Use of horseradish peroxidase for gene directed enzyme prodrug therapy

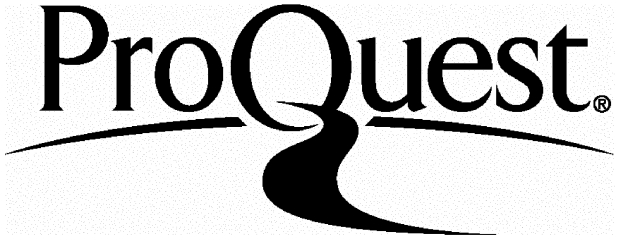
Joanna Tupper

Department of Oncology, University College London
Tumour Microcirculation Group, Gray Cancer Institute

A thesis submitted to the University of London for the degree of

Doctor of Philosophy

2004



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

Abstract

Gene directed enzyme prodrug therapy (GDEPT) is a form of targeted cancer therapy, where an enzyme is used to create a cytotoxin from a prodrug specifically within the tumour. The treatment of solid tumours is often hampered by the presence of hypoxia, which can limit the effectiveness of conventional therapies. Horseradish peroxidase (HRP)-directed GDEPT has been shown to target normoxic and hypoxic cells in monolayers. This study further evaluated HRP for gene therapy of cancer.

The HRP/indole-3-acetic acid (HRP/IAA) combination was shown to retain its activity under the tumour-simulated conditions seen in 3-dimensional tumour cell spheroids. In particular, the halogenated derivative of IAA, 5-Br-IAA, showed specificity against larger spheroids, which contain significant regions of hypoxia.

The use of alternative prodrugs was investigated using paracetamol. The HRP/paracetamol combination was effective in monolayers, even under the severe tumour-associated condition of anoxia.

Following the successful *in vitro* investigations in the FaDu squamous carcinoma cell line, the HRP-GDEPT system was analysed *in vivo*. Characterisation of solid tumours grown from stable FaDu transfectants showed that the indole prodrugs had satisfactory pharmacokinetic profiles, and millimolar concentrations could be achieved in tumour tissue at non-toxic doses.

The potential for the HRP/IAA system to cause delayed growth was determined using indoles, alone, and in combination with radiation. Unfortunately the HRP/IAA combination did not appear to have any reproducible growth delay effects.

The immune response to HRP was assessed by generating murine carcinoma cells expressing HRP or the green fluorescent protein (GFP). However, passaging of the syngeneic cell line *in vitro* was sufficient to increase immunogenicity and decrease tumourigenicity even in the absence of transgene expression.

Gene delivery *in vivo* was attempted in order to circumvent the use of stable transfectants. Electroporation could significantly increase the expression of GFP delivered intratumourally, although expression as a percentage of tumour area remained low.

The data presented demonstrates the continued promise of the HRP-GDEPT system *in vitro*, but more work is required to improve the *in vivo* efficacy.

Contents

Acknowledgements

This thesis would never have been finished without the help and support of many people and the funding from Cancer Research UK. Thank you to Gabi Dachs, Gill Tozer and Peter Hoskin my supervisors, who provided help and advice, plus lots of reading, despite being incredibly busy.

Most people at the GCI have had a hand in getting this thesis so far. The whole of the Tumour Microcirculation group, past and present, never shouted at me for asking annoying questions, and were always ready with advice and friendship; I can't thank you enough. Thanks to Ian and Vivien for watching over me and acting as if sitting on the floor was normal; Sally and Gemma for huge amounts of help with the animal studies; Maja Cemazar for showing me how to work the electroporator; Mike Stratford for incredible HPLC help; Claudia and Chryso for infinite patience; Kevin Sales for spheroid advice by email; Olga, Amel and Mhairi for providing counsel and reassurance.

Thank you to all my friends and family for their support, encouragement and tentative questions about the thesis. My parents for humouring my excitement when I was talking rubbish, and persuading me I could do it. Thank you Jamie for your invaluable support, Jenny and Harriet for wise words and group doubting.

List of figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 9

List of tables

Chapter 1

Chapter 6

Abbreviations

1Me-IAA	1methyl indole-3-acetic acid
5Br-IAA	5bromoindole-3-acetic acid
5-FC	5fluorindole-3-acetic acid
AAV	Adeno associated virus
ADEPT	Antibody directed enzyme prodrug therapy
Ah	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear transporter
AUC	Area under the curve
b.i.d.	Twice daily
CAR	Coxsackie and adenovirus receptor
CB 1954	5-aziridinyl-2,4-dinitrobenzamide
CEA	Carcinoembryonic antigen
CD	Cytosine deaminase
Clp	Plasma clearance
CMDA	4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glamic acid
CMV	Cytomegalovirus
CPA	Cyclophosphamide
CPG2	Carboxypeptidase G2
Cpmax	Maximum plasma concentration
CYP	Cytochrome P450
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
DTNB	5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent)
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EP	Electroporation
F	Bioavailability
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
g	Gravitational force
GCV	Gancyclovir

GDEPT	Gene directed enzyme prodrug therapy
GFP	Green fluorescent protein
GSH	Glutathione
GSSG	Glutathione disulphide
Gy	Gray
HBSS	Hanks' balanced salt solution
HIF	Hypoxia inducible factor
HM Ag	Heterologous membrane antigen
HPLC	High performance liquid chromatography
HRE	Hypoxia responsive element
HRP	Horseradish peroxidase
HSV	Herpes simplex virus type-1
IAA	Indole-3-acetic acid
i.p.	Intraperitoneal
IPA	Ifosphamide
i.t.	Intratumoural
K_a	Absorption rate constant
K_{el}	Elimination rate constant
MOI	3-methylene-2-oxindole
MPA	Metaphosphoric acid
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPQI	*N*-acetyl-*p*-benzoquinoneimine
NTR	Nitroreductase
P450R	NADPH:cytochrome c P450 reductase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PI	Propidium iodide
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
ROS	Reactive oxygen species
Rx	Radiation (external beam X-ray irradiation)
s.c	Subcutaneous
SCID	Severe combined immunodeficiency

SDS	Sodium dodecylsulphate
$t_{½}$	Half life
TCA	Trichloroacetic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TMB	3,3',5,5'-tetramethylbenzidine
TPZ	Tirapazemine
UPRT	Uracil phosphoribosyltransferase
VEGF	Vascular endothelial cell growth factor

Chapter 1

Introduction

1.1 Cancer development and hypoxia

Cancer is characterised by uncontrolled cell growth, invasion of local tissues and metastatic spread. In the development of malignancy, cells accumulate a number of mutations which favour proliferation, or remove growth control. These mutations must remain unrepaired and not lead to apoptosis in order for a tumour to develop.

Tumour survival is critically dependent on the formation of a vascular network (Folkman, 1990). Rapid proliferation of cells alone will not result in tumour formation until the basic requirements for nutrients, oxygen and removal of waste products are met. During the avascular phase, cells rely on the diffusion of oxygen and nutrients for survival. Growth of the tumour is slow and will eventually reach equilibrium between proliferation at the periphery, and loss of cells to necrosis at the centre (Holmgren et al., 1995).

The need for nutrients can be met by neo-vascularisation, yet tumour blood flow is often disorganised, with tortuous elongated vessels, and non-flowing regions (Brown & Giaccia, 1998). This results in the formation of both chronic and acutely hypoxic regions in both tumour and supporting stroma. In addition, anaemia and the formation of methaemoglobin and carboxyhaemoglobin can reduce the oxygen carrying capacity of the blood, exacerbating blood flow abnormalities (Fyles et al., 2000). Cells may be acutely hypoxic due to decreased perfusion of a blood vessel, whilst cells distant from blood vessels may also be hypoxic because the inadequate vascularisation of the tumour results in diffusion limited hypoxia (Thomlinson & Gray, 1955).

The presence of tumour hypoxia has an impact on cancer treatment and patient survival. Hypoxia has been specifically analysed with respect to radiation treatment (Gray et al., 1953). Anoxic cells are approximately 3-fold more radioresistant than cells irradiated under normoxic conditions (Vaupel et al., 1989). The rapid increase in the radiosensitivity of cells in the presence of oxygen (or when it is present milliseconds following radiation exposure) is thought to be due to oxygen reacting with ionised molecules, and the subsequent formation of poorly repairable peroxide

lesions (oxygen fixation hypothesis, (Hall, 1994).

Chemotherapy may also experience reduced effectiveness in hypoxic regions (Chaplin et al., 1989; Grau & Overgaard, 1988; Teicher et al., 1981). Hypoxic cells slow their growth, and regions of tumours distant from blood vessels have a decreased proliferating fraction (Tannock, 1968). This can reduce therapeutic efficacy, since the majority of anticancer chemotherapeutics are primarily effective against rapidly dividing cells. The induction of stress proteins by hypoxia can lead to resistance to commonly used agents such as doxorubicin, etoposide and methotrexate (Hughes et al., 1989; Sanna & Rofstad, 1994; Shen et al., 1987). In addition, delivery of agents to cells distant from functional blood vessels will be limited by diffusion.

Both clinical and animal tumour models have been shown to contain regions of hypoxia. Direct measurements using oxygen electrodes, and indirect measurements using molecular markers and exogenously administered agents, have confirmed hypoxia in clinical tumours. Carcinoma of the cervix is probably the best-described human hypoxic tumour (Brizel et al., 1995; Hockel et al., 1991).

An initial study suggested that hypoxia increased along a linear tract, with the centre of the tumour being the most hypoxic, and oxygenation increasing outwards (Fleckenstein et al., 1990). However, other studies, in both cervical carcinoma (Hockel et al., 1991; Vaupel et al., 1991), and soft tissue sarcoma (Brizel et al., 1994), disputed the simplicity of this model. Chaplin and Hill (Chaplin & Hill, 1995) showed that in a murine tumour model, changes in blood flow could occur independently in different regions of the same tumour, supporting the earlier clinical results.

The presence of hypoxia is an adverse prognostic factor in the majority of solid tumours. Patients with cervical carcinoma containing median pO_2 values of less than or equal to 10 mmHg had increased probability of lymphatic spread (Hockel et al., 1999), and decreased 5 year survival (Haensgen et al., 2001; Hockel & Vaupel, 2001). In tumours of the head and neck, pO_2 tensions less than 10 mmHg were associated with poor loco-regional control, disease-free survival, and overall survival (Brizel et al., 1999), in patients receiving fractionated radiotherapy with or without concomitant chemotherapy.

In vitro exposure of tumour cells to hypoxia can lead to an accumulation of the number of cells in the G1 phase of the cell cycle (Young et al., 1988), which was associated with an inhibition of DNA synthesis. Reoxygenation in this study, and others (Rice et al., 1986), resulted in an increase in S phase cells and the number of cells with increased DNA content (greater than 4C, where C is the normal cellular DNA content).

Experimental models of tumour metastasis have shown that cells exposed to hypoxia *in vitro* initially have decreased metastatic potential when implanted *in vivo* (Young et al., 1988). However, if the hypoxic cells undergo reoxygenation before injection they show increased metastatic ability, which is further increased by extending the hypoxic incubation time (Young et al., 1988). Tumours with a significant hypoxic fraction also appear to metastasise at an earlier stage of growth compared with more oxygenated tumours (De Jaeger et al., 2001).

Hypoxia also results in changes in gene expression leading to a more aggressive phenotype. Expression of the tumour suppressor gene *p53* (Graeber et al., 1996), and the proto-oncogene *bcl-2* (Haensgen et al., 2001; Kinoshita et al., 2001) are altered, with extended hypoxia and reoxygenation resulting in increased growth rate and greater increases in *bcl-2* staining relative to hypoxic incubation. *Bcl-2* has been reported to prevent hypoxia-induced apoptosis (Jacobson & Raff, 1995; Shimizu et al., 1995), which may account for the increased growth rate when this gene was upregulated. It also demonstrates that there is a selective pressure from hypoxia for expansion of variants that have lost their apoptotic potential.

Hypoxia has been shown to increase the mutation frequency of cells (Reynolds et al., 1996; Sandhu et al., 2000), thus suggesting that hypoxia is capable of mediating tumour progression by the induction of genetic instability.

1.1.1 Overcoming tumour hypoxia

One method of circumventing the problem of tumour hypoxia is to attempt to oxygenate hypoxic regions. This has been performed using hyperbaric oxygen (Churchill-Davidson, 1968). This showed some effectiveness, but was discontinued, mainly due to problems with patient compliance. More recent studies have utilised chemical radiosensitisers. These can diffuse from the blood stream and reach hypoxic regions since they are not metabolised by normal cellular respiration. Unfortunately, the high neural toxicity associated with the prototypical drug misonidazole, resulted in

dose limitations, and poor clinical experience (Dische, 1985).

A combination of carbogen (95% oxygen, 5% carbon dioxide) and nicotinamide, a derivative of the B vitamins believed to prevent transient blood flow fluctuations (Chaplin et al., 1990), has been used to overcome both transient and chronic hypoxia. Clinical trials combining this gas mixture with accelerated radiotherapy (ARCON) have indicated some clinical efficacy (Bernier et al., 2000; Hoskin et al., 1999; Hoskin et al., 2003).

An alternative strategy to eliminating hypoxia is to exploit it. Bioreductive drugs can be used as hypoxic cell cytotoxins. These drugs are selectively reduced under hypoxia, often by DT-diaphorase or reductase enzymes. Currently under investigation are tirapazamine (TPZ, (Brown & Wang, 1998)), E09 (Jaffar et al., 1998) and AQ4N (Patterson & McKeown, 2000).

AQ4N is the prodrug of AQ4 which targets topoisomerase II (topo II) (Patterson, 1993), with decreased toxicity to non-cycling cells. The active metabolite persists within cells upon reoxygenation, and will synchronise cell growth (Hejmadi et al., 1996). In contrast to other bioreductive agents, AQ4N is activated by cytochrome P450 (CYP) isoforms (Patterson et al., 1999), and this was confirmed by increased cell kill and *in vivo* activity when cells were transfected with CYP 3A4 (McCarthy et al., 2003). When given alone in tumour models, it has only weak anti-tumour activity, but could substantially increase the effects of radiation (McKeown et al., 1995; Patterson et al., 2000) and standard cancer chemotherapeutics such as cisplatin and cyclophosphamide (Friery et al., 2000; Patterson et al., 2000).

Tirapazamine (TPZ), is a benzotriazine which shows toxicity under conditions of hypoxia (Brown & Lemmon, 1991). Intracellular reductases convert TPZ to a cytotoxic radical, which is back-oxidised to the parent compound by molecular oxygen (Lloyd et al., 1991). TPZ causes both single and double strand breaks (Daniels et al., 1998), and is a topo II poison (Peters & Brown, 2002). It is currently in phase II/III clinical trials in combination with radiation and cisplatin (Craighead et al., 2000; Rischin et al., 2001), with some activity. Modifications to the TPZ structure are being investigated to target DNA and reduce side effects (Delahoussaye et al., 2003; Hay et al., 2003).

E09 is activated by DT-diaphorase (Jaffar et al., 1998), and showed good preclinical effects. E09 displayed some renal toxicity in clinical trials (Dirix et al., 1996), but no therapeutic efficacy. However, E09 was used as a single agent, which is

inappropriate for a bioreductive agent. Other DT-diaphorase bioreductives are being researched (Jaffar et al., 2003).

1.1.2 Hypoxia mediated gene control

Many genes are regulated by hypoxia, including transcription factors, glycolytic enzymes, and growth factors (for review see Dachs (Dachs & Tozer, 2000)). A major part of the cellular response to hypoxia involves the hypoxia inducible factor-1 (HIF-1) transcription factor. The DNA binding activity of HIF-1 is upregulated by hypoxia (Wang & Semenza, 1993). HIF-1 is a heterodimer, consisting of HIF-1α and HIF-1β subunits (Wang et al., 1995). HIF-1α has been found to be overexpressed in a variety of human tumours, and in particular seems to be associated with metastasis and tumour progression (Zhong et al., 1998; Zhong et al., 1999).

The HIF-1α subunit is hypoxia-regulated post-translationally by ubiquitination (Huang et al., 1998; Kallio et al., 1999), and redox-dependent stabilisation of the HIF-1 dimer. In contrast, the HIF-1β subunit, originally described as one of the aryl hydrocarbon receptor nuclear translocator, ARNT, family (Wang et al., 1995), is not regarded as being oxygen sensitive (Pugh et al., 1997).

HIF-1 has a variety of association partners, and efficient hypoxia-induced gene transcription appears to require the formation of a multi-protein complex of HIF-1, the cofactor CBP/p300, and other transcription factors (Kallio et al., 1998). The HIF-1 dimer binds to a cognate hypoxia regulatory element (HRE), located at varying positions relative to the gene of interest. The generalised sequence for an HRE is 5'-(A/G)CGT(G/C)(G/C)-3'.

Antibodies towards HIF-1α have been generated, and have been used to assess expression of the transcription factor in a variety of human tumours. HIF-1α is upregulated in stage 1 endometrial adenocarcinoma, and expression was related to increased angiogenesis and unfavourable prognosis (Sivridis et al., 2002). Expression of HIF-1α is associated with poor survival following radiotherapy in a variety of tumours, including cancer of the cervix (Bachtiary et al., 2003; Burri et al., 2003), head and neck (Hui et al., 2002; Koukourakis et al., 2002), and breast (Bos et al., 2003; Bos et al., 2001; Schindl et al., 2002).

Because of difficulties overcoming hypoxia with conventional therapies, there has been increasing amounts of research into alternatives.

1.2 Gene Therapy

Gene therapy of cancer is now one of the major uses of genetic based treatments. Other uses include pulmonary and cardiovascular diseases (Phillips, 2001), and AIDS (Buchschacher & Wong-Staal, 2001). It was originally developed for the treatment of inherited monogenic disorders such as cystic fibrosis (Flotte & Laube, 2001) and severe combined immunodeficiency disorder (Anderson et al., 1990), but suffered from setbacks, as successful treatment requires the majority of defective cells to be corrected. Trial results are constantly improving, with notable results for X-linked SCID in children. The advantage for cancer gene therapy strategies is that not all cells need to be corrected, due to the bystander effect (see section 1.3.1).

Gene therapy consists of three components; gene delivery, regulation of expression and therapeutic efficacy. Each needs to be optimised for a successful clinical outcome.

1.2.1 Gene delivery

In vitro transfection of cells is relatively straightforward with several protocols available, such as calcium chloride treatment, lipofection, electroporation, and the use of viruses. However, *in vivo* gene delivery has proved to be the major limitation to gene therapy, resulting in greater reliance on the bystander effect (Fig. 1.1 and section 1.3.1). At present, viral delivery methods are the most effective, but also raise safety concerns, and hence there is substantial interest and research into in non-viral methods.

The ideal delivery agent should effectively deliver the exogenous genetic material to the target cells without degradation in the circulation or tissue. Delivery should target the nucleus rather than the cytoplasm. Agents should be capable of delivering fairly large amounts of DNA without a decrease in transfection efficiency. It is also desirable that repeat administration be possible without the induction of an immune response.

Viral Delivery

Retroviruses contain only RNA and not DNA. They can enter the cell via a variety of cell surface receptors (Miller, 1996), and then generate DNA using reverse

transcriptase enzymes. The DNA is then integrated within the host cell genome allowing stable long-term expression. In order to integrate the proviral DNA, the host/target cell must be proliferating.

Retroviruses are rendered replication-deficient by the removal of the *gag*, *pol* and *env* genes, which are replaced by the exogenous genetic material. The viruses are then combined with packaging cells which are capable of producing the proteins necessary for viral assembly, allowing continued production of the virus (Danos & Mulligan, 1988).

Therapies using retroviruses at first used vectors derived from murine leukaemia virus (MLV). As these vectors only transduce actively dividing cells, it was seen as an advantage in cancer therapy providing a certain degree of targeting. Integration into the host genome eliminated the requirement for repeat administrations, thus decreasing the risk of possible immune responses. However, the viral reverse transcriptase is error prone, potentially resulting in genetic instability. Also, integration into the host genome has the potential to result in insertional mutagenesis and activation of oncogenes. This has been highlighted by the cases of two children successfully treated for X-linked SCID with an *ex-vivo* retroviral approach in France, who subsequently developed leukaemia. It would appear that the virus used in the treatment integrated into a cellular genome immediately adjacent to the lmo2 locus (commonly involved in childhood T cell acute lymphoblastic leukaemia, (Davenport J, 2000).

Newer retroviral therapies utilise lentiviruses such as HIV-1, which can integrate into non-proliferating cells (Naldini et al., 1996). For safety reasons, it may be prudent for human use to use lentiviruses of non-human origin.

Adenoviruses are non-enveloped icosadeltahedrons, which enter the cell via endocytosis. They then lyse the endosomal vesicle and the DNA is delivered to the nucleus where it remains episomal, so avoiding permanent change or insertional mutagenesis. Unlike retroviral delivery, cellular replication is not a pre-requisite for expression of the virally delivered DNA (Rosenfeld et al., 1991).

Internalisation of the adenovirus requires binding to the coxsackie and adenovirus receptor (CAR), followed by an interaction with integrins. However, expression of CAR has been reported to be downregulated in several tumour types (Curiel, 1999). Decreased expression of CARs on the surface of the target cell leads

to a decrease in the transduction efficiency, both *in vitro* and *in vivo*, accompanied by a decrease in the efficiency of mutation compensation and molecular chemotherapy strategies (Kim et al., 2002). Redirection of adenoviruses to give CAR-independent transduction could potentially increase transduction efficiencies.

Modification of adenovirus can be performed using two strategies: i) non-genetic retargeting and ii) genetic retargeting involving engineering of viral proteins. These changes can increase the binding capacity, and/or generate tropism for a particular cell type (Douglas et al., 1996).

The wide distribution of the CAR precludes selectivity, and can lead to non-specific gene expression, especially in the liver. Yet its downregulation in cancer cells poses a problem. Adenovirus can be coated in polymers and retargeted to the basic fibroblast growth factor (bFGF) or VEGF receptors resulting in efficient CAR-independent ligand-mediated binding and uptake both *in vitro* and *in vivo* (Fisher et al., 2001). Adenovirus-liposome complexes have also been produced, which in a mouse glioma model displayed greater transduction efficiency and a five-fold decrease in anti-adenovirus antibodies compared with adenovirus alone (Mizuno et al., 2002).

Modification of adenovirus to express an epidermal growth factor (EGF) CAR protein led to efficient gene transfer to pancreatic carcinoma cells (Wesseling et al., 2001). Other modifications include HI loop insertion of peptides, such as α_v integrin-binding peptides (Wesseling et al., 2001), and those identified from phage display to bind to endothelial cells (Nicklin et al., 2001). The main drawback of genetic modification of viruses is that although cells can be effectively targeted, and any potential infectious particles will retain their tropism, the process is more complex than non-genetic coating of particles.

The major problem occurring with adenovirus delivery is an inflammatory and immune response involving both cellular and humoral mechanisms preventing repeat administration (Nagao et al., 2001). This is probably more critical for therapy of inherited disorders such as cystic fibrosis, where repeat administration is required as cells are replaced. In addition, the death of an 18 year old participant in a clinical trial involving adenoviral gene delivery has highlighted the problems associated with viral use (Marshall, 1999).

Herpes simplex viruses are large double stranded DNA viruses which enter the cell through interactions with heparin sulphate residues on cell membranes. As with adenoviruses, HSV can be rendered replication deficient through deletion of immediate early genes. It is possible to remove large regions of the viral DNA and yet retain their ability to infect cells (Sena-Esteves, 2000).

Oncolytic HSV vectors have been utilised as a means of destroying tumour tissue whilst sparing the surrounding normal tissue in brain, colon metastases, gliomas and bladder cancer. A potential problem with HSV vectors is their natural tropism for neuronal tissue, and the potential for latent infection.

Adeno-associated viruses (AAV) are small single stranded DNA viruses which are non-pathogenic. They are capable of infecting both replicating and senescent cells. Although integration into the host genome is likely, viral DNA will insert at a specific locus (Samulski, 1993). This characteristic is lost in gene therapy vectors due to the deletion of *rep* genes in order to remove replication competency.

AAV have been shown to penetrate into both tumour cell spheroids and experimental tumours better than adenovirus (Enger et al., 2002). This is particularly important when looking to target regions of hypoxia that may be distant from feeder blood vessels.

The packaging capacity of AAV is fairly low allowing only small inserts,which is a disadvantage if large genes are to be used. In addition the preparation of viruses requires helper virus in the form of either HSV or adenovirus, which may result in contamination if there is ineffective purification.

More recently work has developed into the use of replication competent viruses (Hemminki & Alvarez, 2002). These would be able to deliver exogenous DNA to a greater number of cells, with potentially increased therapeutic efficacy. Work on the ONYX 015 virus (also referred to as dl1520 or Cl-1042), which is only able to replicate in p53 null cells (Heise et al., 1997), has demonstrated the safety of this approach and given encouraging results both pre-clinically, and in early clinical trials (Kirn, 2001).

Non-viral delivery

Liposomal gene delivery in human gene transfer protocols typically utilises

cationic lipids, which are complexed with negatively charged plasmid DNA. The liposome fuses with the plasma membrane releasing the genetic material into the cell. Since there are no extra macromolecules targeting the DNA to the nucleus, much of the delivered material remains in cytoplasmic vesicles which are degraded before reaching the nucleus (Crystal, 1995).

Liposomes have several advantages over viruses, as they can essentially hold DNA of unlimited size, and are unable to replicate or recombine to form infectious agents. If purely liposomal, with no targeting proteins, then they are unlikely to result in an immune response. However, DNA transfer *in vivo* with liposomes is so far unreliable and inefficient, which has led to the modification of the lipid component with a variety of peptides in order to increase the uptake into cells, release from the endosomal vesicle, and to target specific cells. Integrin-targeting peptides have been developed which contain a poly-lysine chain to bind DNA (Harbottle et al., 1998). In combination with cationic lipids this has been used to successfully transfect bronchial epithelium (Cunningham et al., 2002). Ligands to the epidermal growth factor receptor have been developed for use with lipids to transfect lung carcinoma cells *in vitro* (Yanagihara et al., 2000), and subcutaneous tumours in animals (Ogris & Wagner, 2002; Wolschek et al., 2002), whilst limiting uptake and expression in the liver.

A major area of research is the use of electric pulses to increase gene and drug delivery. The cell plasma membrane is unable to conduct electricity except through the use of ion channels. A high voltage passed through a cell results in the formation of pores, which allow the transfer of macromolecules between the extracellular matrix and the cytosol. These electropores spontaneously reseal. The voltage used, and the duration of the pulse are critical factors in determining the cellular uptake of molecules. Different tissues have differing optimal parameters.

Electroporation can increase the uptake into cells of chemotherapeutic drugs, such as bleomycin which is otherwise membrane transport-limited (Hyacinthe et al., 1999).

Gene transfer *in vivo* with electroporation has been successfully demonstrated in muscle (Rizzuto et al., 1999; Vicat et al., 2000), mammary tumours (Wells et al., 2000), hepatocellular carcinoma (Heller et al., 2000), gliomas (Nishi et al., 1996;

Yoshizato et al., 2000), and colon adenocarcinoma models (Goto et al., 2000). However, levels of expression are still below those obtained using viral vectors.

Cellular delivery

Although bacteria do not transfer genes to the tumour tissue they represent a growing area of research as cellular vehicles. The *Clostridium*, *Bifidobactum*, and *Salmonella* genera have all been studied in this context. It is the anaerobic nature of clostridia that makes them attractive for cancer therapy, since they will colonise the necrotic and hypoxic regions of tumours whilst sparing normal tissue. Spores of *C. acetotbutylicum* have been shown to colonise rat tumours resulting in the secretion of tumour necrosis factor-α (TNF-α) or cytosine deaminase (CD, see section1.3.2) (Theys et al., 2001). Spores of *C. beijerinckii* have been engineered to express nitroreductase, and cytosine deaminase, and tumour specific expression was shown. However, no antitumour activity was seen when the appropriate prodrug was administered, probably due to low levels of viable clostridia. *C. sporogenes* have been engineered to express the CD gene and shown to have potent anti-tumour activity in combination with its prodrug (Liu et al., 2002).

In addition to the use of bacteria, macrophages have been proposed for use as cellular delivery vehicles. Macrophages are the tissue-matured cells derived from circulating monocytes that infiltrate diseased and damaged tissues.

Macrophages which have been activated *ex-vivo*, and then re-infused into tumour bearing mice could result in growth delay (Fidler, 1974), and re-infusion of 3 x 10^9 *ex-vivo*-activated macrophages to cancer patients resulted in only mild side effects. However, only minimal therapeutic benefit was observed (Andreesen et al., 1998).

The advantage of macrophages over other non-modified vectors is their inate ability to "home" to diseased sites. Typically, following systemic administration, macrophages accumulate in the lungs, followed by a return to the circulation and localisation in the liver and spleen. The proportion of macrophages found in diseased sites varies according to the study (see (Burke et al., 2002a) for review).

Macrophages have been successfully used in a spheroid model to carry the cytochrome P450 gene (CYP, see section 1.3.2) into the spheroid (Griffiths et al.,

2000). Upon addition of prodrug clonogenic survival was reduced. In addition, the use of a hypoxia responsive element (HRE, see section 1.2.2) could be incorporated to increase targeting. Macrophages appeared to be more resistant to the activated prodrug than tumour cells, and so prodrug activation would not immediately destroy the activating cell. The use of an HRE is possible since macrophages contain a functional response to hypoxia utilising the HIF pathway (Burke et al., 2002b).

One disadvantage to the use of macrophages for gene delivery is that they need to be transfected *ex vivo*, and this is less effective than transformation of bacterial cells.

1.2.2 Gene expression

For cancer gene therapy strategies, expression of exogenous genetic material needs to be directed towards the tumour and not normal tissues. However, the majority of preclinical and clinical studies still utilise strong constitutive gene expression driven by viral promoters.

Targeting of gene expression can be achieved using tissue or condition specific regulatory sequences. Control of therapeutic genes can be placed under inducible promoters, for example, those derived from the gene for the heat shock protein (Braiden et al., 2000), carcinoembryonic antigen (CEA) promoter (Humphreys et al., 2001), hypoxia response elements (HREs) (Dachs et al., 1997), and radiation responsive promotors (CArG elements) (Datta et al., 1992). The tumour vasculature can also be targeted using the promoter sequence from the vascular endothelial growth factor (VEGF) receptor, flt-1 (Reynolds et al., 2001). Targeting the vasculature may be advantageous since this may result in a large bystander effect. Also, the vasculature is likely to have a more stable genotype than tumour cells, decreasing the likelihood of resistance (Kerbel, 1991).

Tissue specific gene expression

A variety of genes have been identified which are over-expressed in certain cancers. The use of promoter or enhancer sequences from these genes can be used to drive the expression of exogenous genetic material resulting in gene expression primarily in the cancerous growth.

Examples of promoters which have been isolated for use in gene therapy include, those which control expression of the carcinoembryonic antigen (CEA),

prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA). CEA is a glycoprotein over expressed in many carcinomas, but in particular those of the gastric system. Transfection of cells with the herpes simplex virus thymidine kinase (HSV-tk, see section 1.3.2 for details) gene under the control of the CEA promoter selectively sensitised stably transfected lung cancer cells (Osaki et al., 1994) and pancreatic cancer cells (DiMaio et al., 1994) to the gancyclovir (GCV) prodrug both *in vitro* and *in vivo*, with a significant bystander effect. *In vivo* transfection of the cytosine deaminase (CD, see 1.3.2) gene to colon carcinoma, with retroviral (Cao et al., 1999) or adenoviral vectors (Lan et al., 1997) under the control of the CEA promoter, resulted in selective expression and tumour regression after 5-fluorocytosine (5-FC) administration.

Prostate specific antigen (PSA) is involved in semen liquifaction and is expressed preferentially in the prostate. Liposomal transfection of prostate cancer cells *in vitro* with the HSV-tk gene under the control of the PSA promoter effectively sensitised these cells to GCV (Suzuki et al., 2001). Although the PSA promoter could give selective expression of the CD gene in prostate cells *in vitro*, the level of expression obtained was not sufficient to result in cell kill in response to 5-FC (Yoshimura et al., 2001).

An alternative to the PSA promoter is the prostate specific membrane antigen (PSMA) promoter (O'Keefe et al., 1998). This showed selectivity, both *in vitro* and *in vivo*, when used to control the CD gene followed by treatment with 5-FC, resulting in significant cell kill. In addition PSMA is upregulated by traditional hormone therapy (Wright et al., 1996), thus giving the potential for increased response in the clinic in combination with conventional therapies.

