereton M, Strohalm J, Ulbrich K and Duncan R (1995) Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier. *Eur. J. Cancer*, **31A(5)**: 766-770

Sgouras D and Duncan R (1990) Methods for the evaluation of biocompatibility of soluble synthetic polymers which have potential for biomedical use: 1-use of the tetrazolium-based colorimetric assay (MTT) as a preliminary screen for evaluation of in vitro cytotoxicity. *J. Mater. Sci.- Mater. Med*, **1(2)**: 61-68

Shen W-C and Ryser HJ-P (1981) Poly(L-lysine) has different membrane transport and drug-carrier properties when complexed with heparin. *Proc. Natl. Acad. Sci. U.S.A.*, **78(12)**: 7589-7593

Shiah J-G, Sun Y, Peterson CM and Kopecek J (1999) Biodistribution of free and *N*-(2-hydroxypropyl)methacrylamide copolymer-bound mesochlorin $e_6$ and adriamycin in nude mice bearing human ovarian carcinoma OVCAR-3 xenografts. *J. Control. Rel.*, **61**: 145-157

Sievers EL, Appelbaum FR, Spielberger RT, Forman SJ, Flowers D, Smith FO, Shannon-Dorcy K, Berger MS and Bernstein ID (1999) Selective ablation of acute myeloid leukemia using antibody-targeted chemotherapy: a Phase I study of an anti-CD33 calicheamicin immunoconjugate. *Blood*, **11**: 3678-3684

Sikora K (1999) Developing a global strategy for cancer. *Eur. J. Cancer*, **35(14)**: 1870-1877

Simeckova J, Rihova B, Plocova D and Kopecek J (1986) The activity of complement in the presence of N-(2-hydroxypropyl)methacrylamide copolymers. *J. Bioact. Compat. Polym.*, **1**: 20-31

Singer JW, Bhatt R, Tulinsky J, Buhler KR, Heasley E, Klein P and de Vries P (2001) Water-soluble poly-(L-glutamic acid)-Gly-camptothecin conjugates enhance camptothecin stability and efficacy in vivo. *J. Control. Rel.*, **74**: 243-247

Skoog DA and Leary JJ (1992) Infrared absorption spectroscopy In *Principles of instrumental analysis*, Skoog DA and Leary JJ (Eds) p252-291. Saunder College Publishing, Florida, U.S.A.

Sludden J, Boddy AV, Griffin MJ, Robson L, Todd R, Cassidy J, Bissett D, Main M, Brannan MD, Elliott S, Verrill M and Calvert H (2001) Phase I and pharmacological study of CT-2103, a poly(L-glutamic acid)-paclitaxel conjugate. *Proc. Am. Assoc. Cancer Res.*, **42**: #2883

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, **150**: 76-85

Soyez H, Schacht E and Vanderkerken S (1996) The crucial role of spacer groups in macromolecular prodrug design. *Adv. Drug Del. Rev.*, **21(2)**: 81-106

St'astny M, Rihova B, Strohalm J and Ulbrich K (1996) Comparison of the binding kinetics of antibody-targeted *N*-(2-hydroxypropyl) methacrylamide (HPMA)-bound doxorubicin in vitro and in vivo. *J. Control. Rel.*, **42**: 229-236

Stewart DR, Callahan EH, Jacob JE, Rice JR, Shannon KF, St John JV and Nowotnik DP (2000) Preclinical development of the polymer platinate AP5280. *Proc. 4$^{th}$ Intl. Symp. Polym. Ther.*, 57

Subr V, Strohalm J, Ulbrich K, Duncan R and Hume IC (1992) Polymers containing enzymatically degradable bonds, XII. Effect of spacer structure on the rate of release of daunomycin and adriamycin from poly [N-(2-hydroxypropyl)-methacrylamide] copolymer drug carriers in vitro and antitumour activity measured in vivo. *J. Control. Rel.*, **18**: 123-132

Supratek website (2001) www.supratek.com

Takakura Y, Takagi A, Hashida M and Sezaki H (1987) Disposition and tumor-localization of mitomycin-C dextran conjugates in mice. *Pharm. Res.*, **4(4)**: 293-300

Thomson AH, Vasey PA, Murray LS, Cassidy J, Fraier D, Frigerio E and Twelves C (1999) Population pharmacokinetics in phase I drug development: a phase I study of PK1 in patients with solid tumours. *British J. Cancer*, **81(1)**: 99-107

Tokudome Y, Oku N, Doi K, Namba Y and Okada S (1996) Antitumour activity of vincristine encapsulated in glucuronide-modified long-circulating liposomes in mice bearing Meth A sarcoma. *Biochim. Biophys. Acta*, **1279**: 70-74