Environmentally controlled gene expression

Hypoxia is present in many solid tumours (see section 1.1.1), and is responsible for the upregulation of a variety of genes via the HIF-1 system (section 1.1.2). The first hypoxia-controlled gene to be identified was that of erythropoietin (Beck et al., 1991; Pugh et al., 1997; Semenza et al., 1994). Hypoxia responsive elements (HRE's) have since been identified in many genes, including phosphoglycerate kinase 1 (PGK) and lactate dehydrogenase A (Firth et al., 1994), type II hexokinase (Mathupala et al., 2001), VEGF (Forsythe et al., 1996), and glyceraldehyde-3-phosphate dehydrogenase (Graven et al., 1999).

The HRE from the mouse PGK-1 gene has been utilised to regulate expression of the marker gene CD2 at clinically relevant levels of hypoxia (Dachs et al., 1997). Subsequent reoxygenation of cells further increased levels of the gene, which would lead to enhanced expression within regions of transient hypoxia in tumours. Utilising the CD enzyme it was shown that hypoxic induction of CD was sufficient to sensitise cells to 5-FC when cells were returned to normoxia

The use of the HRE from the VEGF gene has been used as a promoter to control expression of the HSV-tk gene (Koshikawa et al., 2000). This construct was shown to increase the sensitivity of cells to GCV after induction under hypoxia *in vitro*, and resulted in regression of subcutaneous tumours. In addition, the combination of HRE's with tissue specific promoters has been used to control expression of marker genes (Modlich et al., 2000) and HSV-tk (Ido et al., 2001).

Ionizing radiation increases expression of immediate-early genes involved in transcriptional control, such as the early growth response (Egr) family (Hallahan et al., 2003; Sherman et al., 1990). In 1992 Datta (Datta et al., 1992) showed that x-ray irradiation resulted in transcriptional activation of the *Egr-1* gene. Experiments involving deletions within the promoter identified the serum response domain as particularly important. This region is also referred to as the CArG box, [$CC(A+T)_6GG$]. CArG elements are radioresponsive, and are also induced by reactive oxygen species (Datta et al., 1993).

The use of CArG elements to control gene transcription has been demonstrated with effective induction of the TNF-α gene resulting in decreased tumour volume (Seung et al., 1995). The use of synthetic CArG elements has also been used in GDEPT *in vitro*, resulting in cell killing using the HSV-tk/GCV GDEPT combination (Marples et al., 2000). HRE and CArG elements have successfully been used to control expression of both marker and therapeutic genes (Greco et al., 2002b).

1.2.3 Therapeutic genes

The choice of genes for cancer treatment will depend upon the type of strategy. In general, there are three main approaches to cancer gene therapy; mutation compensation, genetic immunopotentiation and molecular chemotherapy. All three strategies are large fields. Due to the subject of this thesis emphasis will be placed on

molecular chemotherapy.

Mutation compensation

Molecular biology has identified many genes which are up or downregulated in cancerous cells. It may be possible to abrogate the malignant phenotype by correcting dysfunctional expression of tumour suppressor genes or oncogenes. The archetypal gene for this strategy is *p53* (Nielsen & Maneval, 1998). Pre-clinical studies have demonstrated the reversal of malignancy associated with increased apoptosis when wild type p53 was delivered. However, some cells remain tumourigenic despite expressing wild-type p53. Oncogenes can be targeted by the use of anti-sense RNA which promotes the degradation of the complimentary sequence (Orr & Monia, 1998).

Antibody therapy can also be used for mutation compensation. Intracellular expression of an anti-erbB-2 single chain antibody led to the down regulation of erb-2 expression (Deshane et al., 1995).

A drawback to the mutation compensation approach is the need for highly efficient gene transfer, since any cells which do not express the introduced wild-type gene will be capable of repopulating the tumour mass. At present gene delivery is a major stumbling block in all gene therapy strategies (section 1.2.1).

Genetic immunopotentiation

Genetic immunopotentiation aims to actively immunise against tumour-associated antigens. The advantage of this approach is that the delivery of genes to cells can be attained *ex vivo*, thus reducing the need for an efficient delivery system. Tumour infiltrating lymphocytes were thought to be the ideal delivery system for this approach (Rosenberg et al., 1990). However, when reinjected, localisation to the tumour was poor and expansion *in vivo* required toxic doses of interleukin 2 (IL-2).

Unfortunately, most human tumours do not appear to have a specific antigen or exceptionally high levels of antigens making the choice of target difficult, and potentially reducing therapeutic effects (Schuler & Steinman, 1997).

Molecular chemotherapy

In molecular chemotherapy, or "suicide gene therapy" the gene encoding an enzyme is delivered to tumour cells, followed by the systemic administration of a

prodrug, which is converted to a cytotoxin by the enzyme (gene directed enzyme prodrug therapy or GDEPT). The method of targeting can be specific delivery and/or genetically controlled (see Fig. 1.1).

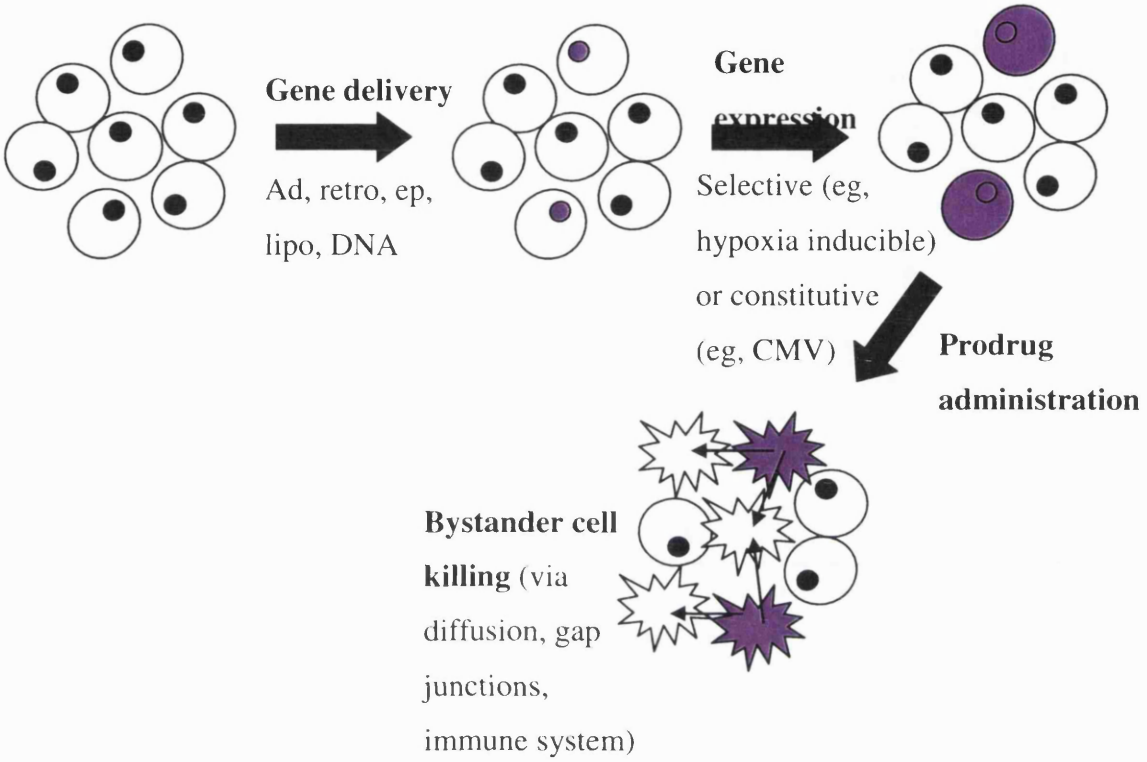


Figure 1.1. Gene directed enzyme prodrug therapy (GDEPT). Gene delivery to tumour cells is followed by gene expression and subsequent administration of a non-toxic prodrug. The therapeutic gene encodes an enzyme that converts the prodrug to a cytotoxin, leading to cell death. Surrounding cells may also be killed due to the bystander effect (see section 1.3.1). Ad, adenovirus; retro, retrovirus; ep, electroporation; lip, liposomes; CMV, cytomegalovirus strong promoter.

1.3 GDEPT systems

An ideal enzyme prodrug combination would be one where the enzyme is monomeric and without post-translational modifications such as phosphorylation or glycosylation. The catalytic activation of the prodrug should be rapid at low concentrations of substrate and under physiological conditions. It should also be sufficiently different from endogenous pathways so that activation of prodrug does

not occur in normal tissues.

Optimisation of the process occurs when conversion of the prodrug to a cytotoxin is a one step process not requiring further metabolic activation. The cytotoxic agent should be able to leave the cell of origin to affect surrounding cells so exerting a "bystander" effect. The active drug's half life should be such that it kills the surrounding cells, but not cause systemic toxicity. In order to maximise the bystander contribution, expression of the enzyme should not in itself be cytotoxic, nor should the gene delivery method cause significant cytotoxicity (Knox, 1999).

1.3.1 Bystander effect

In any gene therapy system delivery of the gene to all target cells is unlikely; currently at best, around 10% of cells may be transfected. Therefore it is important that any cytotoxic agents produced by the transfected cell should be able to kill surrounding cells for tumour growth arrest or regression – the bystander effect.

Using the herpes simplex virus thymidine kinase/gancyclovir combination (HSV-tk/GCV, see section 1.3.2), Moolten (Moolten, 1986) described how mixtures of HSV-tk$^+$ and HSV-tk$^-$ cells sparsly seeded and then treated with GCV resulted in areas of surviving HSV-tk$^-$ cells. However, if the mixtures were plated at high density very few HSV-tk$^-$ cells survived. It has been suggested that this apparent need for cell contact is due to the transfer of phosphorylated GCV between cells via gap junctions. Indeed, the bystander effect in cells with dysfunctional gap junctions treated with HSV-tk/GCV was severely compromised, but could be increased by co-transfection of cells with connexin genes (Grignet-Debrus et al., 2000). The reliance on gap junctional communication for the bystander effect is not a good strategy, since expression of connexins is often decreased in neoplastic tissues, and can also be decreased by hypoxia (Mesnil et al., 1996; Nishida et al., 2000; Touraine et al., 1998).

All current GDEPT strategies rely on a bystander element of cytotoxic species transfer, yet there also appears to be an immune component (Caruso et al., 1993; Haack et al., 2000; Kuriyama et al., 1999a; Kuriyama et al., 1999b; Rainov et al., 2000; Vile et al., 1994b). Reports agree that in immunocompetent animals immunity to parental cells, that is the original non-modified cell line, but not other syngeneic cell lines is conferred. A significant increase in both CD8$^+$ and CD4$^+$ lymphocytes has been reported in both the HSV-tk/GCV and cytosine deaminase/5-fluorocytosine

(CD/5FC) systems. This may be important for metastatic cancers, which may not be targeted by the original gene transfer. Rejection of parental cells suggests that the body may be more capable of mounting an effective immune response against cells which have seeded outside of the original tumour.

A potential method for increasing the bystander effect is the use of the HSV-1 structural protein VP22 (Elliott & O'Hare, 1997). The VP22 protein is unusual in that cells infected with HSV show localisation of the VP22 protein in a diffuse pattern in the cytoplasm (Elliott & O'Hare, 1997). VP22 is able to spread to surrounding cells (up to 200 per infected cell), where it is taken up and transported to the nucleus. Fusion proteins also show this transport property (Elliott & O'Hare, 1997). An HSV-tk-VP22 fusion protein has been constructed, which retained the enzymatic function of HSV-tk and was capable of causing a bystander effect both *in vitro* and *in vivo* (Dilber et al., 1999). However, another system using VP22 failed to show an enhanced bystander effect (Qiu et al., 2004).

1.3.2 Current strategies

Several cancer gene therapy systems are based on existing therapies for bacterial or viral infection. By expressing the viral or bacterial gene responsible for susceptibility to these drugs, tumour cells become sensitive. The advantage with these systems is that prodrug characteristics in humans are often well studied, leading to a more rapid clinical development.

Herpes simplex virus-thymidine kinase/Gancyclovir

This is by far the most studied system of proposed GDEPT strategies. Gancyclovir (GCV) and related compounds are widely used for the treatment of herpes simplex virus (HSV) infections, as they are poor substrates for human monophosphatase kinase, so giving few side effects. GCV is a derivative of acyclovir with the addition of a methoxy group at the 3' carbon acyclic side chain which gives increased activity, especially against HSV. Unfortunately this addition also increases haematopoietic toxicities (Balfour, 1990). After phosphorylation by HSV-thymidine kinase (HSV-tk), GCV undergoes a series of intracellular reactions resulting in the formation of a triphosphate. This competes with deoxyguanosine triphosphate in DNA elongation during cell division resulting in inhibition of DNA polymerase and single strand breaks (Elion, 1983; Reid et al., 1988). This combination of enzyme and

prodrug therefore has some specificity for rapidly dividing tumour cells invading normal quiescent tissue. However, as it is S-phase specific, the cells must be actively dividing at the time of drug administration, which is a potential drawback in tumours containing regions of non-proliferating hypoxic cells.

The exact mechanism of HSV-tk cell kill is still not fully understood, although it involves apoptotic cell death resulting from p53 accumulation, mitochondrial perturbations and activation of caspase cascades (Beltinger et al., 1999; Beltinger et al., 2000; Freeman et al., 1993; Wei et al., 1999).

In vitro studies showed that stable expression of HSV-tk could increase cell sensitivity to GCV up to 2000 fold (Kuriyama et al., 1999a), although results vary according to the assay and cell type used.

The doses of GCV used in animal studies vary from 25 mg/kg twice daily (b.i.d.) to 100 mg/kg b.i.d. (O'Malley et al., 1995) and 150 mg/kg/day (Vile et al., 1994a). The level of toxicity at these doses also varies, which may be dependent on the mouse strain.

Cells stably expressing HSV-tk have been grown as tumours in animal models, resulting in eradication of 80% of GCV treated tumours when 40% of the inoculated cells expressed HSV-tk (Kuriyama et al., 1999c). Growth delay was reported in several cell types including liver metastases (Caruso et al., 1993), murine and human melanoma (Berenstein et al., 1998), and rat glioma cells (Dilber et al., 1999). Inhibition of tumour development has been shown in murine melanoma, (Berenstein et al., 1998) and murine hepatocellular carcinoma (HCC, (Kuriyama et al., 1999c).

In vivo HSV-tk gene delivery by adenoviruses followed by GCV administration has led to growth delay in tumours including murine melanoma, lung and ovarian carcinomas (Wildner & Morris, 2000) and head and neck carcinomas (O'Malley et al., 1995). However when used with a replication competent HSV-1 vector, the level of activity was decreased, probably due to killing of the HSV-1 vector by GCV rather than tumour cells, preventing gene expression and also oncolytic activity (Pawlik et al., 2002).

The main disadvantage of the HSV-tk system is that the highly charged triphosphate is lipid insoluble and therefore incapable of diffusing from one cell to another, so reducing the opportunity for bystander effects. The observed bystander

effect has been proposed to be reliant at least in part, on gap junctions (Dilber et al., 1997; Elshami et al., 1996). It seems that both the level of gap junctional intercellular communication (GJIC) and type of connexin expressed is important (Andrade-Rozental et al., 2000). However, GJIC does not appear to be necessary in all cell types (Bai et al., 1999; Boucher et al., 1998), and free GCV phosphate metabolites have been detected in the culture medium of HSV-tk^+ cells independent of connexin expression (Drake et al., 2000). The bystander effect does appear to be effective between cells of different origin (Arafat et al., 2000), which is promising for studies which aim to target the tumour vasculature, or use macrophages and T cells as delivery vehicles.

The proposal that phagocytosis of apoptotic vehicles from dying cells led to the death of those cells (Freeman et al., 1993), has been partly dismissed as it is probable that uptake occurred after cells had been irreversibly damaged (Hamel et al., 1996), decreasing likelihood that transfer of apoptotic vesicles is important.

In vivo the host immune system is likely to play a role in bystander killing. Infiltration of CD^+ cells and macrophages in addition to the cell killing, resulted in the creation of a cytokine rich environment (Gagandeep et al., 1996). There also appears to be a memory component, in that later challenge with parental tumour cells was successfully combated provided they were of the same origin (Kuriyama et al., 1999a; Yamamoto et al., 1997).

The pharmacokinetic profile of GCV in humans is represented by an open two-compartment model (Morse et al., 1993). The majority of the drug is excreted unchanged in the urine within 12 hours of administration. Oral bioavailability is very low (6% of a single 10 mg/kg oral dose, (deMiranda & Blum, 1983)), resulting in the need for i.v. administration in patients with viral infections. Central exposure to the drug varies, with cerebrospinal fluid (CSF) levels 24-70% of plasma concentrations (Fletcher et al., 1981; Jacobson et al., 1987; Sommadossi et al., 1988).

F	t_{max} (h)	$t_{½}$ (h)	CL (l/h/kg)	V_{ss} (l/kg)	f_e (%)	CSF (%)
3-6%	~1	2-3	12-18	0.5-1	91	24-70

Table 1.1. Pharmacokinetic parameters for oral GCV. F, bioavailability; t_{max}, time to maximum plasma concentration; $t_{½}$, half life; CL, total body clearance; V_{ss}, volume of distribution in the steady state; f_e, urinary recovery of the parent drug; CSF, cerebrospinal fluid concentration as a percentage of that in plasma. (Morse et al., 1993).

The HSV-tk system is currently being tested in clinical trials, with treatment protocols of 5 mg/kg b.i.d. by iv infusion. Trials have shown safety in malignant gliomas using both retroviral producer cells (Floeth et al., 2001) and adenoviruses (Adachi et al., 2000; Trask et al., 2000) for delivery. In addition trials in both prostate (Miles et al., 2001) and ovarian (Alvarez et al., 2000) cancer have shown no severe toxicities. One study in prostate reported an increase in the time for PSA levels to increase (Miles et al., 2001).

Effects so far however, are modest. Ram and co-workers (Ram et al., 1997) and Klatzman (Klatzmann et al., 1998) found that injection of virus producing cells directly into glioblastoma tumours followed by GCV treatment had some effect on tumour growth (partial response), although not in all patients. This response was thought to be mainly due to the bystander effect, as retroviral transduction efficiencies were very low. Sandmair et al (Sandmair et al., 2000) reported that with adenoviral transfection, but not retroviral packaging cells, the outcome (determined as survival) of patients with recurrent glioblastoma tumours could be improved. Trials involving melanoma (Klatzmann et al., 1998) have also had limited success. In addition to poor transfection efficiencies, the slower growth of human tumours compared to xenografts used in animal models, and also the limited dose of GCV tolerated due to bone marrow toxicity may all adversely affect treatment outcome.

Cytosine deaminase/5-fluorocytosine

5-fluorouracil (5-FU) is a widely used anti-cancer agent especially active against colon cancer, but its side effects and high dose levels required for response limit its use. The cytosine deaminase (CD) enzyme of some bacterial and fungal cells is capable of converting 5-fluorocytosine (5-FC) to 5-FU. 5-FU undergoes further

enzymatic conversion to 5-FUTP, which is incorporated into DNA and prevents nuclear processing of ribosomal and mRNA, and to 5-fluorouridine-5'-monophosphate, which irreversibly inhibits thymidylate synthase. The toxicity of 5-FU is not cell-cycle phase specific, but shows decreased *in vitro* cytotoxicity under hypoxic conditions (Dachs, personal communication).

The bystander effect of CD/5-FC is not dependent on gap junctions, as 5-FU is capable of non-facilitated diffusion into, and out of cells (Domin et al., 1993). *In vitro* studies show that the presence of the CD gene can increase sensitivity to 5-FC, with an apoptotic mechanism (Kurozumi et al., 2004), although to what degree, is highly dependent on the cell type used (see Table 1.2).

Dosing schedules for 5-FC in animals are more consistent than those for GCV, with 500 mg/kg b.i.d. being the predominant dose chosen. *In vivo* effects of this system appear to be slightly contradictory. Some studies found that expression of the CD enzyme alone was sufficient to cause an immune response capable of resulting in tumour regression (Haack et al., 2000; Mullen et al., 1994). Tumours grown from stable hepatocellular carcinoma (HCC) transfectants showed 75% eradication by 5-FC, when 20% of the inoculated cells contained the CD gene, similar to results obtained with HSV-tk/GCV (Kuriyama et al., 1999c). Using a colorectal carcinoma cell line (WiDr), Trinh (Trinh et al., 1995) demonstrated that 4% CD expressing cells was sufficient to give a 60% cure rate, while for the same level of regression to be achieved with the HSV-tk/GCV system, 50% of cells needed to be HSV-tk^+ (Trinh et al., 1995). The anti-tumour activity of the CD/5-FC system has been demonstrated in fibrosarcoma (Mullen et al., 1994), glioma (Ge et al., 1997), and carcinoma tumour models (Huber et al., 1993; Kanai et al., 1997; Ohwada et al., 1996).

Cell line	Transfectant type	Assay	Exposure time	IC_{50}	Reference
HCC – murine carcinoma	Stable (retroviral)	MTT	4 day	27 μM	(Kuriyama et al., 1999b)
SW40 – human colon cancer SKBr3 – human breast cancer PANC-1 – human pancreatic cancer	Stable (adenoviral)	Trypan blue exclusion	6 day	60 μM 600 μM 600 μM	(Erbs et al., 2000)
9L – rat gliosarcoma	Transient (adenoviral)	MTT	6 days	0.5 μM	(Adachi et al., 2000)
C6 – rat glioma	Stable (retroviral)	MTT	5 days	3 μM	(Ge et al., 1997)
9L – rat gliosarcoma	Stable (electroporation)	Cell count	5 days	0.2 μM	(Aghi et al., 1998)

Table 1.2. CD/5-FC cytotoxicity *in vitro*. Selection of data showing variation in cell sensitivity to 5-FC. IC_{50} is the concentration of drug required to produce a decrease in cell survival of 50% in CD expressing cells.

As is the case with HSV-tk/GCV, there is a level of immunity against tumour rechallenge in animals treated with CD/5-FC (Consalvo et al., 1995; Haack et al., 2000; Kuriyama et al., 1999b; Mullen et al., 1994). In addition, rats bearing experimental liver metasteses subsequently vaccinated with CD^+ expressing cells subcutaneously or subcapsular followed by 5-FC treatment showed a 70% reduction in volume of the original liver tumour (Pierrefite-Carle et al., 2000).

Because additional enzymatic steps are necessary to convert 5-FC to a

cytotoxin, several groups have assessed the effect of a bifunctional CD/uracil phophoribosyltransferase (UPRT) gene on tumour growth and shown this to be more effective than expression of CD alone (Adachi et al., 2000; Erbs et al., 2000), as the UPRT enzyme catalyses the conversion of 5-FU to UMP and 5-FUMP.

Kinetics of 5-FC in humans show that it is greater than 80% bioavailable in patients with normal renal function (Cutler et al., 1978). A single 500 mg oral dose of 5-FC has the kinetic properties described in Table 1.3. Treatment of fungal infections usually relies on oral administration of 4 g/day, which results in peak serum levels of 88-94 μg/ml (Fass & Perkins, 1971). Unlike GCV, 5-FC will accumulate after multiple dosing. A 2 g oral dose administered every 6 hours will give a steady state plasma concentration of 60-80 μg/ml (Daneshmend & Warnock, 1983). This is greater than IC_{50} levels for tumour cell kill (see Table 1.2).

Two phase one clinical trials have shown the safety of the CD/5-FC combination, one using adenoviral delivery to target colon carcinoma metastatic to the liver (Crystal et al., 1997), and one using a combination of a lytic adenovirus, HSV-tk and CD genes in prostate (Freytag et al., 2002). Some signs of activity were observed, although this study involved intratumoural injection, which is not feasible in all patients. A trial has also been proposed using Salmonella for cellular delivery of the CD gene rather than attempting *in vivo* modification of the tumour cells (Cunningham & Nemunaitis, 2001). Unfortunately, this trial was closed following the treatment of only 3 patients (Nemunaitis et al., 2003). Safety of the *Salmonella* vector, 5-FC treatment and some signs of stable disease in injected sites were observed when there was evidence of tumour colonisation by the bacteria.

Dose	K_a (h^{-1})	K_{el} (h^{-1})	AUC (mg l^{-1} h)	$t_{½}$ (h)	Cl_p (ml min^{-1})
500 mg p.o.	5.08	0.226	54.3	3.1	
500 mg i.v.		54		3.8	154

Table 1.3. Pharmacokinetic parameters of 5-FC in healthy subjects. p.o. oral administration; K_a, absorption constant; K_{el}, elimination constant; AUC, area under the curve; $t_{½}$, half life; Cl_p plasma clearance (after (Cutler et al., 1978).

Although pre-clinical results are very promising it is unlikely that gene therapy would be used as a single treatment modality in patients. Combination with radiotherapy is important especially for 5-FC which can act as a radiosensitiser, and *in vivo* results have shown both a significant bystander effect (Hamstra et al., 1999), and effects at clinically relevant dose regimes of 2 or 5 Gy per fraction over one week (Stackhouse et al., 2000). The dose of prodrug administered appears to be important, as the use of a CD-UPRT fusion gene, in combination with 5-FC and radiation showed a synergistic effect in a 9L gliosarcoma model. However, doses below 500 mg/kg/day were ineffective (Kambara et al., 2002). In an early clinical study in prostate carcinoma utilising a replication competent adenovirus, CD/HSV-tk gene therapy in combination with radiotherapy showed promising results (Freytag et al., 2003).

NADPH:cytochrome c reductase/bioreductives

A potential method of targeting tumour hypoxia is the use of bioreductive drugs. The redox sensitive flavoprotein NADPH;cytochrome c P450 reductase (P450R) is an important activator of many bioreductives. It was shown that the toxicity of tirapazamine (TPZ), a benzotriazene-di-N-oxide, under hypoxia, was strongly correlated with P450R activity in a panel of breast cancer cell lines (Patterson et al., 1995). In addition, sensitivity to TPZ could be restored in a resistant cell line (A549c50) by transfection with P450R (Saunders et al., 2000b).

Co-transfection of P450/P450R to gliosarcoma cells was shown to lead to increased toxicity and tumour growth delay following treatment with cyclophosphamide (CPA) and TPZ compared with either compound alone (Jounaidi & Waxman, 2000). A bystander effect of the P450R/TPZ combination was apparent.

Other bioreductives, including E09 (Saunders et al., 2000a), and RSU1069 (Patterson et al., 1997) show increased activity in cell lines overexpressing P450R.

Placement of the P450R enzyme under the control of an HRE resulted in hypoxia dependent sensitivity to RSU1069 in a human fibrosarcoma model (Patterson et al., 2002). Cells expressing the HRE-P450R construct were grown as solid tumours. When treated with RSU1069 and 10 Gy radiation, 50% tumour free survival was obtained, compared with 100% mortality in empty vector controls. The use of bioreductive drugs and HRE's provides tumour specific targeting of prodrug activation.

The diffusion of TPZ through a multilayered cell model has been used to estimate the penetration of the drug through cells in a tumour cord (Kyle & Minchinton, 1999). It was estimated that levels in cells distant from blood vessels would be approximately 10% of the blood concentration, and that cell kill would be limited to the first 75 μm of tissue surrounding a blood vessel, due to metabolism of TPZ by cells closer to the vessel at intermediate oxygen tensions. This has implications for targeting chronically hypoxic regions which may lie at a greater distance from the vessel than this estimate.

Pharmacokinetics of TPZ have been determined in mouse and man. In mice there is a steep dose lethality relationship, with only 9 mg/m^2 separating the LD_{10} and LD_{50} (doses which lead to death in 10 and 50% of animals respectively)(Graham et al., 1997). An increase in dose leads to a disproportionately high increase in AUC in both mice and men (Graham et al., 1997), although it is more exaggerated in mice. In a phase I clinical trial the MTD was found to be 390 mg/m^2, with dose limiting ototoxicity being the primary symptom, but muscle cramp and nausea also reported (Graham et al., 1997; Senan et al., 1997). Toxicity was generally reported in those patients with the highest plasma AUC (Senan et al., 1997). A dose of 260 mg/m^2 given in combination with cisplatin resulted in a plasma Cmax of 5.97 μg/ml, and an AUC of 811.4 μg/ml min^{-1} (Johnson et al., 1997). The clearance of cisplatin was unaffected by the administration of TPZ. A higher dose of 330 mg/m^2 (Senan et al., 1997) resulted in a plasma AUC of 1026.5 μg/ml min^{-1}. Elimination was observed to be biphasic in man (Graham et al., 1997), which was also observed in mice at doses greater than 0.2 mmol kg^{-1} following i.v. administration (Walton & Workman, 1993).

TPZ is in clinical trials as a bioreductive agent both in combination with chemotherapy and radiotherapy. It has not as yet entered clinical trials for gene therapy.

Nitroreductase/CB1954

The compound 5-aziridinyl-2,4-dinitrobenzamide (CB1954) was found to inhibit the growth of the rat carcinoma WiDr. On this basis a phase I clinical trial was carried out at the Royal Marsden Hospital involving 30 patients in the 1970's (Bridgewater et al., 1995). Unfortunately there was no effect in patients, but this led to further investigation of the sensitive tumour line, and it was found that it expressed high levels of rat NAD(P)H dehydrogenase (DT-diaphorase) able to convert CB1954

to 5-aziridynyl-4-hydroxylamino-2-nitrobenzamide (Knox et al., 1988). CB1954 is however, a poor substrate for human DT-diaphorase. It was demonstrated that this mustard metabolite was capable of causing DNA interstrand cross-linking (Knox et al., 1988), which resulted in cell death.

The group of Anlezark *et al* (Anlezark et al., 1992) assessed the ability of other nitroreductases to metabolise CB1954, and found that the nitroreductase from *Escherichia coli* (NTR) was catalytically superior to the rat DT-diaphorase, and all further experiments have utilised the bacterial enzyme.

The nitroreductase/CB1954 combination is effective against many cell lines *in vitro*, including human melanoma, human mesothelioma (Bridgewater et al., 1995), human ovarian cancer (Bridgewater et al., 1995; Friedlos et al., 1998; McNeish et al., 1998), mouse fibroblast (Drabek et al., 1997), human colon carcinoma (Friedlos et al., 1998; Weedon et al., 2000), human pancreatic carcinoma (Weedon et al., 2000) and human liver carcinoma (Weedon et al., 2000). IC_{50} values using the MTT assay ranged from 1-20 μM depending on the cell line used. A sensitisation of 500 fold was achieved in ovarian carcinoma cells when 80-90% expressed the NTR enzyme (McNeish et al., 1998). The bystander effect has been shown to be due to a freely diffusible metabolite (Bridgewater et al., 1997), and cell killing to be cell cycle independent (Bridgewater et al., 1995). Studies utilising a spheroid model have shown that the bystander effect of the NTR/CB1954 system is effective between cell lines (Benouchan et al., 2003).

Initial *in vivo* studies varied from those of other strategies in that transgenic mice were utilised. T-cell localisation of the NTR enzyme was achieved in mice using the CD2 locus promotor (Drabek et al., 1997). When CB1954 was administered there was a decrease in the cell number of the spleen and thymus to 14-16% of controls. When NTR was expressed in the luminal cells of mice mammary glands (Clark et al., 1997), these cells were selectively destroyed by CB1954 administration. Apoptosis is thought, at least in part, to be the mode of cell death in this system, and by crossing transgenic mice expressing NTR in the mammary gland, with *p53* knock-out mice, single or 3 consecutive daily doses of CB1954 were capable of causing a reduction in luminal cell number (Cui et al., 1999). In addition, staining for apoptosis showed that the *p53* status of the mice did not affect the number of positively stained cells. This is important for cancer therapy as the loss of functional *p53* is associated with early

stage progression in many tumours.

Both human and laboratory animal kinetics have been performed with CB1954 (Chung-Faye et al., 2001; Workman et al., 1986), and the results are summarised in Table 1.4.

A dose escalation study was performed concomitantly with the human pharmacokinetic phase I study (Chung-Faye et al., 2001). This showed that dose limiting toxicities were seen with a dose of 37.5 mg/m^2 when administered iv, presenting as diarrhoea and elevated levels of transaminase. Below this dose, patients tolerated doses every three weeks for a maximum of six cycles. The drawback to this enzyme prodrug combination is the inherent toxicity of mustard compounds.

Species	Dose	Route of administration	C_{pmax}	$T_{½}$ (h)	F (%)
Mouse	50 mg/kg	i.v.	100 µg/ml	1.4-2	
Mouse	50 mg/kg	i.p.	3-5 fold lower than i.v.	1.4-2	85
Dog	25 mg/kg	i.v.	27 µg/ml	2.5-4	
Dog	25 mg/kg	p.o.	3-5 fold lower than i.v.	2.5-4	40
Human	24 mg/m2	i.v.	6.3 µM	A 9 min B 110 min	

Table 1.4. Pharmacokinetic parameters for CB1945. The two half lives shown for humans are due to the bi-exponential delay, thought to be due to hepatic clearance of the drug (Chung-Faye et al., 2001). C_{pmax}, maximaum plasma concentration; F, bioavailability.

Cytochrome P450/cyclophosphamide

The oxazophorines cyclophosphamide (CPA) and iphosphamide (IPA) are cancer chemotherapeutic prodrugs, which must be activated by liver cytochrome P450 (CYP) enzymes. In the rat there are three CYP isoforms involved in CPA hydroxylation, CYP2B1, CYP2C1 and CYP2C11 (Clarke & Waxman, 1989). In addition the CYP3A isoform is required for activation of IPA (Weber & Waxman,

1993). In human liver the CYP2B6 and CYP3A4 forms are catalytically active for both CPA and IPA (Chang et al., 1993). The rat CYP2B1 is the most catalytically efficient isoform (Clarke & Waxman, 1989) identified to date.

Metabolism of oxazophorines gives rise to a 4-hydroxy compound which is in equilibrium with its open ring aldo-tautomer. This breaks down to a phosphoramide mustard and acrolein in equimolar amounts. The mustard is an alkylating agent able to form DNA cross-links in a cell cycle independent manner (Maccubbin et al., 1991).

Expression of CYP is able to sensitise cells to both CPA and IPA in rat gliosarcoma (Chen & Waxman, 1995; Jounaidi & Waxman, 2000), lymphoblastoma (Chang & Waxman, 1993) and human breast carcinoma (Chen et al., 1996) cells *in vitro*. The bystander effect is mediated through a soluble compound, likely to be 4-hydroxy CPA (Wei et al., 1995). It has been proposed (Chen et al., 1996) that acrolein sensitises cells to the mustard. The CYP3A4 enzymes has also been used to sensitise tumour cells to the bioreductive agen AQ4N (McCarthy et al., 2003).

The use of a multicellular spheroid model has demonstrated the ability of macrophages to deliver the CYP gene controlled by an HRE (Griffiths et al., 2000). Both a reduction in clonogenic survival and spheroid size were seen after CPA treatment.

In animal models subcutaneous tumours grown from stable transfectants demonstrated prolonged growth delay after a single 150 mg/kg dose of CPA (Chen & Waxman, 1995; Chen et al., 1996; Jounaidi & Waxman, 2000). This was accompanied by some toxicity, seen as a decrease in body weight. Later studies showed that lower, multiple dosing may be more effective (Browder et al., 2000), and this was demonstrated in a rat gliosarcoma cell line where tumours regressed when given 140 mg/kg/day for six days (Jounaidi & Waxman, 2000).

Tumour response may also be modified by coexpression of cytochrome P450 reductase and administration of bioreductive agents, such as tirapazamine (Jounaidi & Waxman, 2000). This group also demonstrated that despite a requirement of the enzymatic function for oxygen, oxygen concentrations of 1% were sufficient to result in the formation of the active compound. Administration of inhibitors of liver CYP isoforms may be beneficial in targeting prodrug activation to the tumour and reducing global toxicity (Halpern et al., 1989), especially as CPA is capable of auto-induction of the CYP enzymes. The CYP2B1 gene has been used in a replication competent

HSV-1 vector which led to additive effects in a model of liver metastasis (Pawlik et al., 2002).

CPA administration for cancer therapy in humans is dependent on the tumour type. It is usually given in combination, for example in breast cancer the two main regimes involving CPA are "AC" and "CMF". In AC, adriamycin, also referred to as doxorubicin, (60 mg/m^2) and CPA (600 mg/m^2) are administered i.v. every 3 months. With CMF, CPA is administered orally at 100 mg/m^2 on the first fourteen days of every month in combination with the antimetabolite methotrexate and 5-FU (Souhami et al., 1997).

Carboxypeptidase G2/CMDA

Carboxypeptidase G2 (CPG2) is a bacterial enzyme with no human analogue, able to catalyse the conversion of 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glamic acid (CMDA) to the DNA cross-linking mustard 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoic acid (Springer et al., 1990).

Initial *in vitro* studies with monkey kidney cells (COS) and human epithelial cells showed that transfected cells were 11-95 fold more sensitive to CMDA than mock-transfected controls (Marais et al., 1996). In a breast tumour cell line there was only a modest increase in sensitivity (Marais et al., 1997) due to limited CMDA entry into cells. This could be overcome by tethering the CPG2 enzyme to the cell surface (Marais et al., 1997).

There is a significant bystander effect with the CPG2/CMDA system with only 12% of WiDr monolayers required to express CPG2 to obtain 100% cell kill (Marais et al., 1997).

In vivo experiments have utilised surface tethered CPG2 to show tumour regression and cures in 5/6 mice when all cells expressed the CPG2 enzyme (Stribbling et al., 2000). In addition, a significant bystander effect was observed calculated by growth delay and apoptosis staining. The dose regime for this study was a single i.p. administration of 500 mg/kg once per week for 3 weeks. Some non-specific toxicity was associated with CMDA, as demonstrated by a significant decrease in body weight, although this was reversed upon cessation of treatment.

This enzyme prodrug combination is now almost exclusively used for antibody directed enzyme prodrug therapy (ADEPT), in particular with anti-CEA antibodies. Using an ADEPT strategy against colon carcinoma models, tumour response could be greatly enhanced (16-fold) by addition of the vascular targeting agent DMXAA (Pedley et al., 1999). The development of self-immolating drugs may enhance the efficacy of this GDEPT combination (Niculescu-Duvaz et al., 1998).

A phase I clinical trial using this combination for ADEPT (Napier et al., 2000) showed promising results, with one patient showing a partial response and 6 with stable disease for 4 months. Haematological disorders were seen, but were easily managed.

1.4 Horseradish peroxidase/indole-3-acetic acid

Horseradish peroxidase (HRP) is a haem enzyme isolated from the roots of the horseradish plant. It is a globular glycoprotein of which the protein moiety has a mass of approximately 34,000 Da. The remaining mass is composed of the prosthetic haem group, two calcium ions and surface bound glycans. The active site of the HRP molecule resembles that of haemoglobin and myoglobin. Under normal conditions HRP cleaves hydrogen peroxide resulting in the formation of water, and oxygen is retained forming a ferryl group named compound I. Two further steps occur resulting in the formation of substrate radicals which are highly reactive (Fig. 1.2).

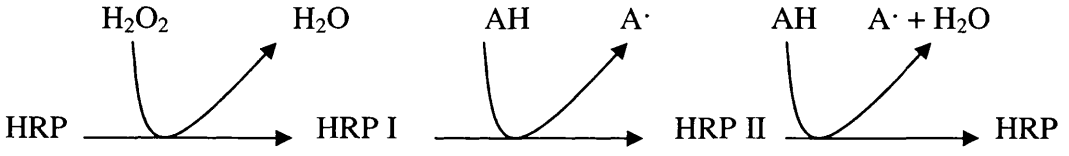


Figure 1.2. The reaction of HRP with substrates. AH, reducing substrate, A· radical.

Indole-3-acetic acid (IAA) is a plant auxin involved in the regulation of plant cellular growth, division and differentiation. It is also a natural metabolite in mammals of the amino acid tryptophan by monoamine oxidase. It is a uremic toxin (Sakai et al., 1995), and a weak agonist of the aryl hydrocarbon (Ah) receptor able to cause gene transcription of products under the control of the Ah receptor (Heath-Pagliuso et al., 1998). Its excretion via urine is increased in upper gastrointestinal tract

cancers, and its use as a marker of disease progression in gastric cancer has been proposed (Kobori et al., 1983).

In mice, 300 mg/kg of IAA led to myotonia (Fuller et al., 1971), hypothermia (Yamada et al., 1985), changes in 5-hydroxy tryptamine metabolism (Yamada et al., 1985), inhibition of experimentally induced oedema (Jones et al., 1995) and teratogenic effects (John et al., 1979). However, IAA has been given to patients and volunteers, with no major toxicities reported after administration of 3-10 g (Mirsky & Diengott, 1956; Rysanek & Vitek, 1959).

HRP has been shown to catalyse the oxidation of IAA in the absence of hydrogen peroxide (Kenten, 1955). The reaction of HRP with IAA (see Fig. 1.2) is characterised by the formation of a radical cation (2), which undergoes scission of the carbon-carbon bond to give a carbon-centred skatolyl radical (3). This forms a peroxyl radical (5) in the presence of oxygen, which then decays to a number of products, including 3-methylene-2-oxindole (8), which can react with cellular nucleophiles such as protein thiols and DNA to form adducts (9). Under anoxic conditions, decarboxylation of the radical cation occurs, and the skatole radical reacts with hydrogen donors (Augusto, 1993).

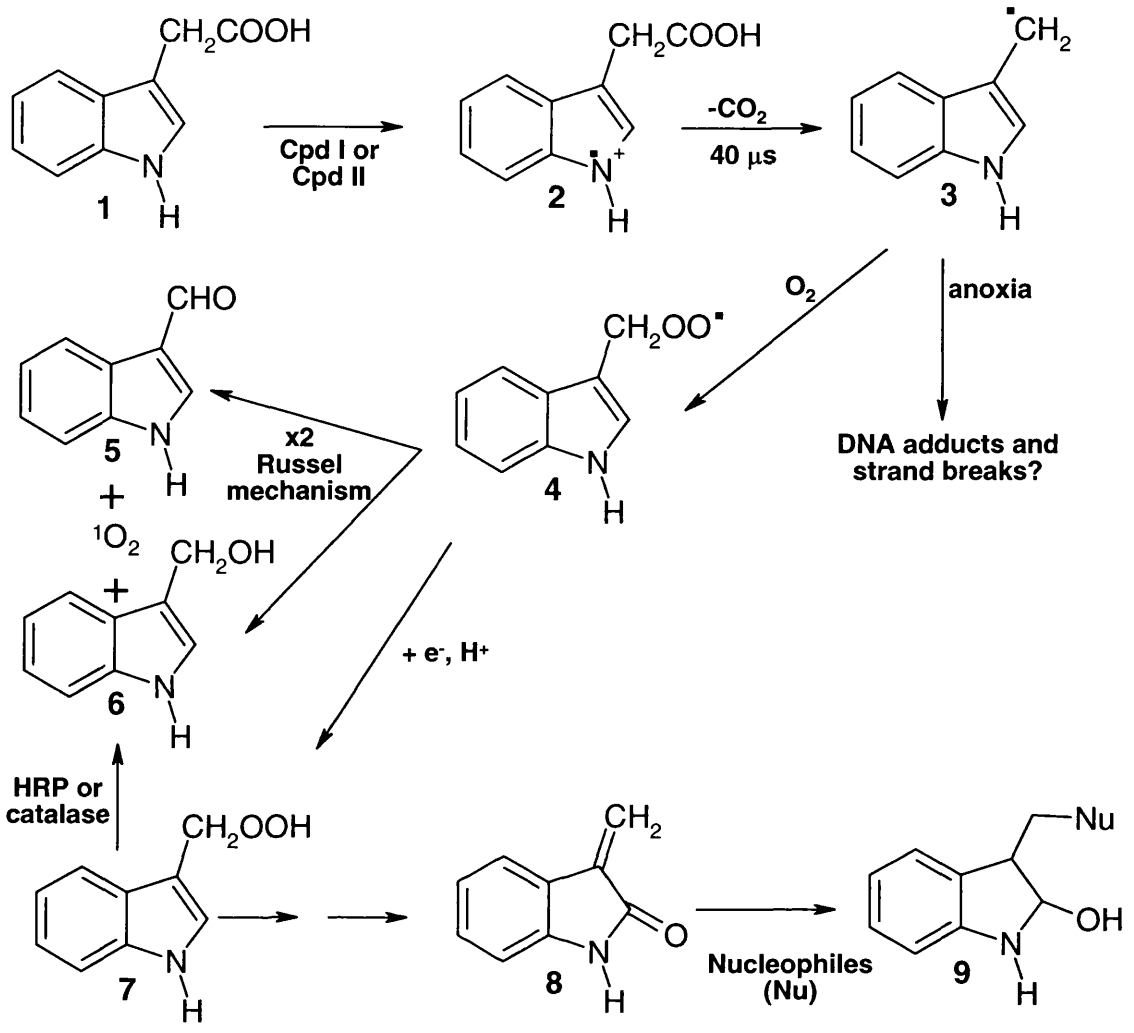


Figure 1.3. Possible mechanisms involved in the cytotoxic activation of indole-3-acetic acid (IAA) by HRP. IAA (1) is oxidised by HRP compound (Cpd) I and II to the radical cation (2), which fragments rapidly to yield the skatole radical (3). The skatole radical promptly reacts with oxygen to form the peroxyl radical (4), which by further steps leads to the major products: indole-3-aldehyde (5), indole-3-carbinol (6), skatole hydroperoxide (7) and 3-methylene-2-oxindole (MOI, 8). MOI can react with cellular nucleophiles to form adducts (9). From (Folkes & Wardman, 2001)

The addition of purified HRP and IAA to hamster fibroblasts (V79) resulted in a decrease in colony formation, whilst neither enzyme or prodrug alone were cytotoxic at the concentrations used or times studied (Folkes et al., 1998; Folkes et al., 1999). The formation of the peroxyl radical was predicted to result in lipid peroxidation, which was indeed the case in phosphatidylcholine-cholesterol liposomes

(Folkes et al., 1998). This could be prevented by the addition of anti-oxidants such as ascorbate, Trolox, α-tocopherol and β-carotene (Candeias et al., 1996). However, lipid peroxidation could not be measured in mammalian cells (Folkes et al., 1999). Trolox was protective against cytotoxicity in this system; however preloading cells with α-tocopherol had little effect. Incubation of activated IAA resulted in DNA strand breaks and adduct formation in a cell free system using plasmid DNA (Folkes et al., 1999).

It is unlikely that non-specific activation of IAA would take place in mammalian tissues, since IAA is a poor substrate for mammalian peroxidases in the absence of hydrogen peroxide (Kobayashi et al., 1980). Myeloperoxidase (MPO) in human promyelocytic leaukaemia lymphocytes (Folkes et al., 1998) and rat white blood cells was less efficient at converting IAA to a cytotoxin than HRP, at therapeutically achievable concentrations of prodrug.

In vitro studies have demonstrated that mammalian cells transiently transfected with the HRP cDNA and then exposed to IAA show reduced colony forming ability compared to green fluorescent protein (GFP) transfected controls (Greco et al., 2000). A strong bystander effect was induced, probably due to the production of a freely diffusible cytotoxin. However, under anoxia, the cytotoxin produced did not remain stable for a sufficient period of time to be transferred in media-switch experiments.

IAA analogues have also been tested in this system, both in oxic and anoxic conditions. The 5-bromoindole-3-acetic acid (5Br-IAA) analogue was found to be highly potent at concentrations at and below 1 mM after 24 hour exposure in air, and in anoxia, a 2 hour incubation at this concentration produced over 2 logs of cell kill (Greco et al., 2001).

The combination of HRP/IAA and radiation has been studied. The HRP/IAA system is capable of reducing glutathione levels (Folkes & Wardman, 2001), and inducing DNA strand breaks (Folkes et al., 1999), which may increase radiation sensitivity. Both IAA and the 1-methylindole-3-acetic acid (1Me-IAA) analogue were able to selectively sensitise HRP expressing human bladder carcinoma cells to the effects of radiation (Greco et al., 2002c). In particular, IAA was able to increase sensitivity under anoxic conditions (Greco et al., 2001), where radical fixation is less likely to occur (Howard-Flanders, 1958).

Control of HRP expression has been placed under the control of a variety of HRE's and CarG elements (Greco et al., 2002b). After the appropriate stimuli, hypoxia and/or 0.7 Gy, there was sufficient HRP activity in transient transfectants to decrease cell survival after 0.5 mM IAA.

Using transient HRP transfectants, mechanisms of cellular death have been investigated (Greco et al., 2002a). Apoptosis was evident in the form of increased annexin V binding, formation of micronuclei and DNA fragmentation. Although the non-specific caspase inhibitor zVAD.fmk decreased the fraction of cells displaying apoptotic features, PARP cleavage could not be detected. It is likely that necrosis and cell cycle arrest also play an important role in the decreased clonogenic ability of cells exposed to the HRP/IAA system.

With regard to the ideal enzyme prodrug characteristics discussed in section 1.2.4 the HRP/IAA combination shows several advantages over existing strategies:

1. The prodrug should not be metabolised by endogenous enzymes, since this may lead to side effects.

IAA is generally a weak substrate for mammalian peroxidases. It is possible that myeloperoxidases are able to produce the skatolyl-type radical, but this required both large quantities of prodrug (10 mM) and a lengthy exposure time of 72 h in human leukaemia cells (Folkes et al., 1998). Other GDEPT prodrugs are also selective for the exogenous enzyme, with the notable exception of CPA.

2. The prodrug should be a better substrate for the delivered enzyme than endogenous compounds. This will ensure efficient catalysis with little antagonism.

IAA and its derivatives are significantly better substrates for HRP than endogenous molecules such as tryptophan (Wardman, in press). The carboxypeptidase G2 (Springer et al., 1994), HSV-tk and CD enzymes also have little or no catalytic activity upon endogenous compounds.

3. The expressed enzyme should be a stable monomeric protein to ensure that correct folding and assembly is easily achieved.

The HRP enzyme is highly stable as demonstrated by its use in many biotechnology applications. The activity may be slightly greater at pH 5 than 7, which may be advantageous with ADEPT strategies, since tumours often have an acidic environment (Griffiths, 1991). However, *in vitro* results showed the intracellular conversion of IAA to a cytotoxin under neutral pH (Greco et al., 2000). HRP is

monomeric as are other GDEPT enzymes. Although hydrogen peroxide is described as a co-factor for the enzyme, the reaction with IAA and derivatives has no need of hydrogen peroxide.

4. The cytotoxin should be formed from a single enzymatic reaction in order to limit potential rate limiting steps.

The production of toxic metabolites from IAA does not require any further enzymatic processes following HRP oxidation, an advantage of this system over the CD/5-FC and HSV-tk/GCV systems where the secondary catalysis by endogenous enzymes is often a limiting step (although this can be improved by the use of a fusion protein). In addition, the toxic IAA metabolite is freely diffusible, unlike the phosphorylated GCV product, so increasing potential bystander effects.

5. The enzyme/prodrug system should be active under the microenvironmental conditions found in solid tumours.

The HRP enzyme is capable of catalysing the formation of cytotoxic products of IAA and its derivatives under low oxygen tensions. The same is not true for HSV-tk/GCV (Greco et al., 2000) or CD/5-FC (Dachs, unpublished results). The CYP/CPA combination is also active under decreased oxygen tensions of 1% (Jounaidi & Waxman, 2000), although this level of hypoxia is not tumour specific.

1.5 Aim of this project

The aim of this project was to further investigate the possibility of using the HRP/IAA combination in a GDEPT strategy. Results from previous work have shown the efficacy of the HRP/IAA system at low drug concentrations and short exposure periods when HRP is added exogenously at relatively high concentrations (Folkes et al., 1998). Intracellular HRP produced by cells transiently transfected with the HRP gene, is able to convert IAA and related indoles to cytotoxic products, although concentrations of indoles required are greater than when purified HRP is added to the culture medium for equivalent levels of cell kill (Greco et al., 2000).

All previous studies have used simple cell monolayer systems to assess cell kill (Greco et al., 2000), the mechanisms of cell death (Greco et al., 2002a) and use of promoters to target specific cell populations (Greco et al., 2002b). The aim of this project was to increase the knowledge regarding the activity of the HRP/IAA system

for GDEPT using a more complicated *in vitro* model than the monolayer. In order to achieve this cells stably expressing the HRP gene needed to be produced.

The *in vivo* properties of the indole prodrugs suitable for GDEPT have not been determined. The pharmacokinetic properties of IAA have been published to a limited degree, however a more complete picture needed to be established, particularly with regard to cancer therapy. The potential toxicity of repeated dosing also needed to be determined. *In vivo* proof of principle for the effectiveness of the HRP/IAA system needed to be established, and also whether the *in vitro* potentiation of radiation could be seen *in vivo*. The effect of host immunity was an area of research since the bystander effect *in vivo* is critical for other enzyme prodrug combinations.

Possibly the most important stage in any GDEPT strategy is the gene delivery. Bystander studies have shown that *in vitro* less than 10% of cells is sufficient to result in significant levels of cell kill (Greco et al., 2000). *In vivo* delivery of the HRP gene would more difficult than simple lipofection, and so the use of electric pulses to enhance delivery was studied.

Chapter 2

Materials and Methods

2.1 Cell culture

FaDu human nasopharyngeal cell carcinoma cells were obtained from the American Type Culture Collection (Manassa, VA, USA), and maintained in Dulbecco's Modified Eagles Medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum (FCS, Sigma, Poole, UK) and 2 mM L-glutamine (Life Technologies). Cells were kept in a humidified incubator at 37°C and 5% CO_2/air. Cells were routinely sub-cultured in 75 cm^2 cantilevered flasks using trypsin-EDTA (Life Technologies).

The murine adenocarcinoma cell line, Carcinoma NT (CaNT), arose spontaneously in the Gray Cancer Institute mouse colony (Hewitt et al., 1976). Cells were maintained in DMEM as described above.

2.2 Large-scale preparation of plasmid DNA

DNA extraction was carried out using the Novagen Mobius 1000 plasmid kit (CN Biosciences, Nottingham, UK).

Bacteria from frozen culture (20 µl) were inoculated into 5 ml of liquid LB broth (Life Technologies) containing the appropriate antibiotic (100 µg/ml ampicillin, or 15 µg/ml kanamycin, Sigma), and incubated at 37°C for 8 h on a shaking platform.

A 0.5 ml sample from this culture was then transferred to 100 ml LB broth in a 500 ml conical flask, and incubated overnight at 37°C with continuous shaking with the appropriate antibiotic.

Cells were harvested by centrifugation in a Sorval RC-5B refrigerated superspeed centrifuge, at 6000 rpm for 10 min in polycarbonate tubes. Supernatant was decanted, and the pellet resuspended in 8 ml of Mobius Bacterial Resuspension Buffer. An equal volume of Mobius Bacterial Lysis Buffer was then added, the tube gently swirled, and incubated at room temperature for 5 min. To this, 8 ml of cold Mobius Neutralisation Buffer was added, and the solution gently mixed. The tube was incubated on ice for 5 min.

A Mobius 100 column was equilibrated by the addition of 10 ml Equilibration

Buffer which was allowed to run freely by gravity.

The neutralysed lystate was centrifuged at 7500 rpm for 2 min and the cleared lysate supernatant added to a Clear Spin Filter unit. This was immediately centrifuged for 3 min at 2000 g, and the flow through added to the top of the equilibrated Mobius Column. The entire volume was allowed to flow through by gravity. When the lysate had cleared the column, 20 ml of Wash Buffer was then added to the column and allowed to travel through. DNA was eluted by the addition of 5 ml Mobius Elution Buffer, and the single fraction collected.

To precipitate DNA, 3.5 ml isopropanol was added to the eluent, gently mixed and centrifuged for 20 min at 11,000 rpm. The supernatant was gently aspirated, the sample washed in 70% ethanol, centrifuged at 11,000 rpm again for 10 min, the pellet air dried, and then dissolved in water.

DNA concentration was calculated from the absorbance of a solution at 260 nm, using the equation:

Concentration (ng/µl) = A_{260}*50*dilution factor

Purity was assessed using the ration of readings at 260 and 280 nm. A range of 1.8-2.0 was deemed optimum, with greater than 2.0 indicating RNA contamination, and less than 1.8 indicating the presence of proteins. DNA solutions were stored at –20°C.

2.3 Transfection of eukaryotic cells

A proportion of cells incorporate exogenous DNA after transfection dependant upon both the method used, and the cell line. Transient transfection leads to expression of transgenes for approximately 1-7 days. In transient transfectants the DNA remains episomal, and is not incorporated into the genome. Hence, the genotype is lost over several generations. By adding a resistance gene to the vector DNA, cells can be grown in the presence of an appropriate antibiotic, and only transfected cells will survive and clones can be isolated. These clones have most likely incorporated the exogenous DNA into their genome and are termed stable transfectants.

In this study, cationic lipids and integrin-targeted peptides were used to transfect mammalian cells *in vitro* (Hart et al., 1998). One day prior to transfection exponentially growing cells were trypsinised and plated in 6 well plates at 4 x 10^5/well, to give 60-80% confluency at the time of transfection.

The transfection mixture was prepared fresh, with a lipofectin (Life Technologies) : peptide : DNA ratio of 0.75:1.8:1 by weight. This resulted in the following mixture:

1.5 µl Lipofectin diluted in 200 µl OptiMEM (Life Technologies)

3.6 µg peptide (0.1 mg/ml, Institute of Child Health, London, UK)

2 µg DNA in 200 µl OptiMEM.

The components were then mixed in order, and allowed to stand at room temperature for 2 h to form complexes. Prior to addition to cells, the mixture was made to 1ml with optiMEM.

Adherent cells were washed twice in PBS, and once in OptiMEM to remove serum. The prepared 1ml of transfection mixture was added to each well, and incubated for 5-7 h in a humidified incubator. Complete medium was added and incubated overnight. Transient transfection efficiencies were assessed the following day.

For stable transfectants, following overnight incubation, cells were trypsinised and plated in 10 cm plates. 24 h later media containing the appropriate selection antibiotic (puromycin 1 µg/ml, Sigma, or 1 mg/ml G418, Life Technologies) was added and cells left to form colonies over approximately 2 weeks. These were isolated and expanded before assessing transgene expression.

2.4 Detection of Green Fluorescent Protein

The enhanced green fluorescent protein (EGFP) is widely used as a marker in gene expression studies. It has the advantages over traditional markers such as luciferase and β-galactosidase in that it does not require the addition of a substrate, allowing real time imaging. EGFP levels were measured using either fluorescence microscopy, or fluorescence activated cell sorting (FACS).

For the former, cells or frozen tumour sections, were viewed using an epi-fluorescent microscope (Nikon TE200), and a 500-510 nm narrow band filter (Glen Spectra, UK). Images were captured and analysed with custom built software (Gray Cancer Institute).

For FACS analysis, cells were harvested using trypsin/EDTA, washed in PBS, and resuspended in Hanks' Balanced Salt Solution (HBSS, Life Technologies). Samples were analysed on a Becton Dickinson FACScan using dedicated software

(CELLQuest for Apple Macintosh, Becton Dickinson, Franklin Lakes, NJ, USA). As cells pass through a laser, they scatter light and emit fluorescence. EGFP has the spectral characteristics of excitation maximum 488 nm, emission maximum 507 nm, and was detected using the FL-1 detector on the FACScan. Cells were gated using forward and side scatter to exclude debris.

2.5 Detection of horseradish peroxidase activity

HRP activity was assessed using a modified version of the 3,3',5,5'-tetramethylbenzidine (TMB) assay as previously described (Greco et al., 2000). The oxidation of TMB results in the formation of a blue colour, which can be detected at 652 nm using a spectrophotometer.

Samples from cell culture, or tumour tissue suspended in 0.5% hexadecyltrimethylammonium bromide, were lysed by freeze/thaw in liquid nitrogen/37°C three times. Samples were centrifuged at 13000 rpm for 15 min. Tumour samples were incubated at 60°C in a water bath for 2 h prior to centrifugation. Supernatants were stored at –20°C until analysis.

Reactions were carried out at room temperature over a period of 10 min, by diluting 40 µl sample in 80 mM phosphate buffer (pH 5.4), 320 mM TMB and 3 mM hydrogen peroxide in a total volume of 2 ml. Absorbance was read every minute at 652 nm on a Hewlett Packard model 8452A diode array spectrophotometer. Over this period, absorbance increased linearly.

Buffers and reagents

Phosphate buffer:

50 mM PO_4 buffer in water, from 100 mM stock (Sigma). Adjusted to pH 6.0.

80 mM PO_4 buffer in water, from 100 mM stock, adjusted to pH 5.4.

Buffers were filter sterilised and stored at 4°C.

0.5% Hexadecyltrimethylammonium bromide:

Hexadecyltrimethylammonium bromide (Sigma)	100 mg
50 mM PO_4 buffer pH 6.0	20 ml

Stored at -20°.

TMB:	
TMB (Sigma)	100 mg
DMSO (Sigma)	1 ml
Sterile water	9 ml
Hydrogen peroxide:	
Hydrogen peroxide 30% w/w (Sigma)	100 µl
Sterile water	9.9 ml
Final reaction mixture:	
80 mM PO_4 buffer	1870 µl
H_2O_2	70 µl
Sample	40 µl
TMB	20 µl

2.6 Protein quantification

Protein levels in cell samples and tumours were assessed using a commercial protein assay kit (Bio-Rad, Hemel Hempstead, UK) against a bovine serum albumin standard. This assay is based on the principle that an acidic solution of Coomassie Brilliant Blue G-250 will shift its maximal absorbance from 465 to 595 nm when it binds protein. The colour change represents the protein concentration, with the extinction coefficient of a dye-albumin solution constant over a 10-fold concentration range.

2.7 Western Blot

Protein levels in samples was analysed using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Cells were lysed by syringing cells in lysis buffer containing SDS. SDS is an anionic detergent which will bind to proteins. Dithiothreitol (DTT) is a reducing agent, which in combination with heat and SDS, will result in the linearisation of globular proteins. Each molecule of SDS will contribute two negative charges, and the number of SDS molecules binding to a protein is proportional to the number of amino acids. Due to the negative charge carried by SDS, proteins can then be run on a polyacrylamide gel by electrophoresis

towards the anode, separated according to size.

Equal quantities of protein were suspended in sample buffer, denatured at 95°C for 5 min, loaded into pre-cast 12% polyacrylamide gels (Invitrogen-Novex, Gronigen, Netherlands) in 1x running buffer (National Diagnostics, Hessle, UK) and subjected to SDS-PAGE at a constant voltage of 125 V.

Proteins were transferred to nitrocellulose membranes (Genetic Research Instrumentation Ltd, Rayne, UK) using an LKB-Pharmacia-Biotech semidry blotter, for 1 h at 0.8 mA/cm^2 membrane surface area.

The membrane was rinsed in 0.1% Tween 20/phosphate buffered saline (PBS-T) and incubated in blocking solution for 2-4 h at room temperature. All incubations were performed on a rotating platform. Membranes were rinsed in PBS-T, and primary antibody in blocking solution was added and incubated overnight at 4°C.

After four 5 min washes in PBS-T, secondary HRP-conjugated antibody was added for 1 h at room temperature. Membranes were then washed in PBS-T 5 times over 45 min, and immunoreactive bands visualised using the enhanced chemiluminescence kit (ECL kit, Amersham Pharmacia Biotech, Amersham, UK) according to manufacturers instructions. Membranes were exposed to diographic film for 20-60 min.

Equal protein loading was confirmed by staining the polyacrylamide gel with Safestain (Invitrogen) for 1 h followed by several washes in water, following transfer.

Buffers and reagents

1 M Tris HCl:

Tris base (Sigma)	12 g
water	60 ml

PH to 6.8 and add 100 ml water

Store at 4°C

Lysis buffer:

water	4 ml
0.5 M Tris HCl pH 6.8	1 ml
10% SDS (Life Technologies)	1.6 ml

Sample buffer (2x):	
1 M Tris pH 6.8	1.25 ml
10% SDS	4 ml
20% glycerol (Sigma)	2 ml
water	2.6 ml
0.05% Bromophenol blue	150 µl
DTT (Sigma)	154.2 mg

Transfer buffer (semi-dry):	
Glycine (Sigma)	2.9 g
Tris Base (Sigma)	5.8 g
10% SDS	3.7 ml
Methanol (Sigma)	200 ml
Water	to 1 L

Membrane blocking solution:	
Bovine serum albumin (BSA, Sigma)	500 mg
Dry powdered milk (DPM)	5 g
PBS-T	50 ml

Primary/secondary antibody solution:	
BSA	0.5 g
DPM	1.5 g
PBS-T	50 ml

2.8 Immunohistochemistry

Localisation of antigens in spheroids and solid tumours, was performed using immunohistochemistry. Samples were prepared as follows; 5 µm sections from samples frozen in OCT were cut using a Bright cryostat, and collected on poly-L-lysine coated glass slides. Slides were air dried, fixed in ice-cold acetone for 10 min, and stored at –20°C. Slides were stained using standard immunohistochemistry procedures or kits (see individual chapters for details). Sections were also submitted to standard hematoxylin and eosin staining; sections were placed in hematoxylin

(Sigma) for 30 s, washed, de-stained in acid alcohol for 5 min, followed by immersion in eosin (yellowish eosin, BDH, UK) for 1 min.