Tozer GM, Prise VE, Wilson J, Locke RJ, Vojnovic B, Stratford MRL, Dennis MF and Chaplin DJ (1999) Combretastatin A-4 phosphate as a tumor vascular-targeting agent: Early effects in tumors and normal tissues. *Cancer Res.*, **59**: 1626-1634

Tozer GM, Prise VE, Wilson J, Cemazar M, Shan SQ, Dewhirst MW, Barber PR, Vojnovic B and Chaplin DJ (2001) Mechanisms associated with tumor vascular shut-down induced by combretastatin A4-phosphate: Intravital microscopy and measurement of vascular permeability. *Cancer Res.*, **61(17)**: 6413-6422

Trail PA and Bianchi AB (1999) Monoclonal antibody drug conjugates in the treatment of cancer. *Curr. Opin. Immunol.*, **11**: 584-588

Trouet A (1974) Isolation of modified liver lysosomes. *Meth. Enzymol.*, **31**: 323-329

Trubetskoy VS and Torchilin VP (1995) Use of polyethylene-lipid conjugates as long-circulating carriers for delivery of therapeutic and diagnostic agents. *Adv. Drug Del. Rev.*, **16**: 311-320

Tungaal JK, Cowan DSM, Shaikh H and Tannock IF (1999) Penetration of anticancer drugs through solid tissue: a factor that limits the effectiveness of chemotherapy for solid tumours. *Clin. Cancer Res.*, **5**: 1583-1586

Twelves C (2002) Clinical experience with MAG-CPT. *Proc. 5th Intl. Symp. Polym. Ther.*, 21

Twentyman PR, Walls GA and Wright KA (1984) The response of tumor-cells to radiation and cytotoxic drugs – a comparison of clonogenic and isotope uptake assays. *British J. Cancer*, **50(5)**: 625-631

UKCCCR guidelines for the use of cell lines in cancer research (2000). *British J. Cancer*, **82(9)**: 1495-1509

Ulbrich K, Subr V, Strohalm J, Plocova D, Jelinkova M and Rihova B (2000) Polymeric drugs based on conjugates of synthetic and natural macromolecules I. Synthesis and physico-chemical characterisation. *J. Control. Rel.*, **64(1-3)**: 63-79

United Kingdom co-ordinating committee on cancer research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (second edition) (1998). *Br. J. Cancer*, **77(1)**: 1-10

Uster PS, Working PK and Vaage J (1998) Pegylated liposomal doxorubicin (DOXIL®, CAELYX®) distribution in tumour models observed with confocal laser scanning microscopy. *Int. J. Pharm.*, **162**: 77-86

Van Putten LM (1987) In *Clinical evaluation of antitumour therapy*, Muggia FM and Rozencweig (Eds) p17. Nijhoff, Boston, U.S.A.

Vasey PA, Kaye SB, Morrison R, Twelves C, Wilson P, Duncan R, Thomson AH, Murray LS, Hilditch TE, Murray T, Burtles S, Fraier D, Frigerio E and Cassidy J (1999) Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: First member of a new class of chemotherapeutic agents - Drug-polymer conjugates. *Clin. Cancer Res.*, **5(1)**: 83-94

Venne A, Li SM, Mandeville R, Kabanov A and Alakhov V (1996) Hypersensitizing effect of pluronic L61 on cytotoxic activity, transport and subcellular distribution of doxorubicin in multiple drug-resistant cells. *Cancer Res.*, **56(16)**: 3626-3629

Vilarem MJ, Riou JF, Multon E, Gras MP and Larsen CJ (1986) The in vitro involvement of topoisomerase II in the activity of aza-ellipticine analogues is not correlated with drug activity on isolated nuclei. *Biochem. Pharmacol.*, **35(13)**: 2087-2095

Vitetta ES, Stone M, Amlot P, Fay J, May R, Till M, Newman J, Clark P, Collins R, Cunningham D, Ghetie V, Uhr JW and Thorpe PE (1991) Phase-I immunotoxin trial in patients with B-cell lymphoma. *Cancer Res.*, **51**: 4052-4058

Wall ME and Wani MC (1995) Camptothecin and Taxol: Discovery to clinic - Thirteenth Bruce F. Cain Memorial award lecture. *Cancer Res.*, **55**: 753-760

Wang C-H, Li J, Teo CS and Lee T (1999) The delivery of BCNU to brain tumors. *J. Control. Rel.*, **61**: 21-41

Wedge SR (1991) Mechanism of action of polymer-anthracyclines; potential to overcome multidrug resistance - PhD thesis. University of Keele U.K.