After staining, and washing in running water, slides were dehydrated through graded alcohols and finally in xylene. Slides were coverslipped using DPX (Surgipath, UK) mounting media, and viewed under standard light microscopy conditions.

2.9 Clonogenic assay

The effect of prodrug treatment upon cells was assessed using clonogenic assays. These use the colony forming ability of cells to determine a surviving fraction following treatment, expressed relative to vehicle treated controls.

Exponentially growing cells were collected from monolayer culture by trypsinisation and plated at low density. Cells were allowed to adhere for 4-6 h. Prodrugs (including paracetamol) were dissolved in Hanks' balanced salt solution (HBSS) and cells exposed in the 37°C incubator.

Following drug exposure, cells were washed in PBS and grown for approximately 10 days in complete media supplemented with feeder cells (V79 cells exposed to 250 Gy ^{60}Co). Colonies were fixed in 0.5% methylene blue w/v in isomethylated spirit (IMS). Colonies greater than 50 cells were scored.

2.10 Animal studies

Animal experiments were carried out in a specific pathogen free animal unit using female SCID (C.B-17crCru-scid/scid homozygotes) or CBA (CBA/Gy f TO) mice, bred in the Gray Cancer Institute. Procedures were performed with approved protocols in accordance with the UK Animals (Scientific Procedures) Act 1986, and the local ethics committee.

All experiments involving genetically modified cells grown as tumours were carried out following Health and Safety Executive (HSE) guidelines and according to the institutional code of practice (Gray Cancer Institute), using protocols approved by the HSE. Mice bearing genetically modified tumours were handled double-gloved in a class II laminar flow hood, and housed in cages fitted with HEPA filters within internally ventilated racking.

Animals were kept on a 12/12 h day/night cycle with food and water available

ad libitum.

2.11 Tumour implantation

Tumours were implanted subcutaneously (sc) in the flank or back of mice under general anaesthesia (metofane, C-vet Ltd, UK). Tumour measurements were made in 3-dimensions using callipers, and volume was calculated using the following formula: $V=(a*b*c*\pi)/6$.

Chapter 3

Development and characterisation of cells expressing horseradish peroxidase

3.1 Introduction

In order to minimise normal tissue damage but retain efficient and effective tumour cell kill, efforts have been made to target cancer therapy. One approach is to use gene directed enzyme prodrug therapy (GDEPT), a multi-phase strategy to treat solid tumours and graft-versus-host disease. Many GDEPT strategies have been developed, including the horseradish peroxidase/indole-3-acetic acid combination (HRP/IAA, (Greco et al., 2000). This combination has been characterised using transient transfectants in a variety of cells lines and been found to be efficient and selective. The combination is effective under low oxygen tensions (Greco et al., 2001), and shows synergy in combination with ionising radiation both under oxic and hypoxic conditions (Greco et al., 2002c).

However, the system has only been assessed using *in vitro* monolayers. The experiments in the following chapter aimed to produce and characterise cells stably expressing the HRP gene or a control marker gene for use in subsequent studies.

3.2 Materials and methods

Details of general materials and methods can be found in Chapter 2. Methods specific to this chapter are described below.

3.2.1 Cell transfection

FaDu cells were transfected using the method described in section 2.3 with either the pssHRP-puro or pEGFP-puro plasmids (kindly donated by O. Greco, Gray Cancer Institute), where expression of the transgene is under the control of the CMV strong viral promoter. The plasmids had the same backbone, derived from thr pCI-puro plasmid (clontech), with ampicillin and puromycin resistance for selection in prokaryotes and eukaryotes respectively.

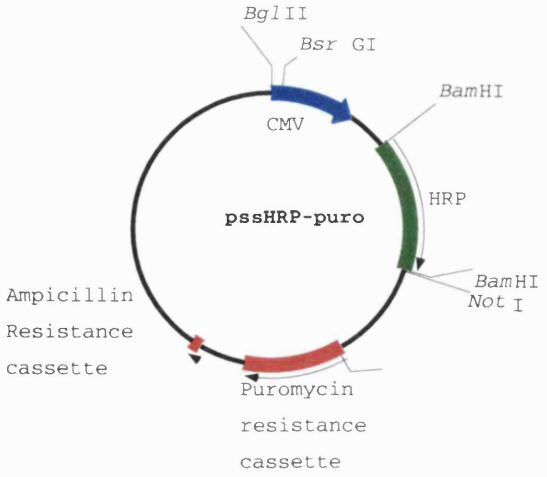


Figure 3.1. pssHRP-puro plasmid used in FaDu cells. pEGFP-puro plasmid was the same with the substitution of the GFP gene for the HRP gene.

3.2.2 Assessment of thiol concentration

Recycling method

The glutathione (GSH) level in cells was measured using a commercially available kit (Cayman Chemical, Ann Arbor, Michigan, USA), following the manufacturers instructions.

Glutathione is a tripeptide (γ-glutamylcycteinylglycine), with a range of functions within plants and animals, one of which is maintenance of the oxidative state of cells.

The recycling assay uses glutathione reductase for the quantification of GSH. Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB) reacts with the sulphydryl group of GSH producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). A mixed disulphide is concomitantly produced, and is reduced by glutathione reductase to recycle the GSH and produce further TNB (see figure 3.1). The rate of TNB production is directly proportional of the recycling reaction, which in turn is directly proportional to the concentration of GSH in the sample.

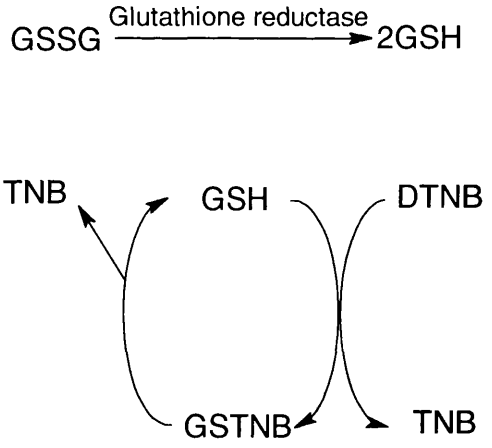


Figure 3.2. Glutathione recycling using Ellman's reagent.

The use of glutathione reductase means that both GSH and GSSG (the disulphide dimer) are measured using this method. To allow distinction between the reduced and oxidised forms of glutathione, samples can be derivatised with 2-vinylpyridine.

$5x10^5$ cells were collected by scraping, and centrifugation. The cell pellet was resuspended and sonicated in phosphate buffer containing 1 mM EDTA. This was centrifuged and the supernatant deproteinated by addition of an equal volume of metaphosphoric acid (MPA, Aldrich, UK), centrifugation, and treatment with 50 µl of 4 M triethanolamine per ml of sample, and stored at -70°C.

Samples were added to a 96 well plate, assay cocktail added and absorbance at 415 nm measured every 5 min for 20 min on a Labtech plate reader.

GSH levels were calculated by producing a curve of absorbance vs time for each standard (i-slope). The gradient of each i-slope was plotted against GSSG concentration to produce a straight line (f-slope). Sample concentrations were calculated using the equation:

[total GSH] or [GSSG] = {(i-slope for the sample)–(y-intercept)}/f-slope*2

Non-recycling method

$2x10^6$ cells were collected. 10^6 were used for the determination of protein thiol and 10^6 for assessment of non-protein thiol.

Non-protein thiol was measured by collection of 10^6 cells from *in vitro* culture. The cell pellet was resuspended by vortexing in 10 ml ice-cold PBS. This solution was centrifuged at 15000 rpm for 5 min. The resulting pellet was vortexed in 1 ml trichloroacetic acid (TCA). The lysate was centrifuged for 15 min at 3000 rpm, and the supernatant decanted into a cuvette, and 0.1 ml DTNB solution added. The absorbance at 412 nm was measured relative to a TCA/DTNB control.

Total thiol was measured in 10^6 cells following a wash in ice-cold PBS. The cells were pelleted, and vortexed in 1 ml lysing solution with 4 ml of 0.5 M phosphate buffer, and 0.1 ml DTNB. The solution was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was removed to a cuvette avoiding the DNA, and absorbance at 412 nm measured relative to a phosphate buffer/DTNB/lysing solution blank.

The molar extinction co-efficient of 1.36×10^4 for a 1 cm pathlength was used.

Buffers and reagents

0.5 M phosphate buffer:

NaH_2PO_4	39 g
Ethylenediaminetetraacetic acid (EDTA)	372 g
dH_2O	to 500 ml

Adjust to pH 7.5 with sodium hydroxide pellets

Trichloroacetic acid (TCA):

TCA	1 g
dH_2O	to 100 ml

DTNB:

DTNB	39.6 mg
0.5 M phosphate buffer	10 ml

Lysing solution:

Sodium hydroxide	2 g
EDTA	372 mg
dH_2O	to 500 ml

3.3 Results

3.3.1 Characterisation of FaDu transfectants

Human nasopharyngeal squamous carcinoma cells (FaDu) were transfected using a receptor-mediated lipofectin method (Hart et al., 1998), with either the HRP or GFP genes, and stable transfectants isolated from puromycin resistant colonies. Cells were analysed for transgene expression, and it was found that 3/25 puromycin resistant colonies retained GFP expression, and 1/8 retained HRP expression. The highest expressing clones were named GFP1 (Figure 3.3) and HRP4D6 (Figure 3.4) and expanded. However, clone FaDu HRP4D6 lost activity of the HRP enzyme over a period of 2 months, and so a subclone named HRP8 was isolated by sparsely seeding HRP4D6 cells, and isolating the resulting colonies. These were used for further work (Figure 3.4).

The expression of transgenes did not affect the growth characteristics of FaDu cells *in vitro* (Figure 3.5).

The percentage of GFP1 cells expressing GFP was assessed using FACS analysis, and found to be approximately 88% (Figure 3.3). The number of HRP8 cells expressing the HRP protein could not be determined by immunocytochemistry due to non-specific binding of the primary antibody. Therefore, the expression of HRP was assessed using the enzyme activity assay.

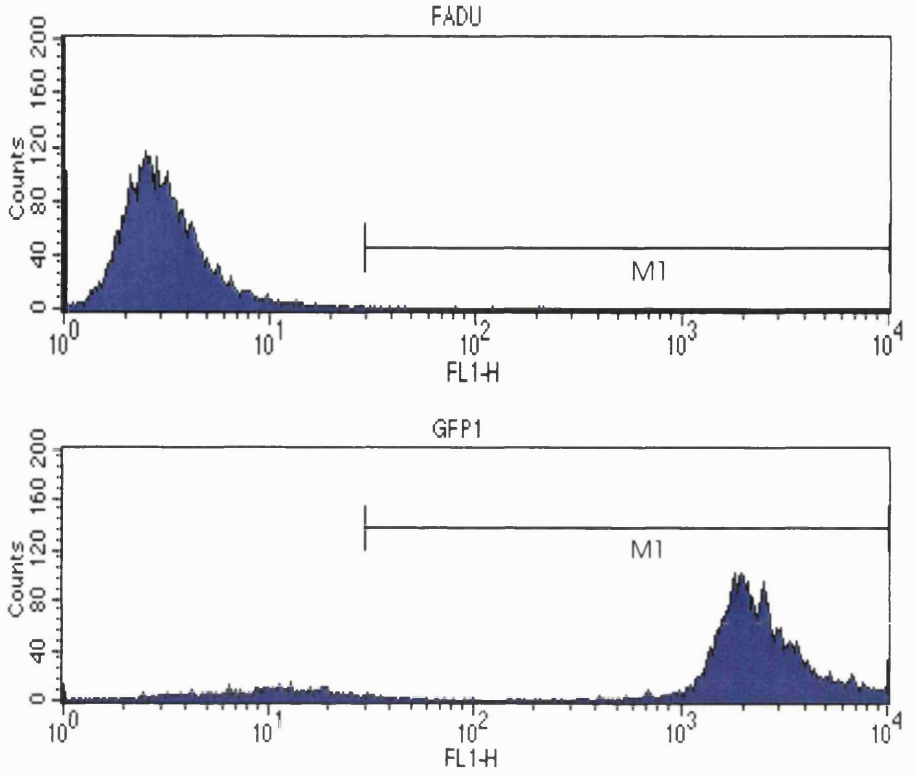


Figure 3.3. FACS results for untransfected FaDu cells (upper panel) and GFP1 clone (lower panel). Cells scored positive are marked using M1.

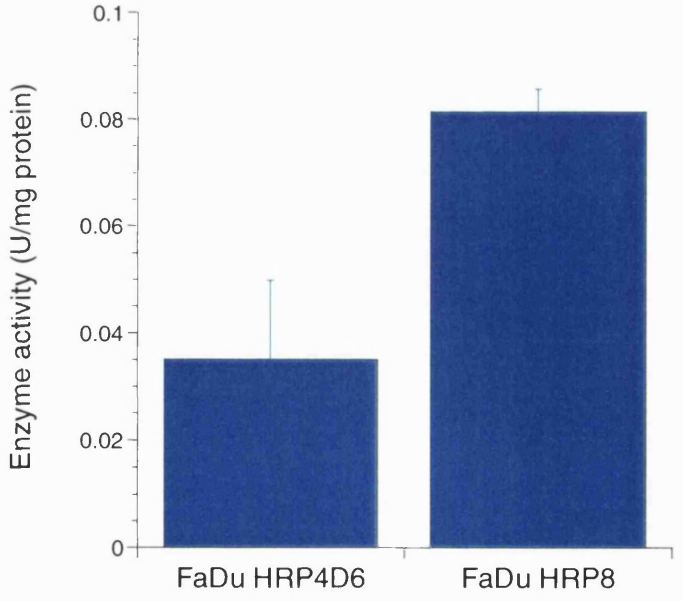


Figure 3.4. HRP activity in FaDu cells stably transfected with the HRP gene. Data are mean ± s.e.m. minimum 3 samples.

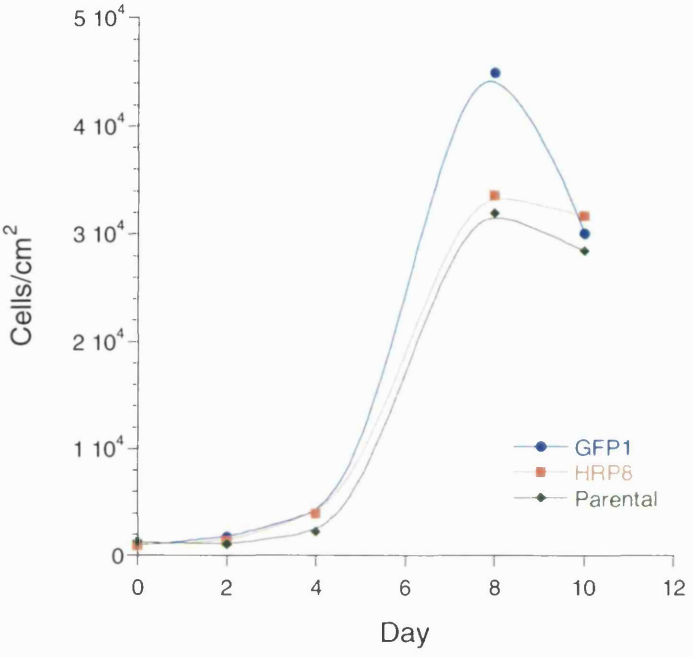


Figure 3.5. Growth of FaDu cells *in vitro*. Data are mean ± s.e.m. 3 experiments.

The thiol concentration of FaDu parental cells and stable transfectants was assessed using two *in vitro* assays, since it has been reported that HRP can oxidise glutathione and this may affect the redox state of the cell. The recycling assay showed that there was a trend towards lower levels of both the oxidised and reduced forms of glutathione in stable transfectants although this did not reach significance (Figure 3.6a). There was no difference between the two stable cell lines.

Using a simple spectrophotometric method (non-recycling), it appeared that there was no difference in thiol levels between parental and transfected cells (Figure 3.6b), and the HRP8 clone had slightly greater levels of glutathione than the parental or GFP cells. The levels of total thiol (protein and non-protein) did not differ between cell lines. Parental cells were treated with paracetamol as a positive control as this will decrease GSH levels. This resulted in a tendency to decreased thiol levels, although not to a significant degree.

Comparison of the two methods showed that the level of thiol estimated by the recycling assay in parental cells was equivalent to that obtained from the spectrophotometric assay when two-fold the number of cells was used. The recycling assay gave much lower thiol concentrations for the GFP1 and HRP8 clones than the

non-recycling method.

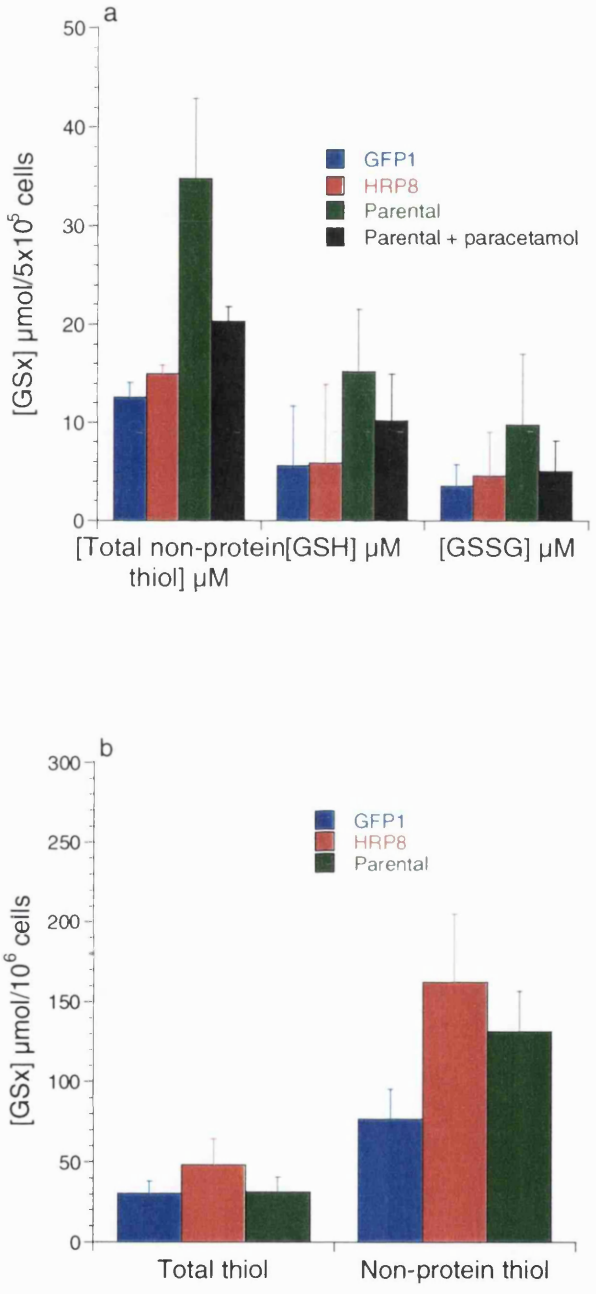


Figure 3.6. Thiol levels in FaDu cells. a) Assessed using Cayman Chemical recycling assay kit. Data are mean ± s.e.m. triplicate samples, 2 independent experiments. b) Assayed using spectrophotometric assay. Data are mean ± s.e.m. duplicate samples, 3 independent experiments.

3.3.2 Efficacy of HRP/indole GDEPT

The sensitivity of FaDu cells to treatment with the indole prodrugs dissolved in 10% ethanol/Hanks' Balanced Salt Solution (HBSS) was assessed using clonogenic assays. Treatment with IAA for 4 or 24 h resulted in decreased clonogenic survival of HRP8 cells (Figure 3.7). 24 h exposure led to a steeper curve, and increased cell kill. There was a shoulder to the curve at approximately 1 mM IAA concentrations.

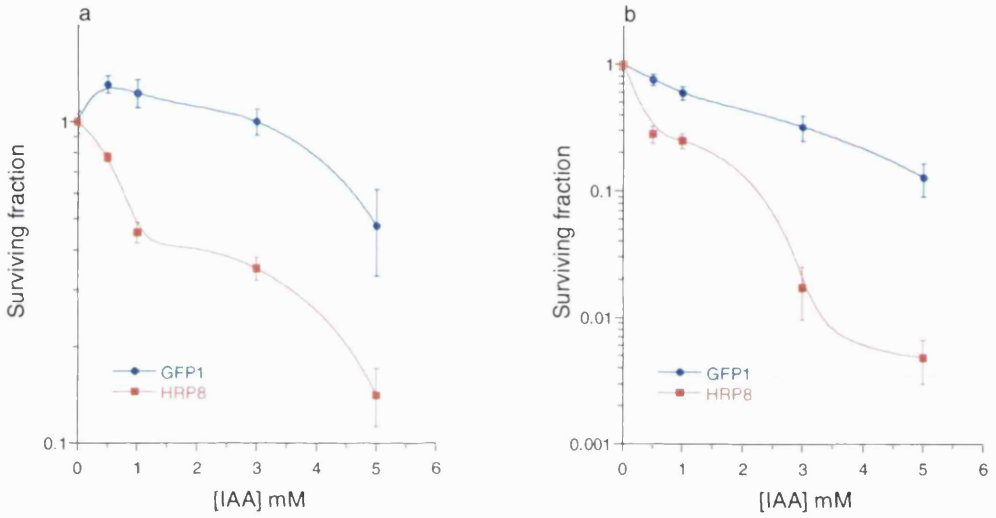


Figure 3.7. Survival of GFP1 and HRP8 cells following a) 4 h, or b) 24 h exposure to IAA. Data are mean ± s.e.m. 3 experiments, triplicate samples.

Comparison of ethanol and dimethylsulphoxide (DMSO) as solvents (10 and 5% final concentration respectively) indicated equivalent levels of cell kill in the HRP8 cell line, although there was some indication of reduced cell kill for HRP8 cells with low IAA concentrations with DMSO as a solvent. Survival of GFP1 controls did not differ between the two vehicles (figure 3.8).

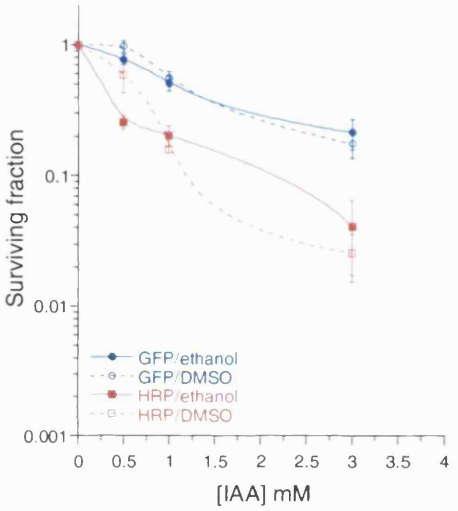


Figure 3.8. Comparison of IAA cytotoxicity in cell exposed to IAA for 24 h using ethanol or DMSO as the solvent. Data are mean ± s.e.m. 3 experiments, triplicate samples.

In addition to the prototypical IAA prodrug, a methylated form of IAA was also utilised. The 1methylindole-3-acetic acid (1Me-IAA) compound also decreased the survival of HRP8 cells following 4 or 24 h exposure periods (Figure 3.9). The level of cell kill seen with 1Me-IAA after 24 h was greater than that seen at equivalent concentrations of IAA. There was a plateau phase between 1 and 2 mM 1Me-IAA following 24 h exposure, after which, survival sharply decreased with approximately 99.9% cell kill at 5 mM concentrations. However, HRP selectivity was lost using 1Me-IAA, thereby reducing the potential therapeutic window. 4 h exposure to 5 mM 1Me-IAA decreased GFP1 survival to levels seen in HRP8 cells. After 24 h, 1Me-IAA reduced GFP1 survival at concentrations greater than 2 mM, with a 2-log decrease in clonogenicity at 5 mM.

The halogenated derivative of IAA, 5bromindole-3-acetic acid (5Br-IAA), was also assessed in this system, and found to be effective at reducing HRP8 clonogenicity only following the longer 24 h exposure period but not after 4 h (Figure 3.10). After 24 h treatment, 5Br-IAA was capable of reducing the survival of HRP8 cells by over 2-logs at just 1 mM. There was no apparent plateau, although GFP1 survival was starting to decrease at this concentration.

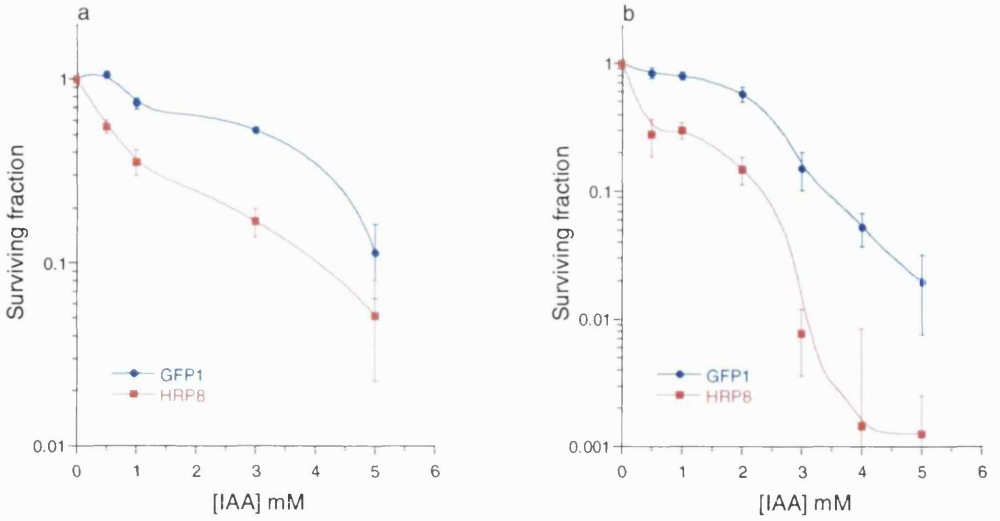


Figure 3.9. Clonogenic survival of FaDu GFP1 and HRP8 cells following a) 4 h or b) 24 h exposure to 1Me-IAA. Data are mean ± s.e.m. 3 experiments, triplicate samples.

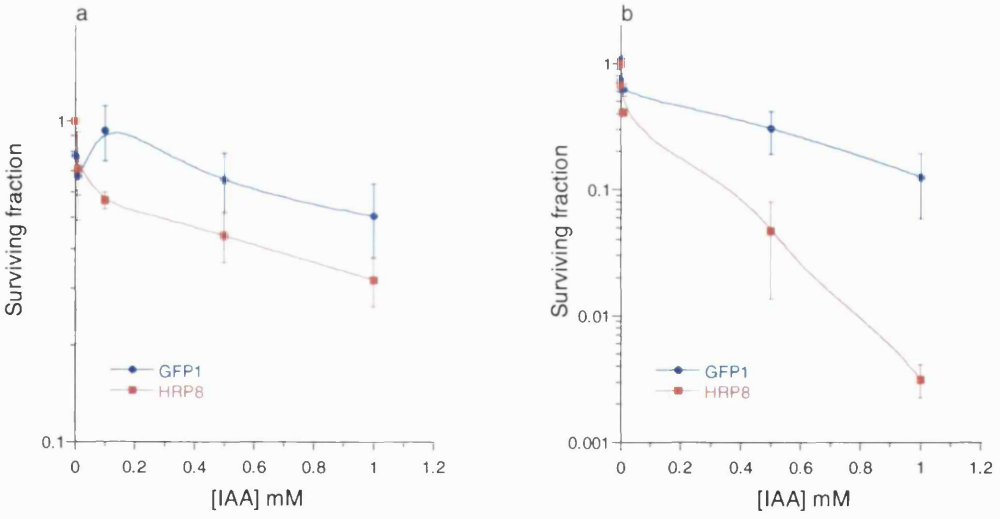


Figure 3.10. Clonogenic survival of GFP1 and HRP8 cells following 4 h or 24 h exposure to 5Br-IAA. Data are mean ± s.e.m. 3 experiments, triplicate samples.

3.4 Discussion

This chapter describes the development and characterisation of FaDu clones stably expressing HRP or GFP for use in subsequent chapters. FaDu cells are a human head and neck cancer cell line of squamous carcinoma origin. In common with a large number of human cancers the cell line does not have functional p53 activity, since it

carries a p53 nonsense mutation at codon 248 (Reiss et al., 1992). The line does retain sensitivity to methotrexate (McGuire et al., 1999). It is widely used in studies using radiation, since it readily forms tumours with a hypoxic fraction quantifiable by oxygen electrodes (Baumann et al., 2001; Urano et al., 2002).

Expression of HRP or GFP was not detected in all colonies resistant to puromycin. This may be due to a variety of causes, such as insertional inactivation/disruption of the transgene or its promoter due to plasmid ring opening resulting in either production of an inactive protein, or no gene expression. The presence of the HRP or GFP protein is not believed to confer a growth advantage on cells, and therefore cells may spontaneously silence gene expression. In the case of HRP, transgene expression may in fact be detrimental (Harman et al., 1986). However, from the growth curves it does not appear that in the HRP8 and GFP1 clones, expression of transgenes is detrimental to growth *in vitro*, although this may be the case only in these particular clones.

Methylation of transgenes and histone proteins has been shown to be important in transgene silencing (Broday et al., 1999; Muller et al., 2000; Pannell et al., 2000; Yan et al., 2003), which is sometimes associated with increased chromatin condensation. Decreased histone acetylation is observed in silenced transgenes (Yan et al., 2003). Insertion of transgenes in proximity to heterochromatin can lead to decreased expression (Saveliev et al., 2003), presumably due to the inaccessibility of the DNA. Insertion of elements with a dominant chromatin opening function can, at least in part, overcome the problems of transgene silencing via a methylation-associated pathway (Antoniou et al., 2003).

The activity of HRP in the HRP8 cell line was approximately the same as that in FaDu cells transiently transfected with HRP (although a different plasmid was used, the promoter and gene sequence were the same (Greco et al., 2000)). However, expression within transient transfectants was restricted to 10-14% of cells. In stable transfectants it was not possible to determine the number of cells expressing the HRP enzyme due to problems with the HRP antibodies. However, it was likely to be considerably greater than 15%, since clones were derived from a single colony. This means that the level of activity per cell was probably less than in transient transfectants.

HRP has previously been shown to oxidise glutathione (GSH) (Harman et al., 1986). GSH is an antioxidant and co-factor for antioxidant enzymes. Its levels are highest in the liver, where it is primarily used to increase the solubility and excretion of lipophilic compounds. In healthy cells, the oxidised form rarely exceeds 10% of the total glutathione (Kosower & Kosower, 1978). GSH levels are controlled homeostatically and self adjusted with respect to synthesis (by GSH synthetase), recycling from GSSG (by GSH reductase), and its utilisation. Since glutathione levels could affect growth and response to both drug and radiation treatment (Biaglow et al., 1989), the thiol levels of stable transfectants were assessed.

Two different *in vitro* assays were employed, essentially showing that there was no difference in GSH levels between transfected and untransfected cells. The recycling method indicated that although stable transfectants had lower levels of total thiol (non-protein) than parental FaDu cells, this difference was not statistically significant (figure 3.5).

Using a simpler method that does not discriminate between the oxidised and reduced forms of GSH, there was no difference between the parental cells and clones. Since a difference in GSH levels was expected, this indicates that either intracellularly produced HRP has no more effect on thiol levels than the marker GFP in these cells, or that the HRP8 clones is able to deal with the oxidation of GSH, and can by some mechanism redress the balance, since GSSG levels were not particularly elevated, so it is unlikely that any mechanism is simply an increased production of GSH.

Exposure of GFP1 and HRP8 cells to IAA resulted in decreased survival of HRP8 cells. The level of cell kill in HRP8 cells showed a pronounced plateau following 24 h exposure. This was also seen in transient transfectants, although to a lesser degree (Greco et al., 2001). In addition, the level of cell kill at high prodrug concentrations was greater in transient transfectants. A possible explanation for these discrepancies is the level of enzyme activity per cell. Transient transfectants are likely to have a much greater level of HRP activity per cell (possibly due to the presence of multiple plasmids, and/or lack of insertional disruptions), and it may be this, rather than the total HRP activity of a population, which is important for cytotoxicity. The cytotoxic product formed by HRP from IAA is freely diffusible and fairly long-lived under oxic conditions. The bystander effect may be able to compensate for lower cell expression numbers.

The effect of vehicle used to dissolve indoles was studied. In previous studies, all indoles were dissolved in ethanol, prior to dilution in salt solution (Greco et al., 2002a; Greco et al., 2000; Greco et al., 2001; Greco et al., 2002c). This may not always be the most appropriate solvent for use *in vivo*, and so DMSO was investigated as an alternative. No difference in the 24 h survival curve was observed if DMSO was used in place of ethanol, so providing an alternative vehicle for further studies.

The exact cytotoxic mechanism of action of IAA metabolites is yet to be fully elucidated. However, the results of experiments with stable transfectants support earlier work that the HRP enzyme is necessary for IAA induced cell death.

Both 1Me-IAA and 5Br-IAA were cytotoxic to HRP8 cells following 24 h exposure periods. 1Me-IAA in common with IAA, displayed the plateau at 1 mM concentrations, although (at high concentrations) greater levels of cell kill were seen. This was accompanied by a decrease in the survival of GFP1 cells, and quite considerable levels of GFP1 cell kill following 24 h exposure. This is in contrast to the results in transient transfectants (Greco et al., 2001). It is unclear why 1Me-IAA should show such cytotoxicity to GFP1 cells, and this may mar the suitability of 1Me-IAA as a prodrug. For any therapeutic strategy it is ideal to have compounds that will not have any effect on non-target tissue. This is very hard to achieve, but the greater the therapeutic window, the lower the potential for side effects and normal tissue damage. The results here indicate that 1Me-IAA may be cytotoxic to, or be converted to a cytotoxin by, normal tissues, at concentrations not much greater than those needed to achieve a therapeutic effect in HRP transfectants.

In agreement with results obtained from transient transfectants, 5Br-IAA was cytotoxic to HRP expressing cells under normoxia only following long exposure periods (Greco et al., 2001). After 4 h exposure there was no decrease in the survival of either cell line. The cytotoxicity of 5Br-IAA was apparent at lower concentrations than either IAA or 1Me-IAA, which is beneficial. Administration of large quantitites of drug could increase the likelihood of normal tissue toxicity. Also, concentrations of prodrug in tumour tissue may be lower than normal tissue due to disturbances in blood flow, and systemic drug administration is preferable rather than direct administration to the tumour or its blood supply.

The amount of cell kill was lower than that seen with standard cancer chemotherapeutic agents (Hall, 2000). However, a direct comparison of the HRP/IAA

system with the herpes simplex virus-thymidine kinase/gancyclovir combination showed that the HRP/IAA combination was superior (Greco et al., 2000).

The results show that stable HRP expressing cells can be produced which retain their sensitivity to indole prodrugs. Stable clones can be used both *in vitro* and *in vivo* to assess responses to indoles in more complex model systems.

Chapter 4

Horseradish peroxidase-directed gene therapy in an *in vitro* 3-dimensional system

4.1 Introduction

Cell monolayers are an experimental model, which is easy to use, and can be manipulated to expose cells to a variety of conditions. However, the clinical situation will never be as simple as a monolayer. Clinical tumours contain a variety of cell types, have cells with different growth rates and at different stages in the cell cycle, under different stresses and signalling factors, in addition to a range of environmental conditions.

A model which is one stage more complex than monolayers is multicellular spheroids. Tumour cells can form a 3-dimensional *in vitro* model, which has been described by a variety of groups to mimic *in vivo* conditions (for review, see (Sutherland, 1988).

Spheroids are characterised by a proliferating outer rim in contact with the growth media, and an oxygen diffusion limited hypoxic layer surrounding a necrotic core. Towards the centre of the spheroid, depending upon its size, there will be alterations in a number of factors, such as pH, glucose concentration, and the concentration of various waste products. Solid tumours are known to have regions of chronic hypoxia at distance from blood vessels, in addition to perfusion limited, acute hypoxia. Spheroids may be a fairly accurate representation of avascular micro-metastases (Knuchel et al., 1988), as well as a model for regions of chronic hypoxia in solid tumour masses.

Spheroids formed from both hepatocytes and tumour cells show altered gene and protein expression compared with the same cells grown as monolayers (Narayanan et al., 2002; Poland et al., 2002). In spheroids derived from a human adenocarcinoma of the colon (HT29), calreticulin proteins were over-expressed compared with monolayers, whilst proteins involved in cell growth, such as Rho, 14-3-3 β, 14-3-3 η and tubulin showed higher levels in monolayer culture. In spheroids derived from porcine hepatocytes, gene expression profiles correlated with

morphological and functional changes associated with differentiation in the maturing spheroids.

Spheroids grown from tumour cells contain subpopulations of cells that are radio- and chemo-resistant (Yuhas et al., 1978). This makes spheroids a good *in vitro* model for evaluating novel therapeutic strategies.

In this chapter a spheroid model has been developed for use with the HRP/IAA combination as an intermediate system between monolayers and *in vivo* tumours.

4.2 Materials and methods

General materials and methods are described in chapter 2. Below are detailed those methods specific to this chapter.

4.2.1 Cell culture

FaDu cells stably expressing the HRP and GFP genes were used as described in Chapter 2, and maintained in monolayer culture prior to use as spheroids.

4.2.2 Spheroid development

Spheroids can be grown using spinner culture methods, or by preventing attachment to the plastic-ware surface. The most common method of attachment inhibition is to coat the bottom of flasks or plates with agar. However, for studies where cells are incubated with a drug, it is important that the culture vessel walls be impervious to be certain of the drug concentration in the media. In this study spheroids were formed through attachment inhibition due to rotation of the culture plate.

Exponentially growing FaDu cells were trypsinised and plated at 10^5/ml in CO_2 independent media (Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies), with 3 ml of cells per well of a 6 well plate. Plates were incubated in a 37°C warm room on a rotating platform (R100, Rotatest rotator) at 75 rpm. Media was replaced on the third day, and subsequently every 2 days. After 3 days visible spheroids had formed, and were measured using a calibrated eyepiece graticule (Graticules Ltd, Kent, UK).

4.2.3 Enzyme activity in spheroids

To assess the level of HRP activity spheroids were washed, trypsinised and syringed to obtain a single cell suspension. Cells were pelleted and resuspended in 0.5% hexadecyltrimethylammonium bromide, and processed as described in 2.5.

4.2.4 Immunohistochemistry of spheroids

Spheroids of varying sizes were exposed to 100 μM pimonidazole for 2 h. Spheroids were washed in PBS, and frozen in OCT. Sections were cut and fixed as described in 2.8.

Pimonidazole staining was carried out using an UltraVision Mouse Tissue Detection System (Stratech Scientific, UK), following the manufacturers instructions, and an anti-pimonidazole IGg1 antibody (Natural Pharmacia Int Inc, USA) diluted 1:100 in TBS with protein block (Dako, UK). Staining was identified using the diaminobenzidine (DAB) chromophore, and slides were counterstained with haematoxylin.

Haematoxylin and eosin stained sections were prepared as described in 2.8.

4.2.5 Spheroid clonogenic assay

Intact spheroids were exposed to prodrugs dissolved in HBSS on the rotating platform for 4 or 24 h. Following exposure, spheroids were washed, trypsinised and syringed to give a single cell suspension, counted, and appropriate numbers of viable cells (trypan blue excluding) plated on 6 cm dishes. Feeder cell supplemented media was added and colonies allowed to form as before.

4.2.6 Viable cell counting

Spheroids were exposed to prodrug as for clonogenic assays (4.2.5). Following exposure, the contents of the well was transferred to a universal, centrifuged at 1000 rpm for 5 min, the pellet washed, centrifuged again, and then subjected to trypsinisation to give a single cell suspension. Viable cells were counted as a percentage of total cells using trypan blue exclusion.

4.3 Results

FaDu cells readily formed spheroids when grown on a continuously rotating platform. Spheroids reached 300 µm diameter after 3 days in culture, and 700 µm after 7-10 days. Spheroids continued to increase in size to approximately 1 mm, at which point growth dramatically slowed, and the necrotic core was very large. Although parental cells appeared to initially form larger spheroids, after 6 days in culture there was little difference between the groups (figure 4.1)

When frozen sections were stained with haematoxylin and eosin, large spheroids showed regions of loosely packed necrotic cells in the centre of the spheroid. In some spheroids, cells on the outer rim appeared to take on a squamous morphology. In addition to the necrosis seen on frozen sections, pimonidazole staining of spheroids for hypoxia, showed an increase in staining, and so a concomitant increase in hypoxia, towards the centre of the spheroid (figure 4.2 a). The staining was evident approximately 100-150 µm from the outer rim. Spheroids kept under anoxia for 2 h prior to pimonidazole treatment stained uniformly brown, as expected (figure 4.2 b). Pimonidazole staining of 300 µm diameter spheroids was negative (results not shown).

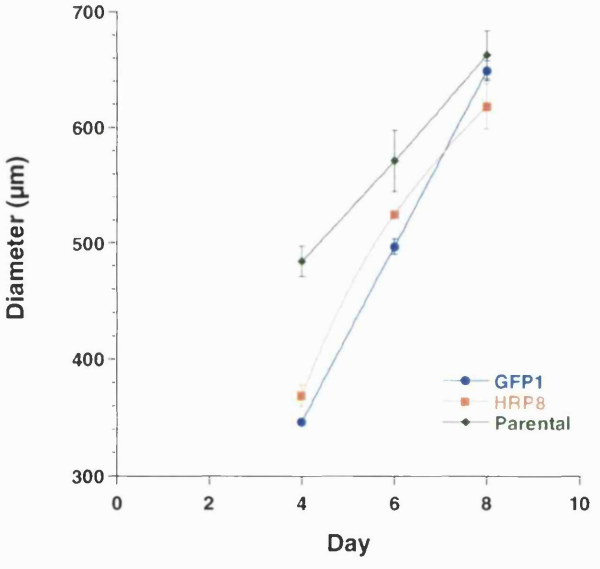


Figure 4.1. Growth of FaDu spheroids on a rotating platform. Data are mean ± s.e.m. 3 experiments, 10 spheroids measured per day.

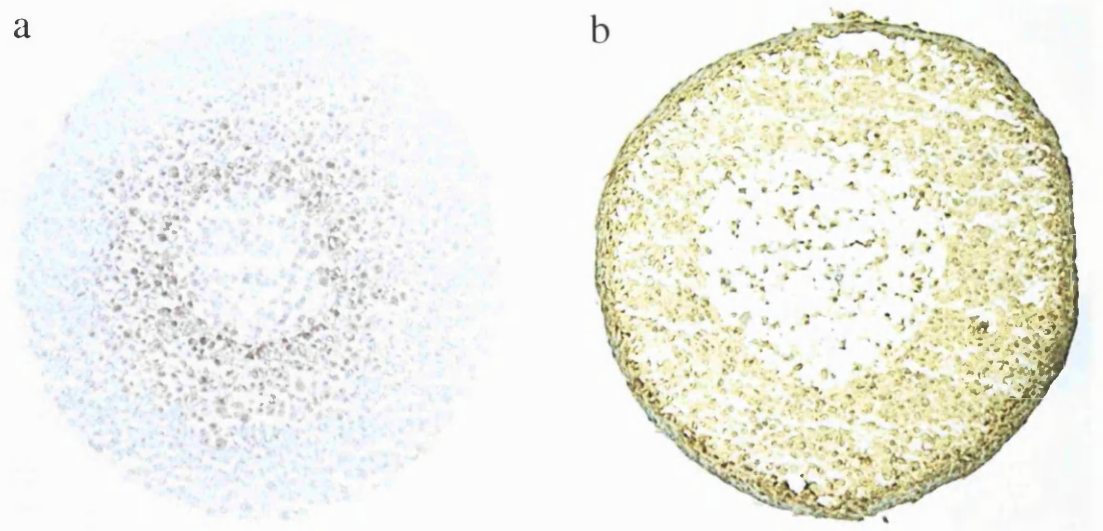


Figure 4.2. 600 μm diameter spheroids treated with Pimonidazole under a) normoxia, or b) anoxia, followed by processing for immunohistochemistry, and staining with the DAB chromophore. Regions of hypoxia are stained brown.

Spheroids were exposed to prodrugs for 4 or 24 h, followed by disaggregation and plating for clonogenic assay. Spheroids of either 300 or 700 μm diameter were used. Spheroids initiated using a mixture of cells, of which 50% expressed HRP and 50% GFP (mosaic spheroids), were also used, in order to study the bystander effect.

Exposure for 4 or 24 h of 300 μm diameter spheroids to IAA resulted in a decrease in the survival of HRP8 transfectants, with virtually no effect on the survival of GFP1 cells, even at high IAA concentrations (figure 4.3 a, b), which in monolayers had resulted in a modest decrease in GFP clonogenicity (chapter 3). Exposure for 4 h of HRP spheroids resulted in greater cell kill than seen in monolayers (surviving fraction 0.02 ± 0.01 in spheroids vs 0.1 ± 0.03 in monolayers at 5 mM IAA). A 4 h exposure of mosaic spheroids to low concentrations of IAA reduced clonogenicity of cells to a similar degree as HRP spheroids. However, at higher prodrug concentrations (above 1 mM), levels of survival became intermediate between GFP and HRP spheroids. Mosaic spheroids showed equivalent levels of cell kill after 24 h exposure except at 5 mM IAA, where clonogenicity of mosaic spheroids was a log greater than that seen in HRP spheroids alone.

Large 700 μm diameter HRP spheroids displayed surviving fractions equivalent to those seen in monolayers, with no effect on GFP controls. In particular, the initial decrease in clonogenicity was very steep (figure 4.3 c, d).

24 h exposure of HRP spheroids to IAA resulted in levels of cell kill

approximately the same as those seen in monolayers (chapter 3) and in smaller spheroids. The plateau between 1 and 3 mM was again evident in the survival curve.

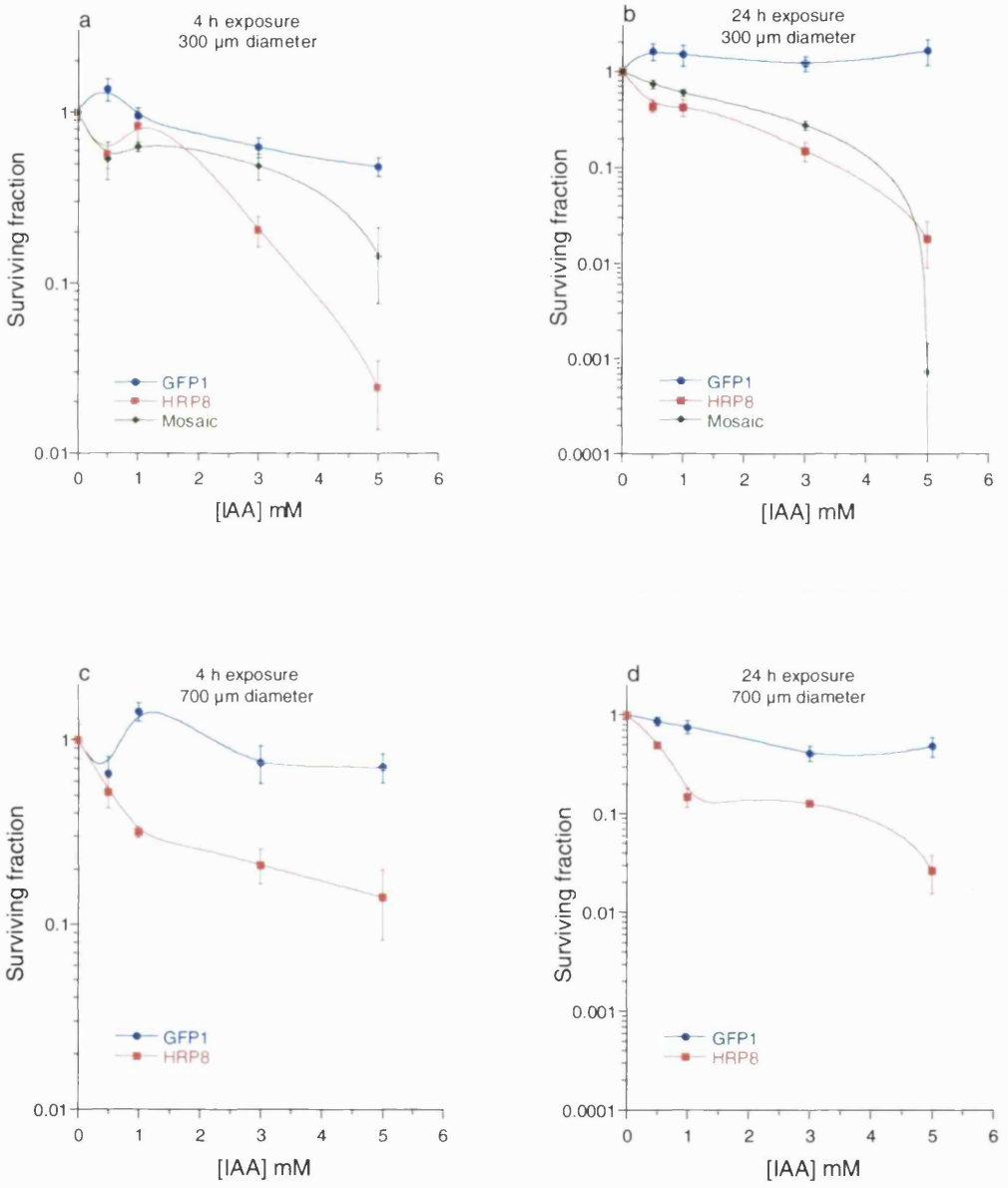


Figure 4.3. Clonogenic survival of spheroid cells following exposure to IAA. a) and b), 300 µm diameter; c) and d), 700 µm diameter. a) and c) 4 h exposure; b) and d) 24 h exposure. Data are mean ± s.e.m. minimum 3 experiments, triplicate samples.

Two IAA derivatives, the methylated and halogenated compounds, were also tested in the spheroid model, as they showed promise in monolayer studies. Exposure of 300 µm diameter spheroids to 1Me-IAA lead to a decrease in the clonogenic

survival of HRP8 cells to approximately 3% at 5 mM concentrations (figure 4.4 a). Mosaic spheroids displayed a similar dose response curve, but with a greater loss of clonogenicity at 5 mM (surviving fraction 0.00035 ± 0.0003, figure 4.4 a). After a 24 h exposure, HRP8 cells had a decreased survival compared with GFP transfectants, with no obvious plateau phase (figure 4.4 c).

Larger spheroids exposed to 1Me-IAA showed decreased survival of HRP8 compared to GFP1 cells (figure 4.4 c and d). This was equivalent to that seen in smaller spheroids, however less than in monolayers. With both 4 and 24 h exposure of 300 and 700 μm diameter spheroids, the survival of GFP transfectants was greater than that seen in monolayer culture after equivalent doses and exposure times (figure 3.7).

In the small 300 μm diameter spheroids, 4 h 5Br-IAA exposure had very little effect on HRP or GFP cell survival (figure 4.5 a). However, in the 700 μm diameter spheroids, HRP8 cell survival was reduced by 50% at 0.1 mM, and by 1 log at 1 mM, with little effect on GFP1. In both 300 and 700 μm diameter spheroids, 24 h exposure to this halogenated indole resulted in a maximum of 1 log cell kill in HRP8 cells, which was markedly less than that seen in monolayers.

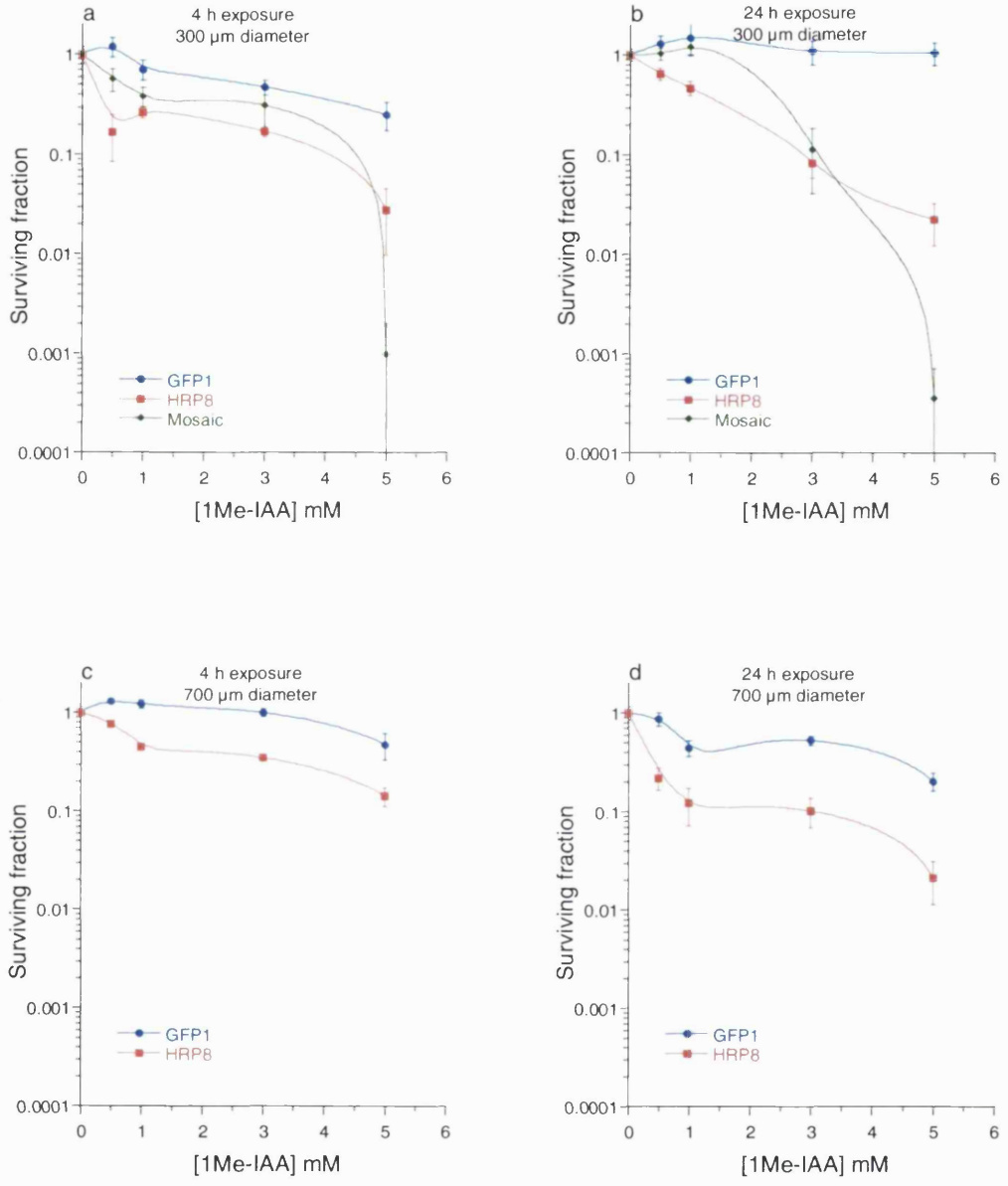


Figure 4.4. Clonogenic survival of cells derived from spheroids treated with 1Me-IAA. a) and b) 300 µm diameter; c) and d) 700 µm diameter. a) and c) 4 h exposure; b) and d) 24 h exposure. Data are mean ± s.e.m. minimum 3 experiments, triplicate samples.

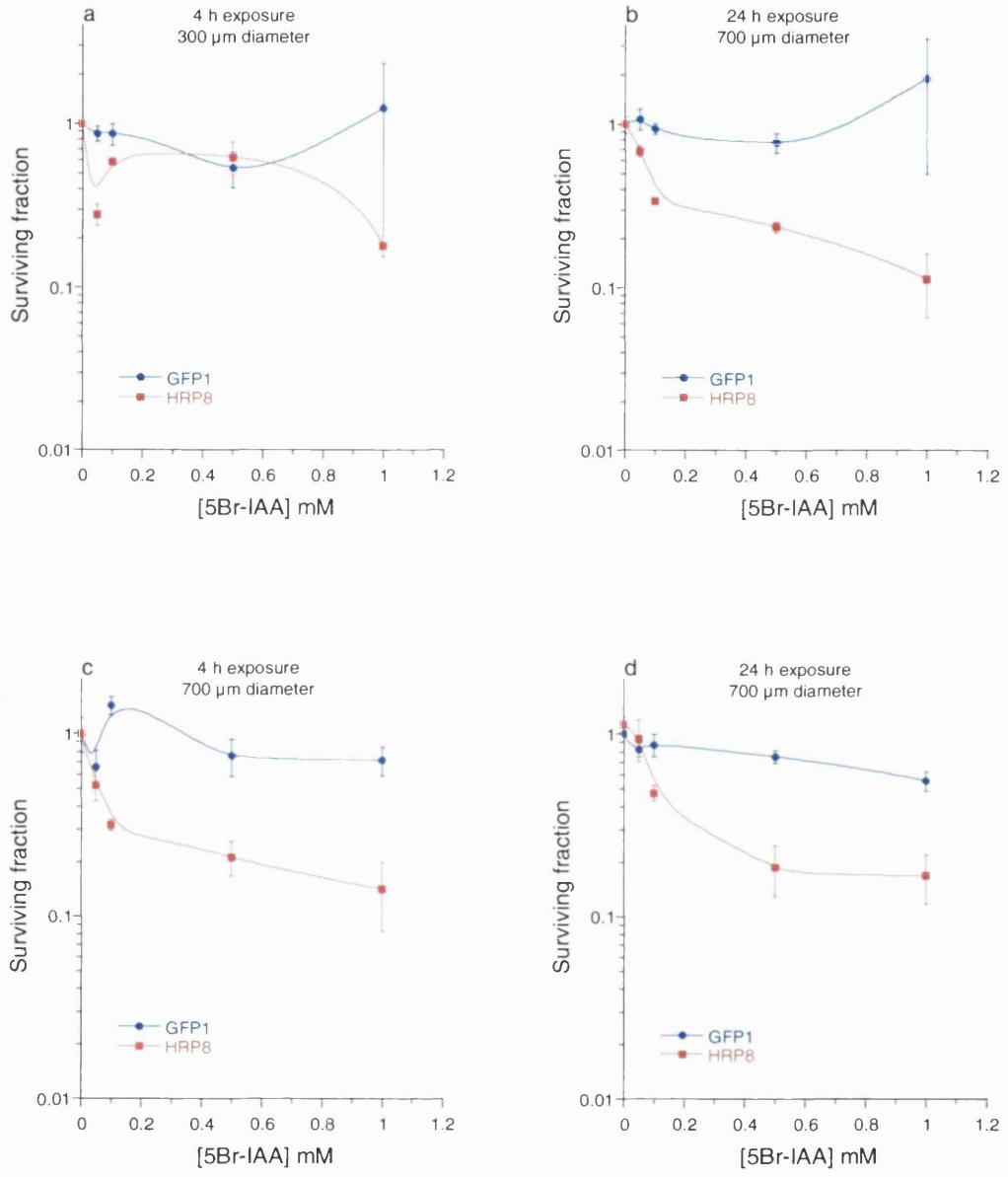


Figure 4.5. Clonogenic survival of cells derived from spheroids treated with 5Br-IAA. a) and b) 300 µm diameter; c) and d) 700 µm diameter. a) and c) 4 h exposure; b) and d) 24 h exposure. Data are mean ± s.e.m. minimum 3 experiments, triplicate samples.

Microscopic analysis of drug-treated spheroids showed significant fragmentation. When cells were exposed to prodrugs for 4 h, disintegration of spheroid structure and loss of cells could be seen under phase contrast microscopy. In particular, 1Me-IAA treated HRP8 spheroids had irregular shapes, and large numbers of free cells within the media. Spheroids composed of GFP1 cells showed few, if any,

morphological changes (figure 4.6).

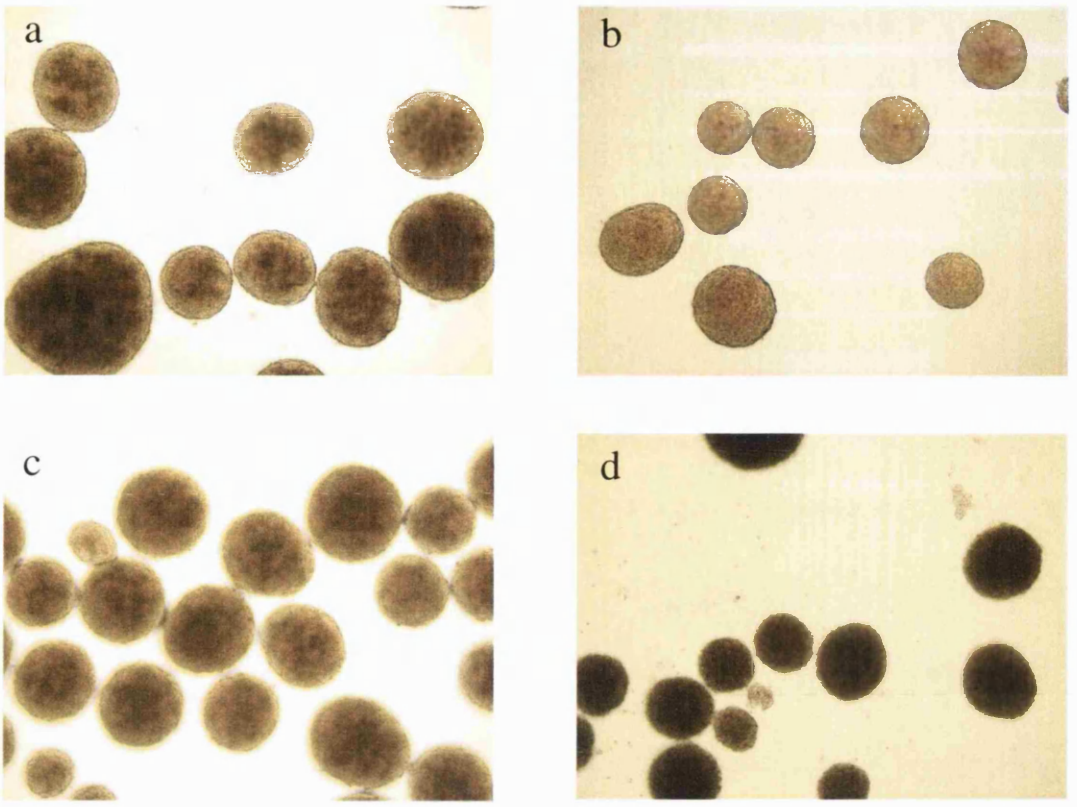


Figure 4.6. Phase contrast images of spheroids. a) GFP1 b) HRP8 spheroids exposed to HBSS for 4 h c) GFP1 spheroids exposed to 5 mM IAA for 4 h. d) HRP8 spheroids exposed to 5 mM IAA for 4h.

Intact spheroids and free cells were collected to look at immediate cell kill in 300 μm diameter spheroids exposed to IAA for 4 h. This showed that for all concentrations of IAA survival of HRP8 cells decreased, whilst survival of GFP1 cells decreased only at 5 mM concentrations (figure 4.7). In particular, at 5 mM IAA concentrations, less than 10% of HRP8 cells collected excluded trypan blue.

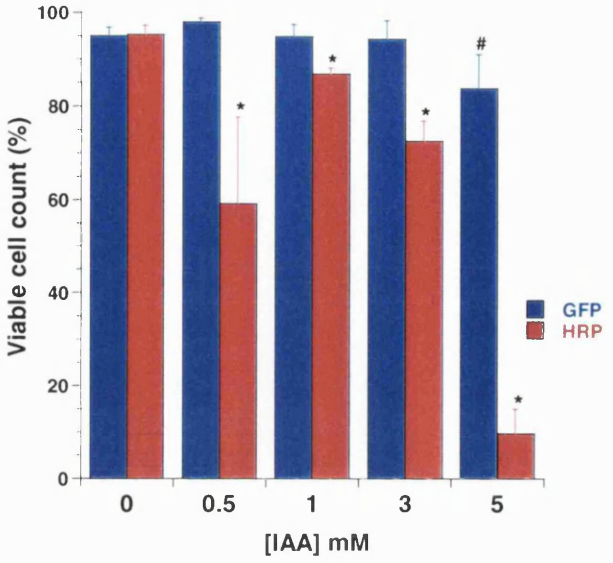


Figure 4.7. Survival of cells obtained from 300 µm diameter spheroids exposed to IAA for 4 h. Data show viable cells as a percentage of the total cell count, mean of 3 experiments ± s.e.m. # $p < 0.01$; * $p < 0.001$ compared with vehicle treated controls.