Weinstein RS (1969) Electron microscopy of surfaces of red cell membranes. In *Red Cell Membrane: Structure and Function*, Jamieson GA and Greenwalt TJ (eds). J.B. Lipincott Company, Philadelphia, U.S.A.

Winnik FM, Winnik MA, Ringsdorf H and Venzmer J (1991) Bis(1-pyrenylmethyl) ether as an excimer-forming probe of hydrophobically modified poly(*N*-isopropylacrylamides) in water. *J. Phys. Chem.*, **95**: 2583-2587

Woods JA, Hadfield JA, Pettit GR, Fox BW and McGowan AT (1995) The interaction with tubulin of a series of stilbenes based on combretastatin A-4. *Br. J. Cancer*, **71(4)**: 705-711

Wroblewski S, Berenson M, Kopeckova P and Kopecek J (2001) Potential of lectin-N-(2-hydroxypropyl)methacrylamide copolymer-drug conjugates for the treatment of pre-cancerous conditions. *J. Control. Rel.*, **74**: 283-293

Yokoyama M, Miyauchi M, Yamada N, Okano T, Sakurai Y, Kataoka K and Inoue S (1990) Polymer micelles as novel drug carrier: adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer. *J. Control. Rel.*, **11**: 269-278

Young RJ and Lovell PA (1996) In *Introduction to Polymers*, Young RJ and Lovell PA (eds). Chapman & Hall, London, U.K.

Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP and Jain RK (1995) Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res.*, **55(17)**: 3752-3756

Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D and Jain RK (1994) Microvascular permeability and interstitial penetration of sterically stabilized (Stealth) liposomes in a human tumor xenograft. *Cancer Res.*, **54**: 3352-3356

Zamai M, Ghiglieri A, Geroni CM, Farao M, Pesenti E, Suarato A, Vandeven M, Gratton E and Caiolfa VR (2001) Camptothecin co-polymer conjugates: A novel perspective in topoisomerase I-based anti-tumour therapy. *Proc. Am. Assoc. Cancer Res.*, **42**: #749

Zunino F, Pratesi G and Pezzoni G (1987) Increased therapeutics efficacy and reduced toxicity of doxorubicin linked to pyran copolymer via the side chain of the drug. *Cancer Treat. Rep.*, **71(4)**: 367-373

# Appendix

## List of abstracts and publications

### Papers

Searle F, Gac-Breton S, Keane R, Dimitrijevic S, Brocchini S, Sausville EA and Duncan R (2001) N-(2-Hydroxypropyl)methacrylamide copolymer- 6 - ( 3 - aminopropyl) - ellipticine conjugates. Synthesis, in vitro and preliminary in vivo evaluation. *Bioconj. Chem.*, **12(5)**: 711-718

Duncan R, Gac-Breton S, Keane R, Musila R, Sat YN, Satchi R and Searle F (2001) Polymer-drug conjugates, PDEPT and PELT: basic principles for design and transfer from the laboratory to clinic. *J. Control. Rel.*, **74**: 135-146

Keane RS, Wilson J, Vojnovic B, Sausville EA, Tozer GM and Duncan R (2002) Extravasation of PK1/FCE28068 in a P22 Carcinosarcoma tumour: preliminary studies using the rat window chamber model. in preparation

Keane R and Duncan R (2002) The effect of drug loading on solution conformation, drug release, haematotoxicity and *in vitro* cytotoxicity of N-(2-hydroxypropyl) methacrylamide copolymer-6-(3-aminopropyl)-ellipticine conjugates. in preparation

### Abstracts

Gac-Breton S, Keane R, Searle F, Sausville EA and Duncan R (2000) HPMA copolymer-aminopropylellipticine: a novel anticancer conjugate. *Proc. 4$^{th}$ Intl. Symp Polym. Ther.*, 60

Keane R, Wilson I, Tozer G and Duncan R (2000) Extravasation of PK1/FCE28068 in a P22 Carcinosarcoma tumour: preliminary studies using the window chamber model. *Proc. 4$^{th}$ Intl. Symp. Polym. Ther.*, 76

Keane R, Gac-Breton S, Searle F and Duncan R (2000) The effect of drug loading on in vitro characteristics of N-(2-hydroxypropyl)methacrylamide-6-(3-aminopropyl) ellipticine anticancer conjugates. *J. Pharm. Pharmacol.*, **52** (Suppl.): 52

Keane R, Sausville EA and Duncan R (2001) Investigation of the effect of drug loading on the properties of HPMA copolymer 6-(3-aminopropyl)-ellipticine: a novel anticancer conjugate. *Anticancer Res.*, **21(Suppl.)**