4.4 Discussion

The growth of cells on a rotating platform prevents adhesion to the culture flask surface, resulting in the formation of multicellular spheroids, which subsequently grow in three dimensions. FaDu spheroids retained expression of transgenes, and were amenable to treatment with indole prodrugs. Consistent with reports of spheroids derived from a variety of other cell lines (Buffa et al., 2001; Olive, 1995; Raleigh et al., 1987), FaDu spheroids developed a hypoxic centre and subsequently a necrotic core when they reached approximately 500-600 µm. This has been reported to be accompanied by alterations in the cell cycle distribution of cells, increases in the levels of carbon dioxide at the core, and also the concentration of other waste products, such as lactic acid. This makes spheroids a more accurate model for the *in vivo* tumour situation than standard monolayer cultures, and in particular of avascular micro-metastases (Knuchel et al., 1988).

Reductions in HRP8 cell survival for small and large spheroids exposed to IAA were approximately equivalent for equal exposure periods. This indicates that the size of the spheroid does not detrimentally affect the HRP/IAA system. IAA has been

shown to be active in combination with HRP under anoxic conditions (Greco et al., 2001). However, in large spheroids, hypoxia will be associated with altered pH and concentrations of waste products, which may affect cellular behaviour. It may be that, in the 700 μm diameter spheroids, only the outer proliferating cells are metabolising the conversion of IAA to the cytotoxin. In that case, the level of cell kill indicates that the active metabolite is capable of diffusing through the spheroid and killing quiescent cells. This is consistent with media transfer experiments showing that the bystander compound is freely diffusible.

Spheroids exposed to the methylated indole, 1Me-IAA, showed a decrease in cell survival after 4 and 24 h exposure periods, in both small and large spheroids. The level of cell kill was the same as that seen in monolayers (chapter 3). Interestingly, exposure of GFP1 spheroids to 1Me-IAA did not result in significant levels of cell kill, unlike the results seen in monolayers. It is not clear why this should be the case.

The 5Br-IAA compound has a different profile of toxicity when comparing monolayers with spheroids. It was previously reported that in monolayers transiently expressing HRP, 5Br-IAA caused a marked decrease in cell kill after a 2 h incubation period, but only when cells were exposed under anoxia (Greco et al., 2001). This suggested the involvement of a short-lived toxic radical species produced under anoxia. In the current study, monolayers (chapter 3) and small spheroids showed no decrease, whereas large 700 μm diameter spheroids displayed a significant decrease in cell survival following 4 h incubation (figure 4.5 c). It is possible that this is due to the activation of 5Br-IAA in hypoxic regions, and subsequent bystander cell killing. It has been hypothesised that under oxic conditions methylene-2-oxindole (MOI) is involved in the cytotoxicity of IAA (Folkes & Wardman, 2001). However, under anoxia, MOI is not formed, and it is likely that an, as yet, undetermined, toxic metabolite is involved. It is possible that an intermediate radical is able to abstract bromine from the aromatic ring to give an aryl radical.

The clonogenic assays are likely to underestimate the amount of cell kill in the spheroid model, since only whole spheroids are collected, and only viable cells plated. As can be seen from figure 4.7, the viable cell number decreases with increased IAA concentrations. Since only viable cells are counted for plating in the clonogenic experiments, it is only secondary cell kill that is measured. The loss of cells due to

spheroid fragmentation was evident from microscopic examination.

Any drug which targets hypoxic regions will need to be able to diffuse to cells distant from blood vessels in order to reach the target area. Diffusion of prodrugs was not measured directly in the spheroid model. However, if 5Br-IAA is, as predicted, activated under hypoxia, then 4 h incubation was sufficient for the drug to reach the hypoxic fraction within 700 μm diameter spheroids. This indicates that the indole prodrugs would be able to reach cells distant from functional blood vessels.

The cell kill seen after IAA treatment for 24 h in mosaic spheroids, mirrored that seen in 100% HRP8 spheroids, except at 5 mM IAA, where increased levels were seen. This indicates that at this time point, the bystander effect can be effective at targeting HRP^- cells. The increased cell kill at 5 mM may be a result of spheroids remaining intact, and subsequent collection of a greater number of affected cells, rather than the loss of these cells to the media.

After 4 h exposure however, mosaic spheroids showed intermediate levels of cell kill. 2 h incubation of IAA with HRP8 cells in monolayer culture followed by transfer of media to HRP^- cells, resulted in level of cell kill equivalent to HRP^+ cells with IAA, except at concentrations above 3 mM, where lower cell kill was seen in cells exposed to pre-activated IAA (O.Greco, unpublished data). This is similar to the results seen in the mosaic spheroids.

The spheroid model shows some of the characteristics associated with solid tumours, which makes it appropriate for use *in vitro* to mimic *in vivo* conditions. Use of the spheroid model has confirmed that indole prodrugs are suitable for use with the HRP enzyme for gene therapy, and the combination is likely to retain activity under the heterogeneous conditions seen in tumours. In particular the halogenated indole is particularly promising due to the low concentrations required, and specificity for hypoxic activation, which would provide targeting and may reduce non-specific drug activation.

Chapter 5

Paracetamol: a novel prodrug for use with the horseradish peroxidase enzyme for gene therapy

5.1 Introduction

IAA and related indoles are proven prodrugs for HRP mediated gene therapy as described in the previous chapters. However, the HRP enzyme is able to catalyse the oxidation of a variety of other compounds, including the widely used analgesic agent paracetamol (acetamidophenol, acetaminophen). Although widely regarded as an anti-inflammatory, paracetamol has only a weak inhibitory action on the two cyclo-oxygenase enzymes that are the traditional targets for non-steroidal anti-inflammatories.

Paracetamol is well tolerated at therapeutic doses, with a bioavailability of up to 90 % (Rawlins et al., 1977). The majority of ingested paracetamol is excreted by the kidneys as either a glucuronide or sulphate conjugate, with a small proportion excreted as mercapturic acid.

In cases of overdose the normal detoxication pathways become saturated, and hepatic necrosis occurs as a primary consequence. Glutathione levels fall and there is a corresponding increase in binding paracetamol to hepatocytes (Timbrell, 1996). It is thought that microsomal cytochrome P450 enzymes (CYP), in particular CYP2E1 and CYP1A2, catalyse the conversion of paracetamol by a 2-electron oxidation, to the cytotoxin *N*-acetyl-*p*-benzoquinoneimine (NAPQI, (Patten et al., 1993).

Treatment of cells with paracetamol or NAPQI results in similar consequences, provided cells contain sufficient levels of CYP activity to produce NAPQI. There is an increase in levels of intracellular calcium (Tsokos-Kuhn, 1989), which can lead to cytoskeletal changes and activation of endonuclease activity. Glutathione levels *in vivo* can decrease by as much as 80% (Timbrell, 1996). The formation of protein adducts is evident and correlates with centrilobular necrosis *in vivo*, but doesn't seem to be correlated with cell death *in vitro*. NAPQI seems to interfere with mitochondrial function resulting in an initial increase in reactive oxygen species (ROS), followed by a decrease, possibly due to damage to the mitochondrion (Manov et al., 2002). There is also an increase in reactive nitrogen species (RNS).

In addition to causing death through necrosis, apoptosis and direct damage to DNA have been demonstrated *in vitro*. Paracetamol can inhibit DNA synthesis by destroying the tyrosyl free radical on ribonucleotide reductase (Hongslo et al., 1990; Richard et al., 1991), which may account in part for the observed changes in cell cycle progression (Djordjevic et al., 1986). The mechanism of apoptosis in cells is unclear since there are contradictory reports as to the involvement of some signalling molecules (Bae et al., 2001; Knight & Jaeschke, 2002), which may be dose or cell type specific.

Paracetamol has been proposed as a prodrug for GDEPT with the gene encoding the CYP1A2 enzyme. The combination of CYP1A2 over-expression in fibroblasts and administration of 0.1-20 mM paracetamol resulted in a decrease in the number of viable cells, and a strong bystander effect *in vitro* (Thatcher et al., 2000).

HRP is able to convert paracetamol to the cytotoxic compound NAPQI via a 1-electron oxidation resulting in the formation of a semiquinone, which can disproportionate to form NAPQI (Figure 5.1, (Potter & Hinson, 1987; Potter & Hinson, 1989; Potter et al., 1986). This reaction is equivalent to the 2-electron oxidation performed by CYP enzymes in the liver. However, the intermediate step can be reversed in the presence of GSH or NADPH (potentially further reducing GSH levels and increasing oxidative stress). The semiquinone may form polymerisation products, although the presence of NADPH will prevent this from occurring (Keller & Hinson, 1991). Oxidation of paracetamol will lead to the formation of substantial quantities of oxidised GSH (GSSG), and thiol dependent oxygen uptake by the cell (Ross et al., 1985). The formation of GSSG is likely to be through oxygen dependent mechanisms rather than by simple dimerisation.

In this chapter, the ability of the HRP enzyme when expressed by human carcinoma cells, to convert paracetamol to a cytotoxin was investigated. In addition the effects on cell cycle, and potential of using anti-oxidants and/or enzyme inhibitors to limit tumour non-specific activation were looked at.

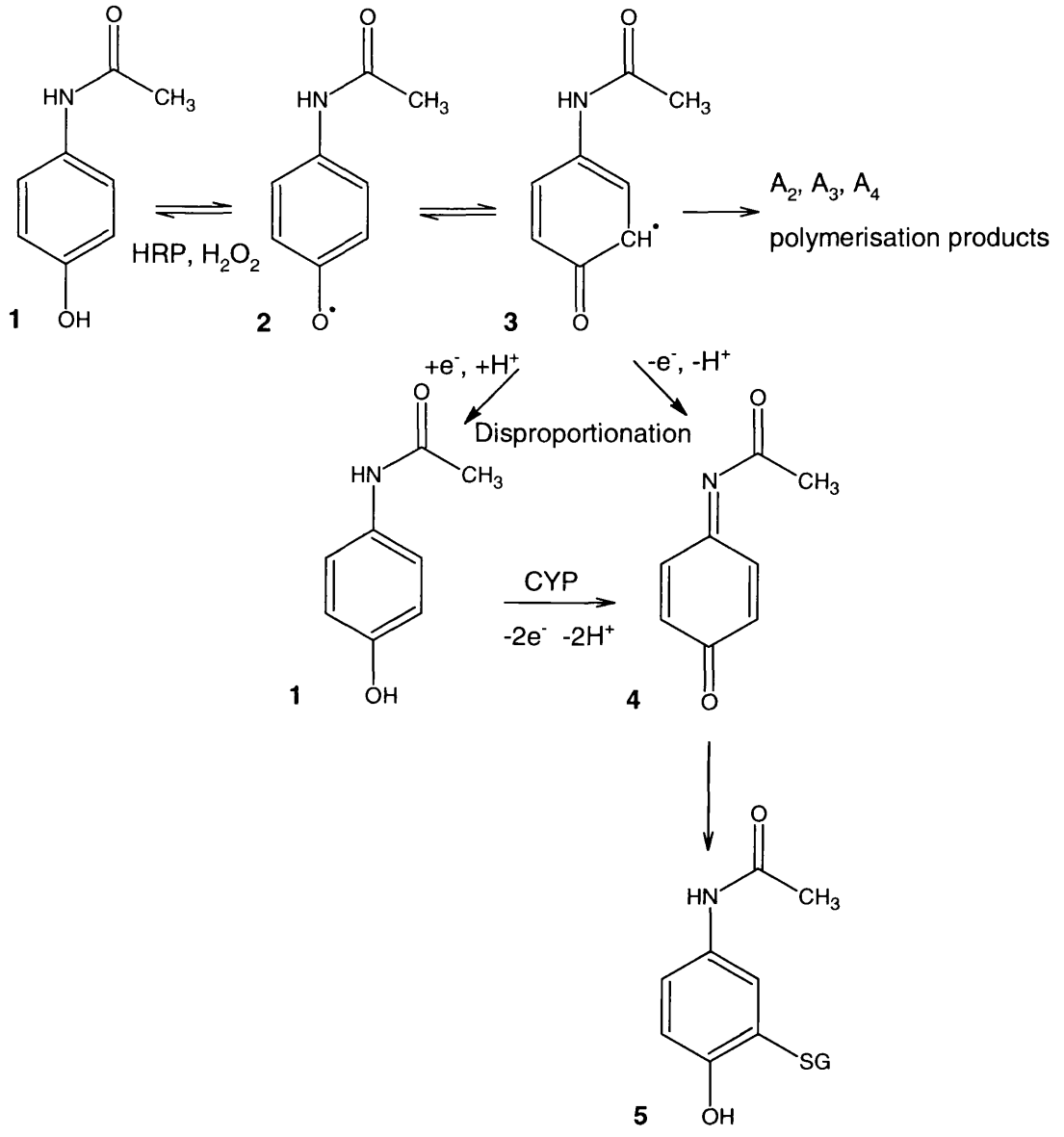


Figure 5.1. Reaction between HRP and paracetamol. Paracetamol (**1**) is oxidised by HRP to form the semiquinone (**2**,**3**), which can revert back to paracetamol via reduction by either GSH or NADPH, the later resulting in the formation of superoxide. The semiquinone cannot react with oxygen to form the quinone, but can disproportionate to form *N*-acetyl-*p*-benzoquinoneimine (**4**), which reacts with cellular components such as thiols (**5**).

5.2 Materials and Methods

FaDu clones were used as described in Chapter 3. Clonogenic assays were performed as described in Chapter 2. Mosaic clonogenics were performed by plating a 50/50 mixture of GFP1 and HRP8 cells.

5.2.1 Anoxic exposure of cells to prodrug

Experiments carried out under anoxic conditions were performed in an anaerobic glove cabinet (DON Whitley Scientific Ltd, Shipley, UK) with 5% CO_2, 5% H_2, 90% N_2 and palladium catalyst. The catalyst combines any incoming oxygen with hydrogen to form water, which is removed using silica gel. All fluids were incubated in the chamber for at least 18 h prior to use. Cells were grown on Permanox dishes (Nalge Nunc International, Loughborough, UK), which contain less dissolved oxygen than ordinary laboratory plastic-ware.

Exponentially growing cells were plated, and allowed to attach for 4 h. Dishes were then moved to the anaerobic glove cabinet. Media on cells was replaced with pre-incubated anoxic media after cells were transferred to the chamber. After 2-3 h media was removed and cells exposed to prodrug. After drug incubation for 4 or 24 h, cells were removed from the chamber, washed and grown in feeder-cell supplemented media as described in chapter 2.

5.2.2 Exposure of cells to prodrug with antioxidant

Cells were plated as for clonogenic assay as described in chapter 2. Following cell attachment to the culture plates, media was removed and replaced with either fresh media or medium containing 1 mM *N*-acetyl cysteine (NAC, Sigma). Cells were left for an hour, after which paracetamol or paracetamol plus NAC in Hanks' balanced salt solution (HBSS) was added for 24 h. Cells were washed and feeder-cell supplemented medium added as before.

5.2.3 Cell cycle analysis

Analysis of cell cycle was carried out using propidium iodide (PI) staining of cells. PI stains the DNA, and the amount of staining seen as fluorescence, reflects the DNA levels within a cell. Cells undergoing mitosis (G_2/M) contain roughly twice the amount of DNA as those in G_0/G_1, whereas cells undergoing DNA synthesis (S phase)

contain intermediate amounts.

Following exposure to prodrug for 24 h, cells were washed and allowed to recover for 24 h in complete medium. Attached a floating cells and media were then harvested, centrifuged and the cells fixed in ice-cold 70% ethanol for 1 h on ice. PBS was then added, cells pelleted and resuspended in PBS containing 20 μg/ml PI and 20 μg/ml RNAse A (Sigma). Cells were incubated at 37°C for 30 min and samples were then subjected to FACS analysis using the FL-3 A setting to determine PI fluorescence levels. Cell debris was gated using forward and side scatter (FSC and SSC).

5.3.4 Enzyme inhibition

Purified HRP enzyme (0.4 ng, Sigma), was incubated in the presence of varying concentrations of furafylline (0-250 μM, Sigma), for varying periods of time (10-40 min) in 80 mM phosphate buffer (pH 5.4). 320 mM TMB and 3 mM hydrogen peroxide were then added to give a total volume of 2 ml. Absorbance was read every minute at 652 nm on a Hewlett Packard model 8452A diode array spectrophotometer.

5.3.4 Western blotting

Western blotting was performed as described in chapter 2. For detection of the CYP1A2 enzyme, a primary rabbit anti-human CYP1A2 antibody was used (Serotec, UK, diluted 1:10000). Induction of CYP enzymes was performed with 8 h exposure to 10 nM 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, Promochem, Welwyn Garden City, UK).

5.3 Results

5.3.1 Efficacy of the HRP/paracetamol combination

Exposure of FaDu clones to paracetamol under oxic conditions for 4 h (figure 5.2a) or 24 h (figure 5.2b) resulted in a decrease in the survival of HRP8 clones compared to GFP1. 4 h exposure to paracetamol decreased survival by approximately 40% at 1 mM concentrations. Levels greater than this did not further decrease survival. Following 24 h exposure there was a log of cell kill at 10 mM paracetamol concentrations. However, at this concentration there was also a decrease in the survival of GFP1 cells to 0.3 ±0.03. The mosaic clonogenic results showed almost

equivalent cell kill to experiments performed using only HRP8 cells. At 10 mM paracetamol concentrations however, the level of cell kill was intermediate between HRP8 and GFP1 survival.

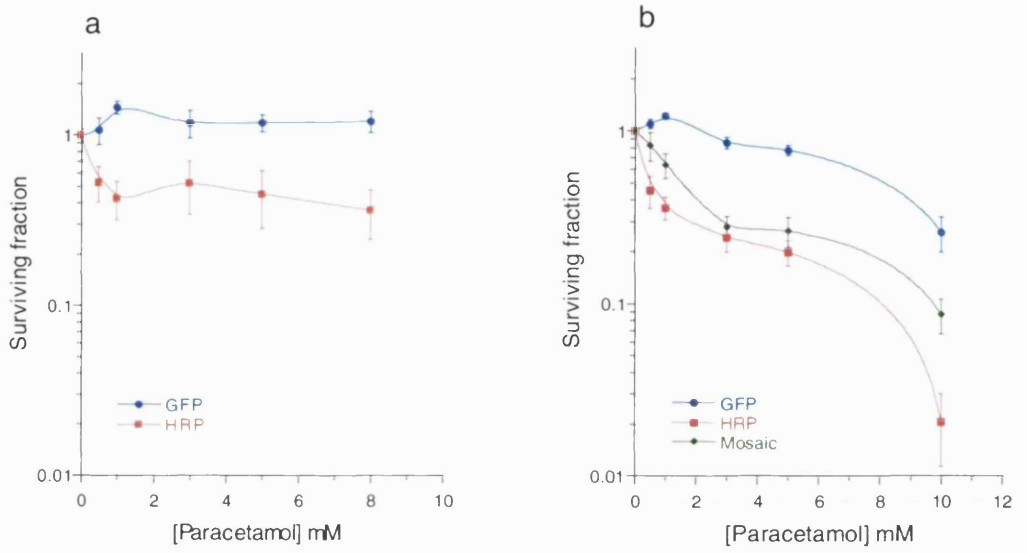


Figure 5.2. Clonogenic survival of FaDu cells following a) 4 h or b) 24 h exposure to paracetamol in normoxic conditions. Data are mean ± s.e.m. minimum of 3 separate experiments, triplicate samples.

Exposure of cells to paracetamol under anoxia also resulted in a decrease in HRP8 cell survival (figure 5.3). 4 h treatment resulted in a curve similar to those seen for IAA exposure (Chapter 3), with a plateau between 1 and 5 mM, followed by sharp decrease in survival. At 10 mM, survival of GFP1 cells was also decreased (figure 5.3a). Exposure of cells to paracetamol under anoxia for 24 h resulted in greater than 99% decrease in survival of HRP8 cells at 10 mM concentrations with no evident plateau (figure 5.3b). As with experiments performed under oxic conditions, the survival of a mixture of HRP8 and GFP1 cells (mosaic) was equivalent to that seen when only HRP8 cells were plated. There was no major difference in the survival curves when cells were exposed to paracetamol under oxic or anoxic conditions.

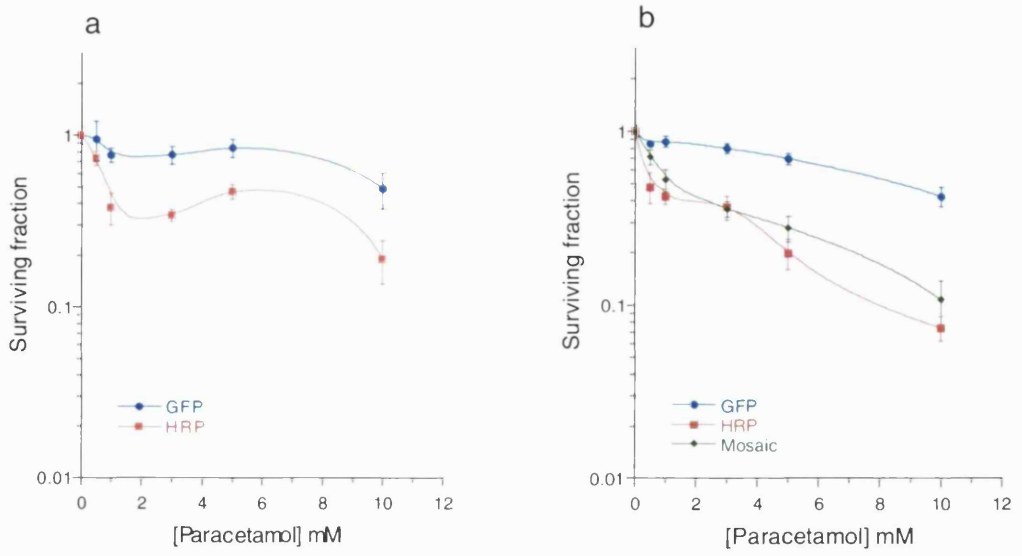


Figure 5.3. Clonogenic survival of FaDu cells following a) 4 h or b) 24 h exposure to paracetamol under anoxia. Data are mean ± s.e.m. minimum of 3 experiments, triplicate samples.

To determine the effect of antioxidants on this GDEPT combination, cells were exposed to paracetamol with or without *N*-acetyl-cysteine (NAC) for 24 h (figure 5.4). There was no difference in the survival of either GFP1 or HRP8 cells when treated with paracetamol alone or in combination with NAC.

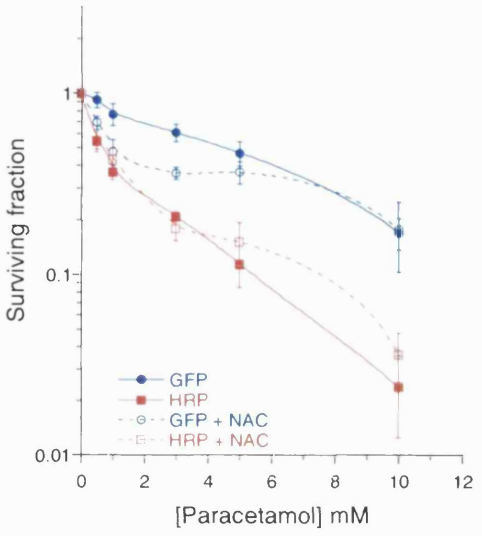


Figure 5.4. Clonogenic survival of cells exposed to paracetamol for 24 h alone, or in combination with NAC. Data are mean ± s.e.m. 3 independent experiments, triplicate samples. Paracetamol and paracetamol with NAC exposure was carried out simultaneously.

5.3.2 DNA fragentation following paracetamol exposure

The effect of paracetamol treatment on cell cycle distribution was assessed using FACS analysis. Propidium iodide staining of cells showed a small but significant increase in the sub G_1 cell population of HRP8 cells following incubation with 1mM paracetamol for 24 h compared with untreated controls (figure 5.5), suggesting apoptosis as one mode of cell death. There was also a small decrease in the number of G_2/S cells indicating a decrease in proliferation. In contrast, there was no statistically significant change in the cycle distribution of GFP1 cells treated with paracetamol (figure 5.5). Exposure of cells to 3 mM paracetamol did not increase the sub G1 population of HRP8 cells further (data not shown).

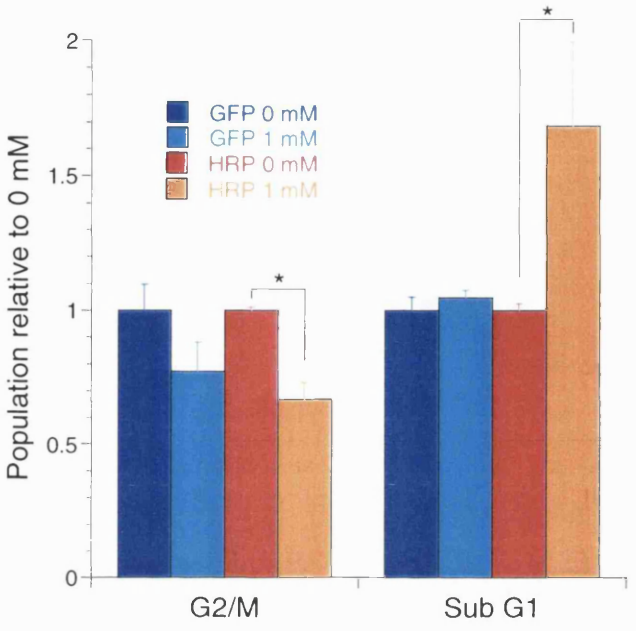


Figure 5.5. Propidium iodide staining of DNA. Cells were exposed to 1 mM paracetamol for 24 h and allowed to recover for 24 h before staining with PI. Data are mean ± s.e.m. 3 experiments, duplicate samples. *p<0.05.

5.3.3 Effect of furafylline on HRP activity

Furafylline is a known inhibitor of CYP enzyme activity, and its effect on HRP activity was determined using the TMB assay. In experiments using purified HRP enzyme, incubation for 10 min with furafylline led to a maximal decrease of 25% in the enzyme activity up to concentrations of 25 μM (figure 5.6a). However, concentrations of furafylline required to decrease the enzymatic activity of purified HRP enzyme by half (IC_{50}) were not attainable due to the limited solubility of the inhibitor. When exposed to 25 μM furafylline for 10 min, activity showed a 25% decrease in activity. This was not decreased further by increasing the exposure period up to 40 min (figure 5.6b).

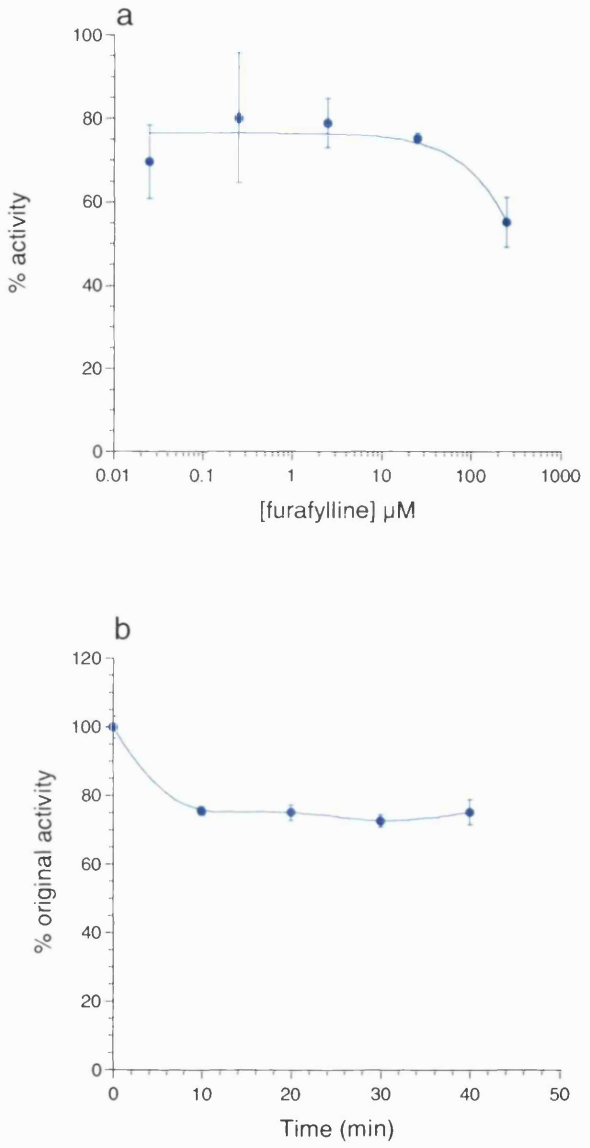


Figure 5.6. Effect of furafylline inhibition on activity of purified HRP enzyme. a) Concentration-response curve. b) Time-response curve to 25 µM furafylline. Data are mean ± s.d. duplicate samples.

5.3.4 Presence of endogenous paracetamol activating enzymes

The CYP enzymes can metabolise paracetamol to a cytotoxin. Hence, endogenous levels of cytochrome P450 1A2 were assessed using western blot (Figure 5.7). The level of expression in FaDu GFP1, HRP8 and parental cells was undeterminable (Lanes 1, 2 and 3). Following induction with TCDD, levels were similar in all cell types, but remained low (lanes 4, 5 and 6).

Figure 5.7. Expression of CYP1A2 in FaDu cells. Lane 1, FaDu parental; lane 2, FaDu GFP1; lane 3, FaDu HRP8. Lanes 4,5,6, as 1-3 with TCDD pre-treatment.

5.4 Discussion

The results in this chapter show the ability of intracellularly produced HRP to catalyse the reduction of paracetamol to a cytotoxin resulting in cell death.

Exposure of cells to paracetamol for 4 or 24 h resulted in a dose dependent decrease in the clonogenicity of HRP8 cells. However, at doses above 8 mM for 24 h, a decrease in GFP1 clonogenicity was observed (figure 5.2). When treated under the severe tumour associated condition of anoxia, the HRP/paracetamol combination retained its activity. To our knowledge, this is the first demonstration of cell kill by paracetamol catalysed by intracellular HRP.

The reaction between HRP and paracetamol has been well characterised using purified enzyme (Potter & Hinson, 1987; Potter et al., 1985; Potter et al., 1986), and the production of NAPQI has been demonstrated. In cases of paracetamol overdose, it is the saturation of detoxication pathways (mainly type II conjugation reactions), which results in the formation of substantial quantities of NAPQI (see (Timbrell, 1996) for review). This can lead to a variety of cellular effects, which combined with a decreased potential to balance the cellular redox state, can result in necrosis, apoptosis and loss of hepatic function.

The production of NAPQI by HRP does not require oxygen (Figure 5.1), and the formation of NAPQI from the semiquinone does not occur spontaneously in the presence of oxygen. Therefore, the cytotoxicity under anoxia is not unexpected and could be advantageous under tumour conditions. However, the precise involvement of the quinone in HRP/paracetamol cytotoxicity has not been determined, and the recycling of the semiquinone back to paracetamol by GSH and NADPH may be important, since the oxidation of these molecules could alter the redox state of the cell, rendering it more sensitive to changes in the environment. In addition, following the oxidation of NADPH, superoxide radicals may be produced. These may play a

role in the cytotoxicity of paracetamol in HRP8 cells under oxic conditions, although this would be dependent on the prior exhaustion of GSH stores. The action of polymerised paracetamol on cells is unknown, as is the level of its production in cells, when the presence of NADPH may prevent polymer formation (Keller & Hinson, 1991).

The cytotoxicity of paracetamol at the concentrations used in this study did require the presence of the HRP enzyme, since GFP1 controls showed a decreased survival only at very high prodrug concentrations. As shown in chapter 3, the glutathione levels in both HRP8 and GFP1 cells was equivalent, or if anything, higher in HRP8 clones. Therefore, it is unlikely that the HRP8 cells were intrinsically more sensitive to GSH depletion by endogenously produced paracetamol products than GFP1 cells, indicating that the HRP enzyme itself was important for the effects seen. In addition, levels of CYP1A2 were extremely low (as shown by others in a range of cell lines), and expression of HRP did not appear to upregulate this enzyme, nor make induction by a dioxin more pronounced (figure 5.7).

Unlike the reaction with IAA, paracetamol activation by HRP does require hydrogen peroxide. However, as shown in the results, cytotoxicity in cell culture is achievable in the absence of exogenously added peroxide, indicating that sufficient hydrogen peroxide is present in metabolising cells.

Addition of the anti-oxidant *N*-acetyl cysteine (NAC) did not significantly affect the survival curves of either GFP1 or HRP8 clones (figure 5.4). The addition did appear to increase the susceptibility of GFP1 cells to paracetamol at low concentrations. NAC is the acetylated and more stable form of the amino acid L-cysteine, a precursor in the formation of GSH. The lack of a protective effect on paracetamol induced cytotoxicity may have a number of explanations. It is possible that the concentration of NAC used was not sufficiently high, or that a longer pre/post incubation period would be required to see an effect. Increased levels of NAC extracellularly in the media may therefore not have resulted in increased intracellular GSH levels. Alternatively, the damage produced by the addition of paracetamol may have been independent of GSH.

The use of paracetamol as a prodrug for GDEPT has previously been proposed using the CYP1A2 enzyme (Thatcher et al., 2000). In that study, 6 h incubation

decreased the survival of V79 fibroblasts stably expressing CYP1A2, with a 50% decrease in cell survival occurring between 1 and 4 mM. In our study following 4 h exposure to paracetamol, slightly less than 1mM paracetamol was required to reduce the clonogenic survival of HRP8 cells by 50%, although the clonogenic assay used here is likely to be more sensitive than the assay used by Thatcher *et al* (counting of viable cells). This group also demonstrated a large bystander effect, with only 5% of cells required to express the CYP1A2 isozyme for effective cell kill. If NAPQI is the cytotoxin produced by both enzyme systems, then a bystander effect would be predicted for the HRP/paracetamol combination. This was demonstrated in mixing experiments, where HFP8 and GFP1 cells were plated together and exposed to paracetamol for 24 h under oxic or anoxic conditions. The cell kill observed was greater than that which would be expected for killing of HRP8 cells only.

The pharmacokinetics of paracetamol shows considerable interpatient variability. The reported time to maximal plasma concentration (C_{pmax}) ranges from 20 min (McGilveray & Mattok, 1972) to 1.4 h (Heading et al., 1973) following ingestion in tablet form. At therapeutic doses, paracetamol does not significantly bind to plasma proteins (Gazzard et al., 1973), and tissue: plasma concentration ratios of 1:1 have been reported.

Following a therapeutic dose of 1 g, a C_{pmax} of 9 μg/l (approximately 0.06 mM) has been reported (Hong et al., 1994), slightly less than the 20 μg/ml reported by (Prescott, 1980). However, in both cases it can be seen that maximal concentrations within tumour tissue (assuming a 1:1 relationship) after this dose would not reach those needed to effect cell death by either the HRP or CYP1A2 enzymes.

In order to increase the amount of paracetamol that could safely be ingested, liver toxicity would need to be minimised. This could be achieved by inhibiting metabolism by endogenous P450 enzymes, or by potentiating the protective mechanisms in the liver.

Furafylline is a potent inhibitor of CYP1A2, one of the endogenous enzymes responsible for paracetamol activation, with an IC_{50} of 0.07 μM (Sesardic et al., 1990). Following administration to humans, plasma steady state levels can reach 5.8 μM (Tarrus et al., 1987), well above the IC_{50} for CYP1A2. This concentration of furafylline decreased activity of purified HRP by less than 25% (figure 5.6a). Hence it could be envisaged that doses of paracetamol greater than 1 g could be safely

administered whilst CYP activity is inhibited using furafylline to increase tumour concentrations, therefore resulting in HRP-induced cytotoxicity specifically in the tumour.

Increasing liver GSH levels and protection of this organ from paracetamol overdose may be achieved by orally administered methionine or NAC (Aebi & Lauterburg, 1992; McLean & Day, 1975; Prescott et al., 1980), compounds used to treat cases of paracetamol overdose. Using NAC *in vitro* (figure 5.4) there appears to be little protective effect against HRP/paracetamol induced cell death. Hence NAC treatment to reduce liver toxicity, with little effect on HRP-induced toxicity, may also be a promising GDEPT approach.

Paracetamol is used safely and routinely in the clinic, so is an attractive prodrug for GDEPT, since its properties in man are well characterised. In combination with HRP it can affect cell kill in both oxic and anoxic conditions. This combination may be useful for hypoxia targeted GDEPT. Unfortunately, the level of cell kill currently obtained is unlikely to result in observable growth delay. It may be that the use of the HRP enzyme in combination with paracetamol is unsuitable for further development. More work is required to analyse this new GDEPT combination, specifically, means to increase paracetamol dosage without increasing liver toxicity need to be explored further.

This work has demonstrated that HRP can be used to convert compounds other than IAA and its analogues to cytotoxins, and is active under reduced oxygen tensions.

Chapter 6

Characterisation of the FaDu tumour model and indole prodrugs

6.1 Introduction

The HRP/IAA system has been demonstrated to be active in monolayers and spheroids *in vitro*. However, the system has not been tested *in vivo*. Although monolayers and spheroids can be manipulated to approximate the conditions seen in solid tumours, they remain far less complex than the neoplastic conditions seen in patients. Animal models remain the most realistic experimental approximation to the clinical situation. It is therefore necessary for novel therapeutics to be evaluated *in vivo*.

The pharmacokinetic profile of a drug will play a role in determining its efficacy. The distribution of a drug is dependent on many factors, including its lipophilicity, and binding to plasma proteins. The tissue exposure is generally greater in tissues with a large blood supply, such as the liver, especially as it has a role in the metabolism of many endogenous and exogenous compounds. The pharmacokinetic profile of indoles will determine the level in tumour and therefore the potential cell kill. The stable clones obtained in chapter 3 and characterised *in vitro* were used to form subcutaneous tumours in an animal model. The indole prodrugs were also characterised *in vivo*.

6.2 Materials and methods

Tumour implantation, measurement, and assessment of transgene expression were carried out as described in chapter 2.

6.2.1 Pimonidazole staining

Pimonidazole hydrochloride in phosphate buffered saline (PBS) was injected intraperitoneally (i.p.) to mice at 60 mg/kg. 90 minutes later animals were sacrificed, tumours excised and processed as described in 2.8 and 4.2.4.

6.2.2 Assessment of hypoxic fraction

Sections from at least 3 levels through the tumour were analysed using Image J software. Regions of positive staining were manually highlighted, and pixel area expressed as a percentage of total section area.

6.2.3 Blood vessel staining

Tumours were excised and processed as described in 2.8. Frozen sections were washed in Tris buffered saline (TBS) and exposed to primary antibody diluted 1:200 (Serotec rat anti-mouse CD34, MCA1825) for 1 h at room temperature. Following washing, sections were incubated with secondary antibody (Dako, biotinylated rabbit anti-rat) 1:200 for 1 h. StreptABComplex (Dako) was made up and used according to the manufacturers instructions. In 5 ml TBS, 1 drop of both solution A and solution B were added and allowed to stand for 30 minutes. This was added to the slides after rinsing off excess secondary antibody, and incubated for 1 h at room temperature. Vector red was incubated until colour developed, and slides counterstained with haematoxylin, dehydrated and mounted.

6.2.4 Chalkley point counting

An eyepiece graticule (Graticules Ltd) consisting of 21 random spots was used in a Nikon light microscope. Under x100 magnification the field of view was moved randomly over the section, and the number of dots in contact with positively staining blood vessels counted. This was repeated at least 20 times per section area.

The vascular index was calculated by averaging the scores for each section. 3 sections were cut at 3 levels through the tumour (approximately 2-3 mm apart), and the results pooled.

6.2.5 Pharmacokinetics

In order to analyse the distribution of indoles between tissues (pharmacokinetics, as opposed to pharmacodynamics, which is the effect of the drug on the body) following a single administration, high performance liquid chromatography (HPLC) was used to analyse levels in a variety of tissues in mice.

Reverse phase HPLC consists of a non-polar stationary phase and a polar eluent. The non-polar surface extracts lipophilic analytes from the eluent, resulting in the formation of an organic-rich layer at the particle surface, which is where the

partitioning occurs. By altering the pH of the eluent, the ionisation state of solutes can be altered, and so, their retention time in the column, with the most polar molecules eluting in the shortest time.

Female SCID mice bearing FaDu tumours received a single dose of IAA in 10% ethanol or 5% DMSO, or 5Br-IAA in 10% ethanol intraperitoneally (i.p.). At specified times after administration animals were sacrificed and blood and tissues removed. Whole blood was collected in lithium-heparin tubes (Sarstedt, UK), centrifuged for 1 min at 12,000 g, and plasma removed to tubes containing ascorbate to give a final concentration of 0.1%.

Tissues were weighed, homogenised in 9 volumes of 0.1% ascorbate solution, and transferred to microfuge tubes. All samples were stored at –80°C prior to HPLC analysis.

Protein was precipitated by the addition of an equal volume of acetonitrile to the sample, vortexing and then centrifugation at 4°C 10,000 g for 10 min. Supernatant was removed to sample vials (Waters), and loaded in to an autosampler (Waters 717, UK) attached to a HiChrom RPB 100 x 3.2 mm reverse phase column. Eluent A was composed of 10% acetonitrile, 20 mM ammonium acetate at pH 5.1, eluent B consisted of 75% acetonitrile/water. The column was run with a 0-50% gradient of B over an 8 min period with a flow rate of 1 ml/min and a re-equilibration time of 5 min. Detection was with a Waters 996 diode array detector at 260 nm.

In order to check the recovery of IAA from protein precipitated samples, control plasma, or water was spiked with 5 mM IAA. An equal volume of acetonitrile was added, samples were vortexed and then centrifuged for 10 min at 10,000 g at 4°C. The supernatant was removed and run in the HPLC column as described above.

Buffers and reagents

0.1% ascorbate:

Ascorbate	100 mg
dH_2O	100 ml

Ammonium acetate:	
ECD grade Acetate	1.54 g
Acetic acid	400 µl
Acetonitrile	100 ml
dH_2O	to 1 l

75% acetonitrile:	
Acetonitrile	750 ml
dH_2O	to 1 l

6.3 Results

6.3.1 Characterisation of FaDu tumours

FaDu cells from *in vitro* cell culture implanted subcutaneously (s.c.) at 10^6/mouse in the flanks of female SCID mice formed solid tumours, with a take rate of 100%. GFP1 and HRP8 clones also formed solid tumours when implanted s.c., also with a 100% appearance rate.

The growth rate of the parental and transfected cell lines was assessed using serial calliper measurements (figure 6.1). The parental and HRP8 tumours grew at the same rate, with tumour doubling times of approximately 2 and 3 days respectively. The growth rate of GFP1 tumours was noticeably slower (doubling time of 8 days) in these immunodeficient animals. This is in contrast to growth *in vitro* where all three clones grew at similar rates (chapter 3).

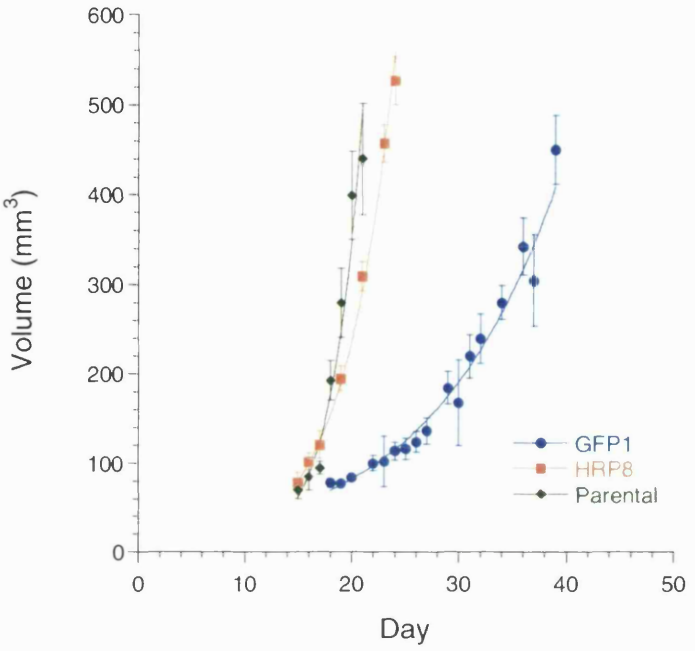


Figure 6.1. Growth of FaDu tumours grown subcutaneously from 10^6 cells in the flanks of female SCID mice. Data are mean ± s.e.m. 5 animals per group.

The expression of transgenes in tumours was assessed. GFP was visualised using frozen, unfixed 10 μm thick sections. GFP1 tumours showed diffuse, often punctate expression of GFP throughout the tumour that was difficult to quantitate (figure 6.2). Expression did not appear to be restricted to the periphery or central regions. HRP8 tumours showed greater activity in the TMB assay than tumours grown from either parental or GFP1 cells (figure 6.3). Due to problems with obtaining effective antibodies, immunohistochemistry to visualise HRP was unsuccessful, and so the number of cells expressing the enzyme was impossible to quantitate.

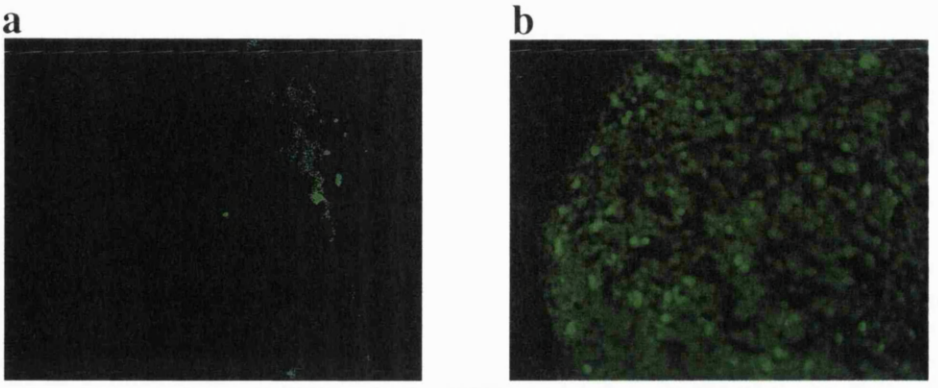


Figure 6.2. Expression of GFP within GFP1 tumours. a) Parental FaDu tumour b) GFP1 tumour. Original magnification x10.

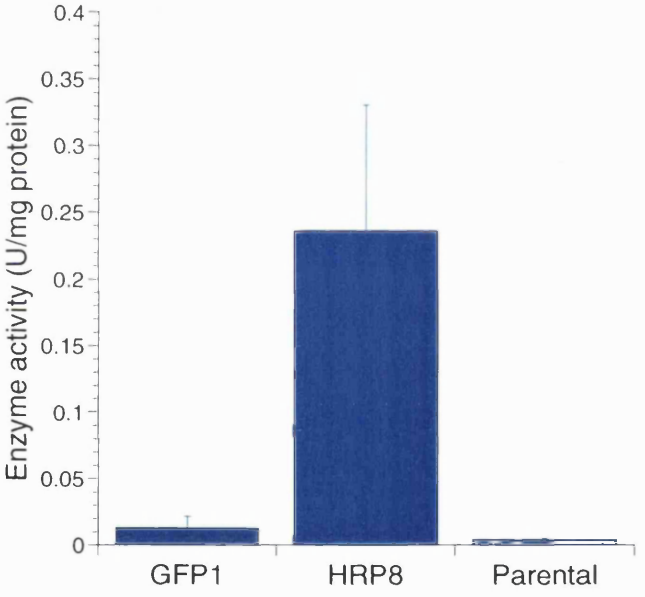


Figure 6.3. HRP activity within FaDu tumours assessed using the TMB assay. Tumours were excised at approximately 6 mm mean diameter. Data are mean ± s.e.m. 5 tumours per group.

Tumours were analysed histologically to determine whether transgene expression changed tumour characteristics. To assess regions of hypoxia within tumours, Pimonidazole staining was utilised (figure 6.4). Staining was present throughout the tumours, and when expressed as a percentage area showed little difference between the 3 tumour lines (table 6.1). Staining was not restricted to the

centre of tumours, but could be seen both peripherally and centrally. Positive staining was visible in small isolated regions, and also covering larger areas.

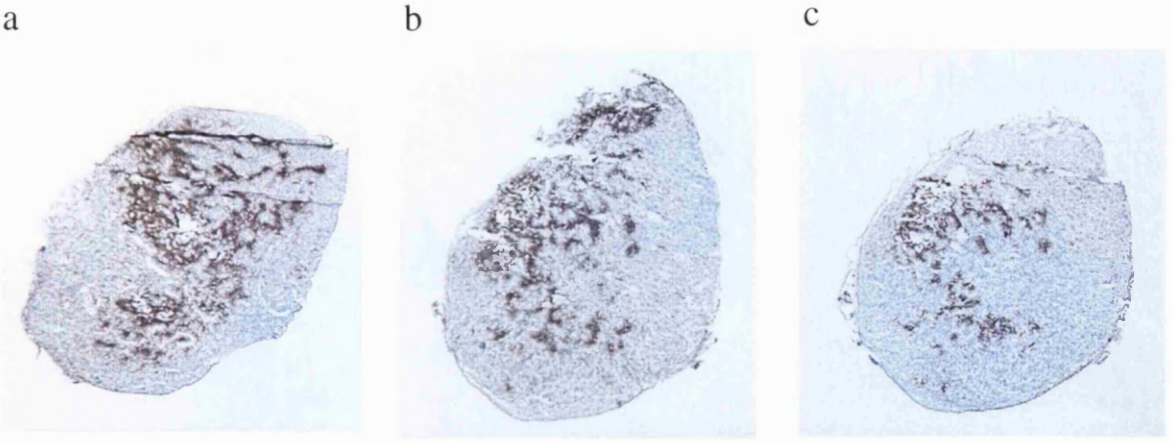


Figure 6.4. Tumour sections stained with Pimonidazole to show regions of hypoxia (brown). a) GFP1 b) HRP8 c) parental FaDu tumours. Tumours were approximately 6 mm mean diameter when excised.

Parental	7.66% ± 1.3
GFP1	8.18% ± 3.6
HRP8	5.22% ±1.0

Table 6.1. Regions of hypoxia in FaDu tumours. Data are % area positively staining for pimonidazole, mean 3 sections per tumour ± s.e.m., 4-8 tumours per group.

Tumours were also stained immunohistochemically for CD34 to determine vascular density (figure 6.5). This recognises the murine CD34 cell surface glycoprotein expressed by endothelial cells, and also by haematopoietic progenitor cells in the bone marrow. Using Chalkley point counting, it was shown that the vascular index of GFP1 tumours was significantly lower than that of parental or HRP8 tumours, although the tumours had roughly equivalent hypoxic fractions (figure 6.6).

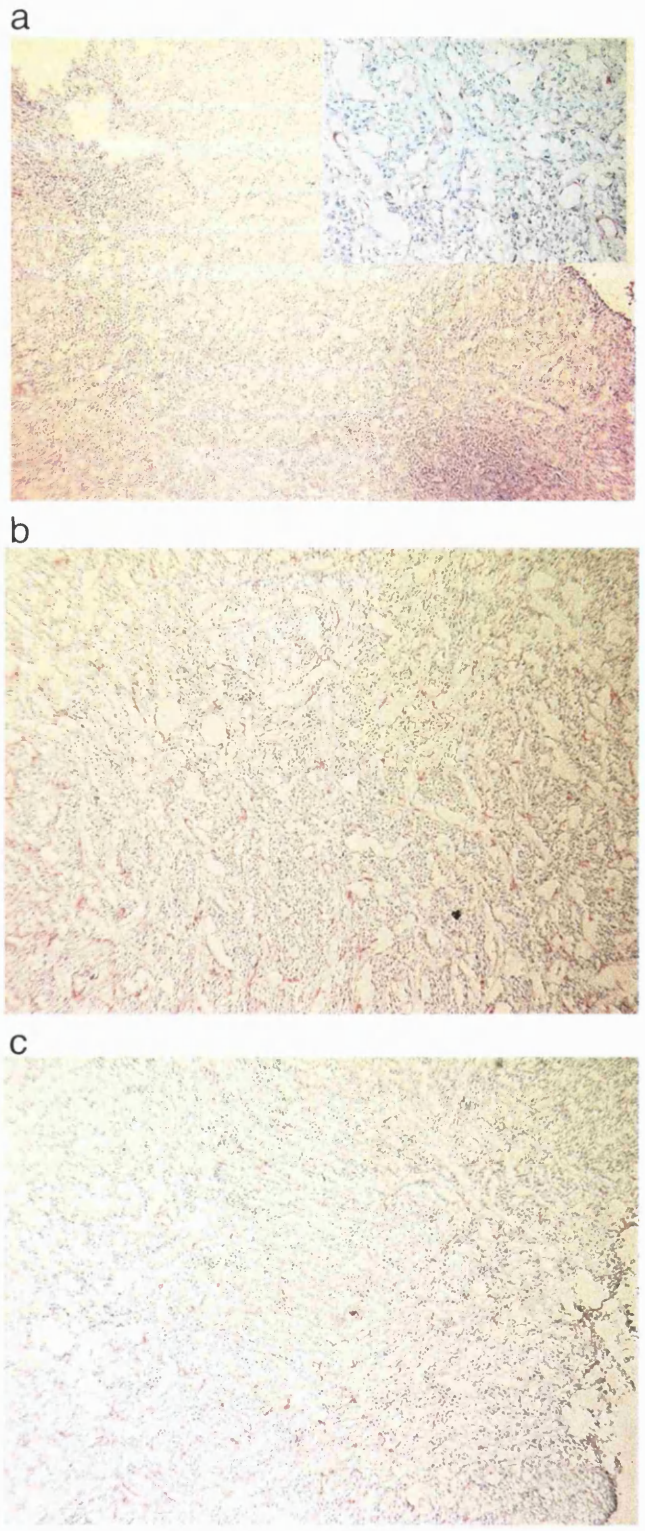


Figure 6.5. CD34 staining of tumours. a) GFP1 b) HRP8 c) parental FaDu. Insert shows high magnification of vessels (in red).

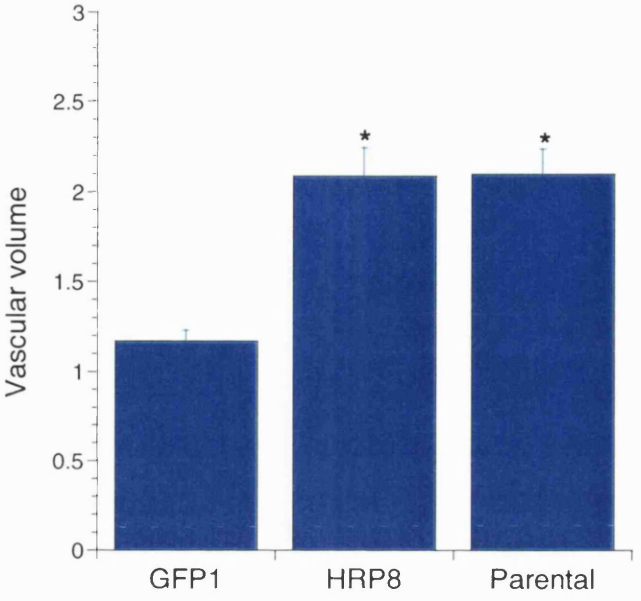


Figure 6.6. Quantitation of vascular density in FaDu tumours. *p<0.01 versus GFP1.

6.3.2 Characterisation of prodrugs

The maximum tolerated dose (MTD) was determined in order to prevent potential drug induced side effects during dosing. Prodrugs were administered at a range of doses intraperitoneally (i.p.) for 7 days, and animal health monitored during this period and for one month subsequently.

The weights of animals receiving IAA at 200 mg/kg, 5Br-IAA 200 mg/kg and 1Me-IAA 200 mg/kg, did not significantly decrease during or after treatment (figure 6.7). Animals appeared in good health, with no diarrhoea, piloerection or hunching behaviour.

Following 4 days administration of IAA at 300 mg/kg animals displayed myotonia (decreased muscle tone and decreases in reaction time), and appeared to be hypothermic. Animals were sacrificed at this time (figure 6.7).

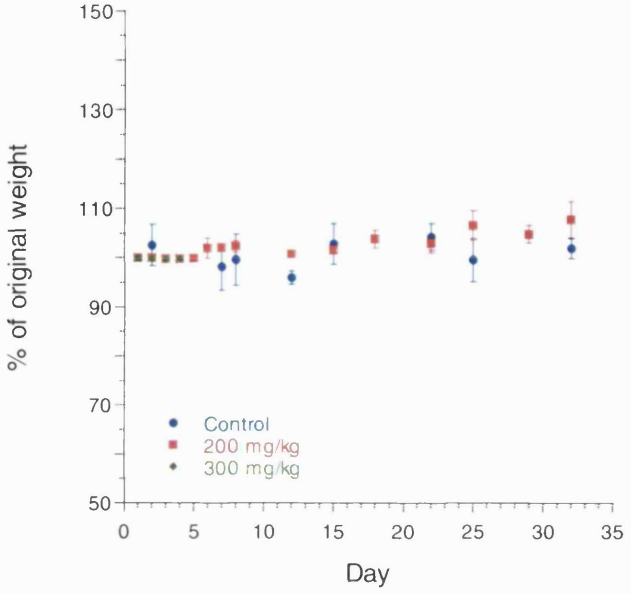


Figure 6.7. Weights of animals receiving IAA for 7 days, with one month follow-up. Data represent percentage of original weight, 3 animals per group ± s.e.m. Results for 5Br-IAA and 1Me-IAA were similar (not shown).

Pharmacokinetic studies were performed for IAA and 5Br-IAA. HPLC analysis of samples resulted in a characteristic peak for IAA (figure 6.8).

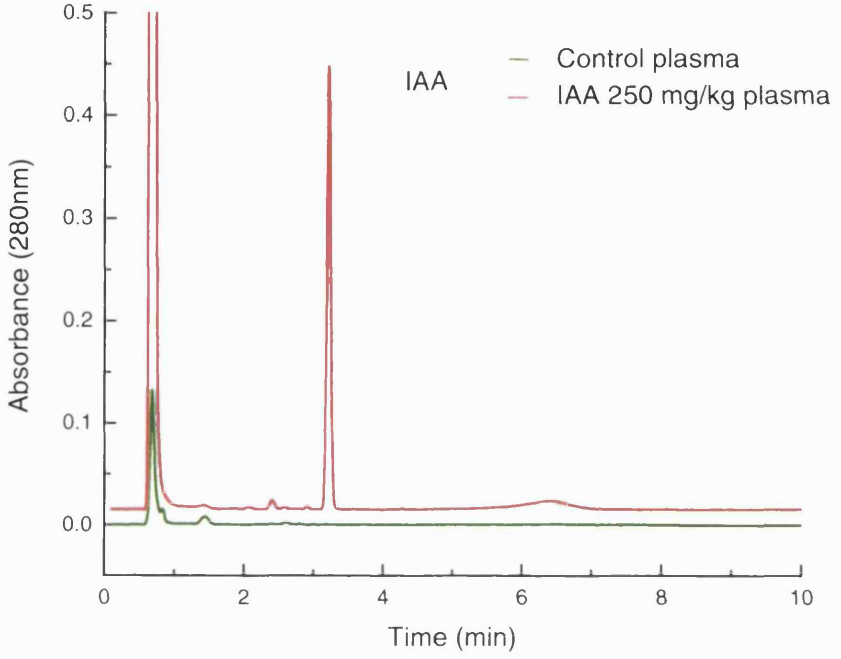


Figure 6.8. Profile of IAA in the plasma, 20 min following administration of 250 mg/kg i.p. Green line shows plasma from control animal.

Administration of a single dose of 250 mg/kg IAA in ethanol resulted in a profile resembling a first-order process (figure 6.9a). Plasma, liver and skeletal muscle concentrations peaked 10 minutes following administration, remained relatively constant for a short absorption phase, with a peak plasma concentration of 1.1 mM (± 0.1) at 20 minutes. At the 4 h sampling time, there was a large variation in IAA concentrations between animals. The pharmacokinetic parameters for IAA are described in table 6.2.

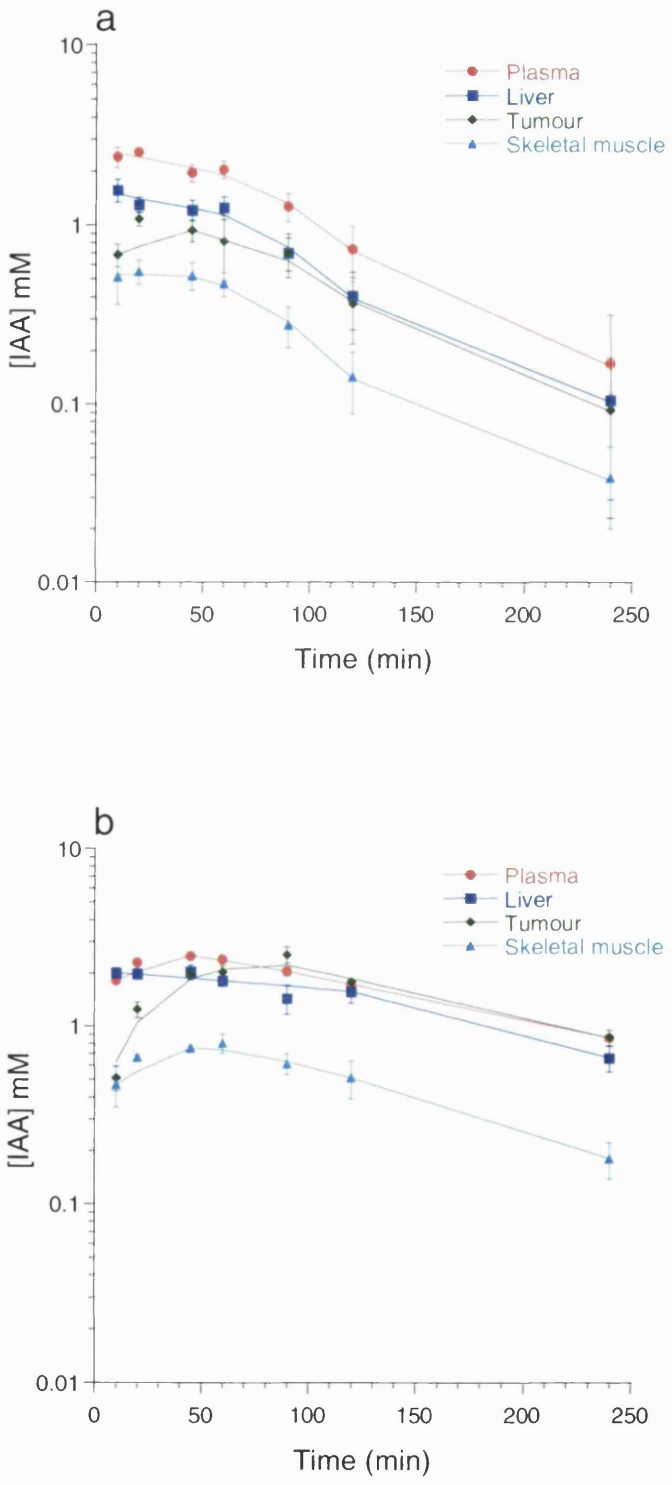


Figure 6.9. Pharmacokinetic profile of single administration of IAA at 250 mg/kg using a) ethanol or b) DMSO as the solvent. Data are mean ± s.e.m, 3-4 animals per time point.

The effect of solvent was looked at using DMSO in place of ethanol. The pharmacokinetic profile of IAA in DMSO (figure 6.11b) was markedly different from that seen when ethanol was used. With the exception of the liver, all tissues showed a short absorption phase. In the tumour this lasted 90 min.

Plasma concentrations reached 2.5 mM (± 0.05), and the peak tumour concentration was 2.5 mM (± 0.3), greater than the maximal concentration achieved using ethanol. The tumour $AUC_{0\text{-}240'}$ for IAA in DMSO was more than twice that seen when IAA was administered in ethanol. The plasma $t_{½}$ was also extended, and data more closely fitted an exponential decrease in tissue concentrations.

Drug	Plasma $t_{1/2}$ (min)	Plasma K_{el} (min^{-1})	Plasma $AUC_{0\text{-}240'}$ (mmol l^{-1} min)	Tumour $AUC_{0\text{-}240'}$ (mmol l^{-1} min)	Liver $AUC_{0\text{-}240'}$ (mmol l^{-1} min)	Muscle $AUC_{0\text{-}240'}$ (mmol l^{-1} min)
IAA 10% EtOH	23	0.03	257	117	149	57
IAA 5% DMSO	69	0.01	403	373	336	117
5Br-IAA 10% EtOH	87	0.008	367	179	212	70

Table 6.2. Pharmacokinetic parameters of indoles following a single i.p. administration of IAA 250 mg/kg, or 5Br-IAA 150 mg/kg. Data are from 3-4 animals per time point. $t_{½}$ half life, K_{el} elimination constant, AUC area under the curve (calculated using Origin graph package software).

The halogenated prodrug 5Br-IAA was administered in 10% ethanol at 150 mg/kg (figure 6.10 and table 6.2). As for IAA, concentrations were initially highest in the plasma and liver. However, whereas elimination of IAA from the blood and the liver was at approximately the same rate, 5Br-IAA was eliminated from the liver at a faster rate than from the plasma. At 240 minutes, tumour levels were greater than those in the liver.

Tumours showed an absorption period of approximately 1 h, to give a maximal concentration of 1.0 mM (± 0.01), before slowly being eliminated. Skeletal

muscle again showed the lowest prodrug concentrations. There was less inter-animal variation following administration of 5Br-IAA in ethanol, than IAA in ethanol.

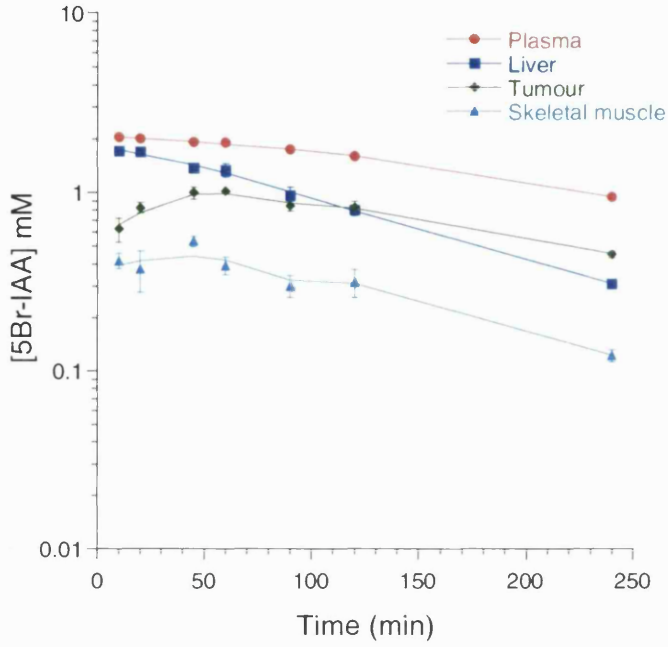


Figure 6.10. Pharmacokinetic profile of 150 mg/kg 5Br-IAA. Data are mean ± s.e.m. 3 animals per time point.

6.4 Discussion

The work in this chapter shows the characterization of prodrugs and stable FaDu transfectants *in vivo*.

FaDu cells formed solid tumours when implanted s.c. in the flanks of female SCID mice. GFP1 and HRP8 cells also formed solid tumours, although the growth rate of GFP1 tumours was markedly slower than parental cells. It may be that the GFP1 cells were, for some reason, less able to deal with the stresses experienced *in vivo*, such as nutrient deprivation, altered pH and fluctuating oxygen tensions (Brown & Giaccia, 1998) which are unlikely to occur *in vitro*. GFP1 cells, when grown as spheroids, did not appear to have a significant growth disadvantage (figure 4.2). However, this may not have been apparent at diameters less than 1 mm.

Although the hypoxic fraction did not appear to be different in GFP1 tumours from parental, the vascularisation was significantly lower, and this may have contributed to the slower growth rate and increased doubling time.

GFP and the modified enhanced green fluorescent protein (eGFP) have been reported to produce a strong T cell mediated immune response (Stripecke et al., 1999) which can lead to both growth delay and spontaneous regression of tumours. SCID mice are unable to mount an effective immune response since they are hypogammaglobulinaemic, and lack both B and T cells, thus the immunogenicity of GFP is unlikely to be a problem in these animals.

Both GFP1 and HRP8 cells retained transgene expression when grown as solid tumours. GFP expression was not uniform throughout the tumour area. In the absence of selection pressure, cells may switch off expression of exogenous DNA. The presence of hypoxia may also affect the level of fluorescence. Correct folding of the GFP protein to form the fluorophore requires the presence of oxygen (Heim et al., 1994). In T24 bladder carcinoma cells expressing GFP, fluorescence under 0.3% oxygen was significantly lower than under physiological oxygen tensions (2.5-5% (Coralli et al., 2001)), and protein levels were also decreased. Although in the study by Coralli *et al* experiments *in vivo* could not correlate the presence of hypoxia (visualized using pimonidazole staining) with regions of low or absent fluorescence, it is possible that the fluorescence seen in GFP1 tumours may be influenced by the presence of hypoxia. The spatial distribution of the GFP fluorescence however, seems to render this unlikely to be the sole cause.

In order to quantitate GFP expression, FACS analysis may have been an alternative to fluorescence microscopy. However, the background yellow fluorescence of tumours may affect results due to problems differentiating between high background fluorescence, and low green fluorescence (G.U. Dachs, personal communication).

HRP8 tumours retained HRP expression measured using the TMB assay. Both parental and GFP1 tumours exhibited measurable peroxidase activity. Different tumours have been shown to have varying amounts of peroxidase activity (Parkins et al., 2000), which may be related to the presence of neutrophils and other leukocytes. The TMB assay will detect myeloperoxidase activity (MPO, (Hotter et al., 1997),

although this enzyme is unable to effectively catalyse the oxidation of IAA. In addition, the presence of haemoglobin in the sample can affect the results, which is not a problem when harvesting cells from *in vitro* cell culture, but will be important and potentially highly variable in tumour samples.

The 2-nitromidazole compound, pimonidazole, was used to enable immunohistochemical evaluation of hypoxia. Binding of the reduced form increases steeply below 10 mmHg, and is visualized with antibodies raised against the reductively activated conjugate. Parental, GFP1 and HRP8 tumours all showed regions of hypoxia. These regions are important, since they may be radioresistant (Gray et al., 1953). They also form a target for therapy, since chronic hypoxia is a tumour specific condition (Brown & Giaccia, 1998).

In a multiple dosing strategy, 300 mg/kg IAA appeared to have severe adverse effects, with animals appearing flaccid and cold. Myotonia and hypothermia were also seen in male albino (Fuller et al., 1971) and male ddY mice (Yamada et al., 1985) after a single administration of 300 mg/kg IAA. It was found by Fuller *et al*, that derivatives of IAA, 5-hydroxyindole-3-acetic acid and 5-methoxyindole-3-acetic acid, had no myotonic effects at 300 mg/kg. Ventricular administration of IAA did not have any effect on muscle tone (Yamada et al., 1985), and the muscular effects were proposed to be a result of direct activity on the muscle, rather than a centrally mediated effect. In contrast, hypothermia induced by IAA appears to have a central mechanism. Doses of 200 mg/kg/day were therefore chosen for further studies.

High performance liquid chromatography (HPLC) analysis of a single dose of 250 mg/kg IAA showed that the prodrug is able to quickly distribute between most tissues (figure 6.11). Of all the tissues studied, the liver received the greatest exposure, which is not unexpected due to its large blood supply and role in metabolism.

IAA is reportedly excreted in the urine (Kobori et al., 1983) when produced endogenously. Kidney levels of IAA have not been assessed, although it is possible that they would be relatively high, as is the case for 5fluoroindole-3-acetic acid at 50 mg/kg in CBA mice (Folkes et al., 2002).

The profile of IAA differed according to the solvent used. When administered in DMSO, the pharmacokinetic profile more closely resembled a single compartment model. The plasma half life was three times longer when DMSO was used in place of ethanol, and all tissues received a greater exposure to the prodrug. It is possible that DMSO can alter the distribution of the prodrug in someway.

Tumour levels of IAA remained above 0.5 mM for approximately two hours when administered in ethanol, and greater than 4 hours when dissolved in DMSO (figure 6.11). *In vitro* exposure of HRP8 cells to 0.5 mM IAA resulted in a 20% decrease in survival of monolayers (figure 3.6) and almost a 50% decrease in the clonogenicity of HRP8 cells grown as spheroids (both 300 and 700 μm diameter, figure 4.3).

Levels of IAA in skeletal muscle peaked at 0.5-0.7 mM. This is 300 times lower than the concentration required to cause an increase in muscle twitch force and delay in relaxation time in isolated cat soleus muscle (Fuller et al., 1971). However, it seems unlikely that muscle concentrations achieved following 300 mg/kg IAA, which resulted in the side effects observed in muscle, are 300 times greater than those obtained after 250 mg/kg. It is possible that, in this study, myotonia was seen after four administrations due to an accumulation of IAA in the muscle. However, the pharmacokinetics do not particularly support this hypothesis, as muscle levels decreased over the sampling period of 4 h. Nor do the MTD data, where seven doses of 200 mg/kg results in a greater cumulative exposure than four 300 mg/kg administrations. It may be that a critical concentration of IAA must be reached in the muscle, or that repeat exposure sensitizes the muscle in some way.

5Br-IAA has a different kinetic profile to IAA (figure 6.12). The drug is very slowly eliminated from the blood, with a half life four times that of IAA. Liver levels decrease fairly rapidly, and after two hours fall below levels in the tumour.

Tumour concentrations reached 1 mM for approximately 30 minutes, and remained above 0.5 mM for four hours. This concentration was sufficient to decrease clonogenicity of HRP8 cells in 700 μm diameter spheroids to 0.2 (± 0.05). Levels in the liver were greater than 1 mM for ninety minutes. After four hour exposure of cells, concentrations greater than 1 mM resulted in non-specific toxicity of GFP1 cells, which may indicate a potential for liver damage.

Considering 5Br-IAA was administered at 100 mg/kg lower dose than IAA, it shows promising pharmacokinetics. Choice of solvent may be important, as DMSO

seemed to increase the tumour exposure to IAA without increasing peak liver, plasma or muscle concentrations. Use of 5Br-IAA with DMSO may provide a more lengthy exposure of tumour and increased tumour AUC.

Comparison of the pharmacokinetic data for IAA with existing GDEPT prodrugs shows that IAA has a considerably shorter half life (GCV, 2-3 hours (Morse et al., 1993), 5-FC, 3-4 hours (Cutler et al., 1978), CB1954, 1.4-2 hours (Chung-Faye et al., 2001)). This will require the activation of IAA in a more rapid manner than existing strategies. In transient transfectants, IAA can lead to cytotoxicity in two hours (Greco et al., 2000), and cytotoxicity in V79 cells incubated with purified HRP and IAA occurred after one hour (Folkes & Wardman, 2001). The decrease in surviving fraction after short incubations may not be sufficient to result in measurable growth delay *in vivo* after a single administration, but both multiple dosing and dosing twice daily (which is used for both HSV-tk/GCV and CD/5-FC) would increase tumour exposure.

In addition to dosing strategies, modification of the prodrug could be used to allow increased dosage with decreased normal tissue toxicities. Polymer conjugation of prodrugs will alter their pharmacokinetic properties, as the increased size of the molecule hinders leakage from the blood vessels. However, the tortuous, irregular and often immature vessels within tumours can display increased permeability, resulting in the phenomenon of "enhanced permeability and retention" of large molecules (Matsumura & Maeda, 1986). Immunogenicity of compounds is usually reduced, whilst increasing circulation times (Delgado et al., 1992). Toxicity to the reticuloendothelial system should be monitored, due to scavenging of large molecules (Seymour et al., 1987).

Cleavage of the polymer-drug linkage can be targeted to require specific enzymes and so further reduce potential side effects (although this is possibly a more useful feature for direct cytotoxic agents). For example, linkage could be through the use of a bioreductively cleaved bond, so releasing the prodrug under conditions of hypoxia.

Chapter 7

Assessment of the *in vivo* efficacy of the HRP/IAA system

7.1 Introduction

The HRP/IAA system has been proven to work in a GDEPT system *in vitro*, but the efficacy *in vivo* is dependent upon a number of factors which are not present in *in vitro* model systems. Delivery of transgenes is far more complicated *in vivo*. In addition, cells within a tumour will be cycling at different rates, resulting in differential uptake and expression of genes. The administration of prodrug *in vitro* results in the exposure of the majority of cells, depending upon the model used. *In vivo*, distribution and metabolism of the prodrug will affect the exposure of tumour cells. Poor blood supply or binding to plasma proteins may result in tumour tissues receiving only a small proportion of the administered drug.

This chapter describes the use of stable transfectants to assess the potential growth effects of this GDEPT system.

7.2 Materials and methods

Cells and tumour models were used as described in chapter 2.

7.2.1 *Ex-vivo* clonogenic assay

Clonogenic assays can be used to measure cytotoxicity *in vivo* as well as *in vitro*. They can measure the loss of viable cells from solid tumours by analysing the ability of tumour cells to form colonies *ex vivo*. Rather than exposing monolayer cells to drugs and/or radiation, treatment is administered to the animal, followed by tumour excision, isolation of tumour cells, and growth *ex-vivo*.

Animals bearing GFP1 or HRP8 tumours received a single i.p. administration of prodrug when tumours reached approximately 6 mm in diameter. 24 h later, tumours were excised, weighed and cross-scalpeled. The tumour was then incubated with 10 ml DMEM (without FCS), containing 1 mg/ml protease (Sigma), 0.5 mg/ml collagenase (Sigma) and 0.5 mg/ml DNAase (Sigma) on a rotating disc at 37°C for 1 h. Enzymes were neutralised by the addition of 10 ml DMEM (containing FCS), and

large debris allowed to settle. The supernatant was removed and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in 3-6 ml media (depending upon pellet size), and viable cells counted using trypan blue exclusion. Viable cells were plated with feeder cells as in 2.9 and colonies allowed to form over 10 days in an incubator.

7.2.2 Radiation treatment

The administration of radiation to tumours is an effective treatment in the clinic, and is used in a variety of tumour types. X-rays are situated on the electromagnetic spectrum with a wavelength of 10^{-8}-10^{-12} m. X-rays can directly damage DNA if they pass through the nucleus of a cell. In addition (and probably resulting in a greater level of damage), they can react with molecules in the cell leading to the formation of radicals, and secondary DNA damage. The presence of oxygen is required for the generation of secondary radicals, and the importance of hypoxia in radiation treatment has been recognised since the 1950's (Gray et al., 1953).

Tumours were irradiated using a Pantak X-ray generator. A stream of electrons is accelerated through an electric field. X-rays are released when the electrons hit a tungsten plate. Aluminium and copper filters are used to block lower energy X-rays, and a filter is used to make the energy field more uniform.

Local tumour irradiation was carried out without anaesthetic using specially designed lead jigs, exposing only the tumour bearing rear dorsum to a horizontal X-ray beam (Sheldon & Hill, 1977). 6 mice were simultaneously irradiated with 240 kV X-rays to give a total dose of 2 Gray (Gy) per tumour (divided into 2 doses of 1 Gy to each side of the tumour) per day to give a total dose of 10 Gy. Radiation was administered 20 min following prodrug in 5 fractionated doses on consecutive days. Prodrug administration was continued daily for a total of 21 days (see figure 7.1). 2 Gy fractions were chosen as being clinically relevant.

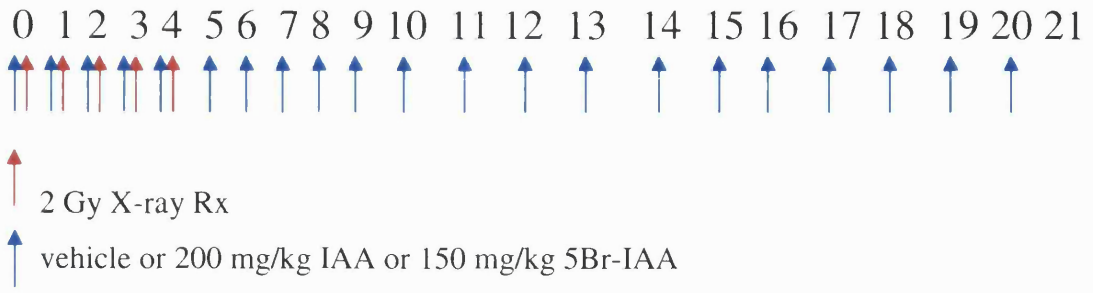


Figure 7.1. Schematic diagram of radiation and prodrug treatment schedule.

7.3 Results

7.3.1 *Ex vivo* clonogenic analysis of HRP GDEPT efficacy

Ex vivo clonogenic assays were used to determine cell kill in response to a single administration of prodrugs to animals bearing tumours derived from GFP1 or HRP8 cells (figure 7.2). A dose of 200 mg/kg IAA gave a small reduction in HRP8 cell survival (60%) compared with vehicle treated controls. 1Me-IAA decreased the clonogenicity of HRP8 tumour cells by 65%, and 5Br-IAA led to levels of cell kill less than the other prodrugs (45%). Administration of all prodrugs to animals bearing GFP1 tumours did not decrease cell survival, and apparently led to an increase in clonogenicity of cells.

However, the plating efficiency in all experiments was extremely low (less than 0.001%), and hence an alternative method was investigated, as this plating efficiency indicates that an atypical cell fraction is being observed. A soft agar assay (Smith et al., 1978) was used to attempt to increase the colony forming ability of cells. FaDu cells still failed to form colonies of sufficient size, or of a reproducible level (results not shown).

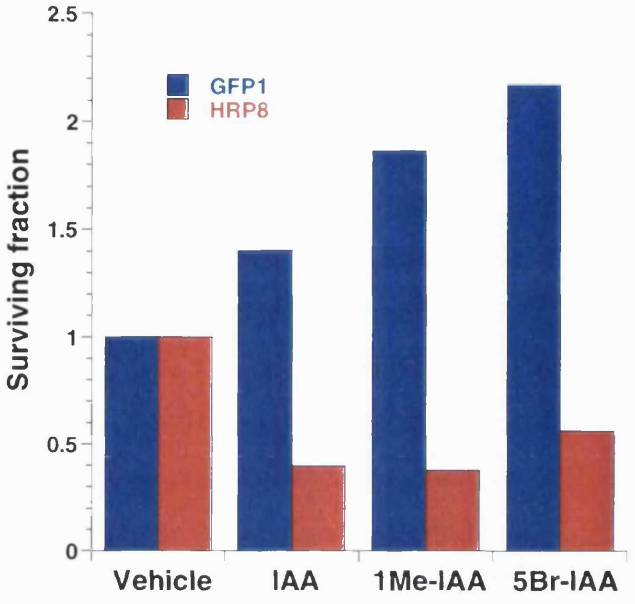


Figure 7.2. Clonogenic survival of cells isolated from FaDu tumours 24 h after administration of prodrugs. Data are mean 2 animals per group.

7.3.2 Tumour growth delay to analyse HRP GDEPT efficacy

Tumour bearing animals were treated with prodrug when tumours reached 5-6 mm in diameter. Growth delay experiments with a 7 day dosing period were performed with all 3 prodrugs. Administration of IAA at 200 mg/kg for 7 days gave a small growth delay of approximately 5 days in HRP8 tumours (figure 7.3). There was no effect of vehicle on tumour growth, and IAA did not have any significant effect on the growth of GFP1 tumours, although growth seemed to be slightly accelerated.

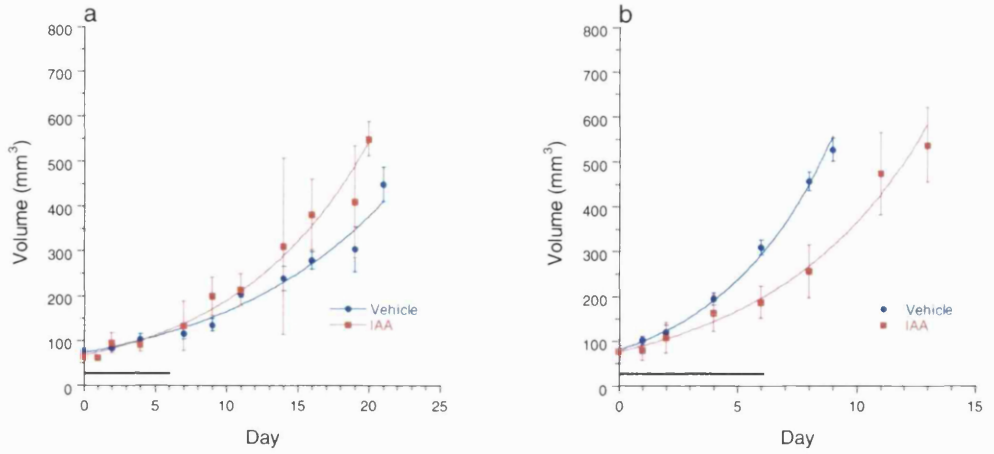


Figure 7.3. Growth of a) GFP1 or b) HRP8 tumours, following 7 days treatment with IAA 200 mg/kg. Data are mean ± s.e.m. 5 animals per group.

The 1Me-IAA compound had no effect on the growth of HRP8 tumours, nor did it alter the growth rate of the GFP1 tumour compared to vehicle alone (figure 7.4).

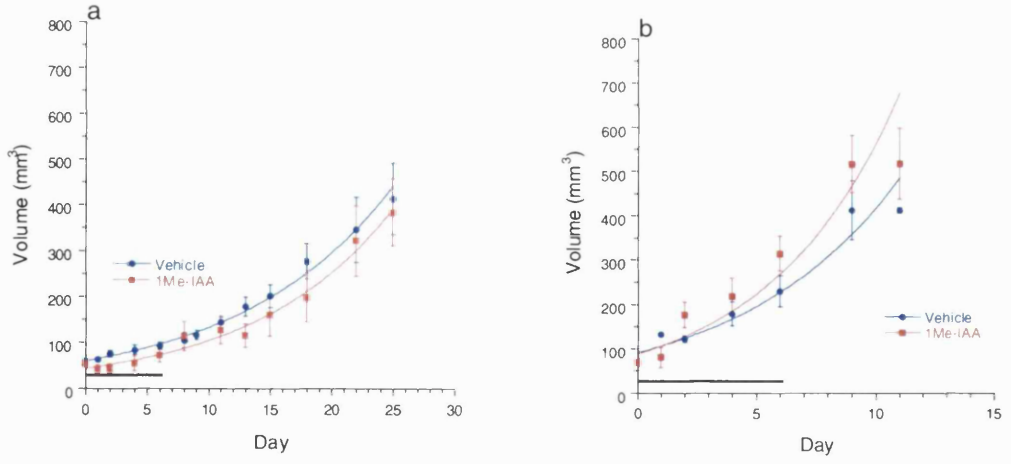


Figure 7.4. Growth of a) GFP1 or b) HRP8 tumours, following 7 days treatment with 1Me-IAA 200 mg/kg. Data are mean ± s.e.m. 5 animals per group.

The halogenated indole, 5Br-IAA, resulted in approximately 5 days growth delay in HRP8 tumours when administered at 200 mg/kg (figure 7.5). However, following three 5Br-IAA administrations, one HRP8 animal was found dead, and two GFP1 animals were found dead following four administrations. It would seem that

this dose of 5Br-IAA although without effect in non-tumour bearing animals, is toxic to those with tumours.

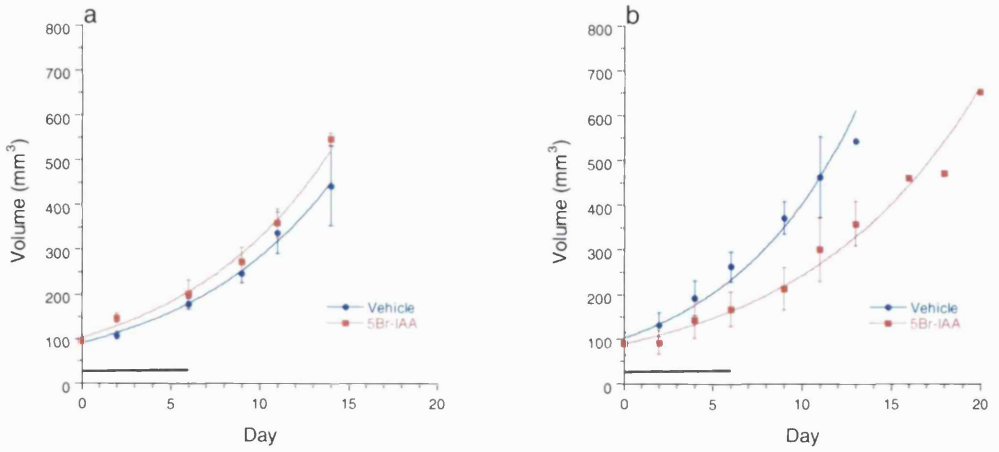


Figure 7.5. Growth of a) GFP1 or b) HRP8 tumours, following 7 days treatment with 5Br-IAA 200 mg/kg. Data are mean ± s.e.m. 5 animals per group, decreasing to 4 on day 3 for HRP8 and 2 on day 4 for GFP1.

In order to achieve a greater exposure of drug and reduce drug toxicity, administration of prodrug was attempted twice daily (b.i.d.) 5Br-IAA at 50-150 mg/kg in 10% ethanol (figure 6.6). This appeared to slow growth in HRP8 tumours, especially at the lower dose of 50 mg/kg. However, there were adverse effects, and all animals at this dose had to have treatment stopped after 4 days due to unacceptable losses of body weight (10%) although in most cases this recovered upon cessation of treatment (figure 7.7). This loss of body weight was also seen to a lesser degree in vehicle treated animals.

Animals bearing GFP1 tumours also displayed decreased body weights with b.i.d. dosing, although to a far lesser degree than HRP8 tumour bearing animals. The tumour growth also appeared to be slightly slower in 5Br-IAA treated groups.

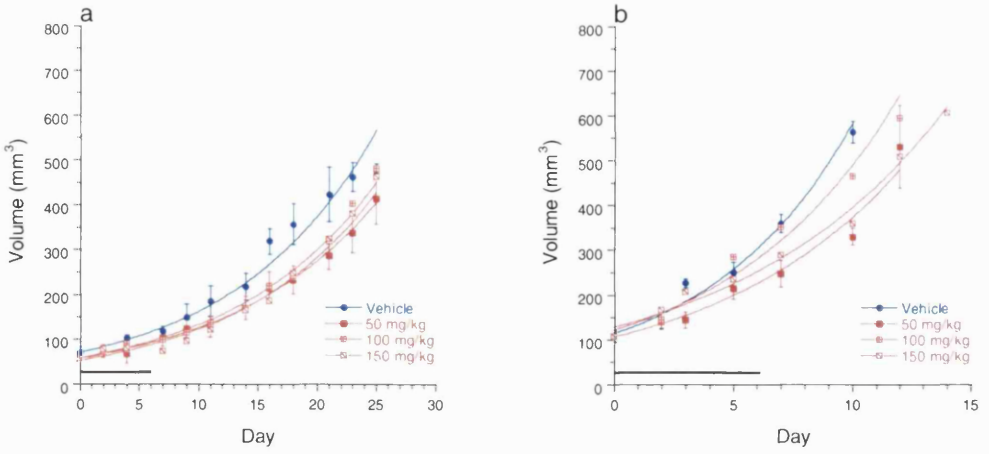


Figure 7.6. Growth of a) GFP1 tumours and b) HRP8 tumours, following 7 days treatment with 5Br-IAA b.i.d. at the doses indicated. Data are mean ± s.e.m. 5 animals per group. Bar represents period of treatment.

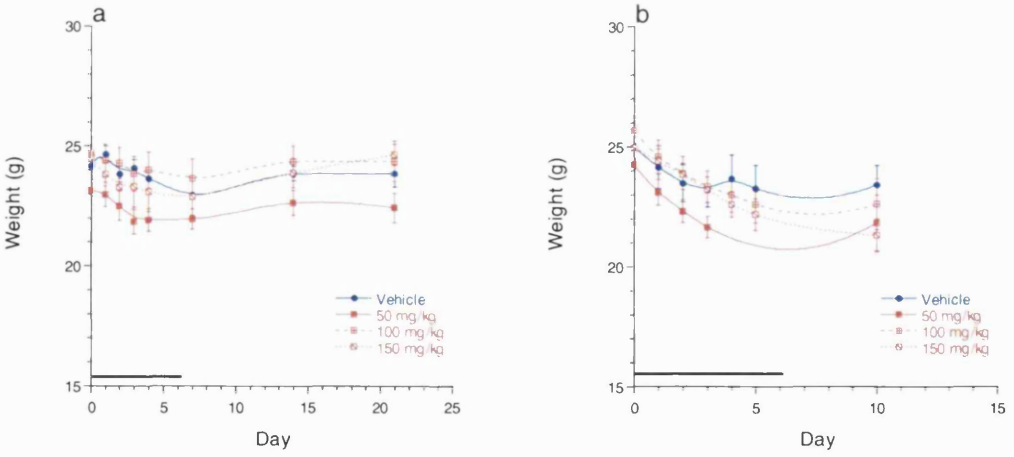


Figure 7.7. Weight of animals with a) GFP1 tumours and b) HRP8 tumours, following 7 days treatment with 5Br-IAA b.i.d. at the doses indicated. Data are mean ± s.e.m. 5 animals per group. Bar represents period of treatment.

Due to the discrepancies between growth delay experiments and due to differences in pharmacokinetic behaviour (Chapter 6), growth delay experiments were repeated to directly compare the effect of vehicle on prodrug activity using either 10% ethanol or 5% DMSO. No difference was detected between vehicle and indole treated for either GFP1 or HRP8 tumours (figure 7.8).

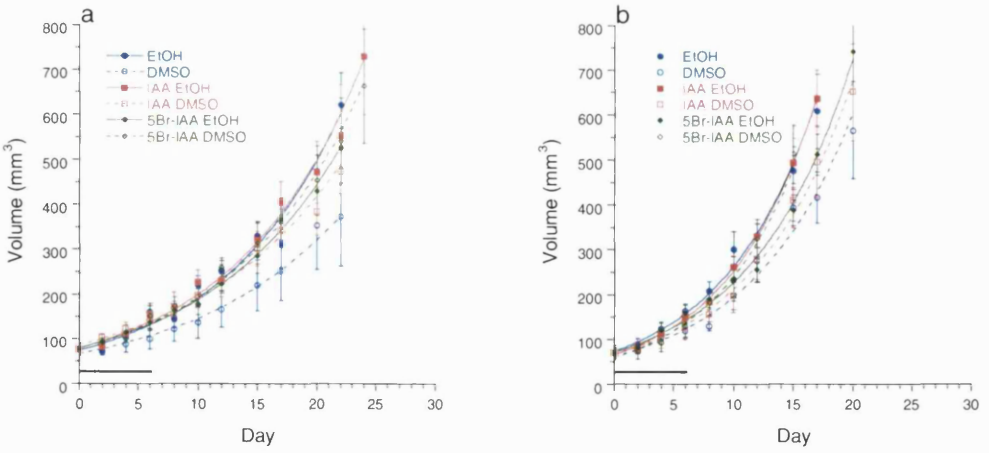


Figure 7.8. Growth of FaDu a) GFP1 or b) HRP8 tumours following 7 days treatment with prodrug. Data are mean ± s.e.m. 5 animals per group.

7.3.3 Combination of HRP GDEPT with radiotherapy

Gene therapy is unlikely to be used as single treatment modality in the clinic, and will most likely be combined with radiotherapy. Hence, interaction between the HRP gene therapy approach and fractionated radiotherapy was investigated. Fractionated radiation was administered 20 minutes following prodrug administration. FaDu parental tumours served as controls rather than GFP1 tumours since GFP tumours showed a significant reduction in tumour growth without treatment. Radiation alone resulted in approximately 25 days of growth delay in both parental and HRP8 tumours (figure 7.9). Tumours remained at the initial treatment size for approximately 1 week following the cessation of radiation treatment, then increased but with a slower growth rate than tumours not exposed to X-ray irradiation, probably due to an effect on the supporting tissue of the tumour, such as the vasculature. Prodrug treatment appeared to slow growth slightly in parental tumours, although this was not significant.

HRP8 tumours treated with indoles (IAA and 5Br-IAA) for 21 days showed a tendency to decreased tumour volume compared with vehicle treated controls, however this was not significant. Addition of indoles to radiation treatment did not further enhance the effectiveness of this therapy

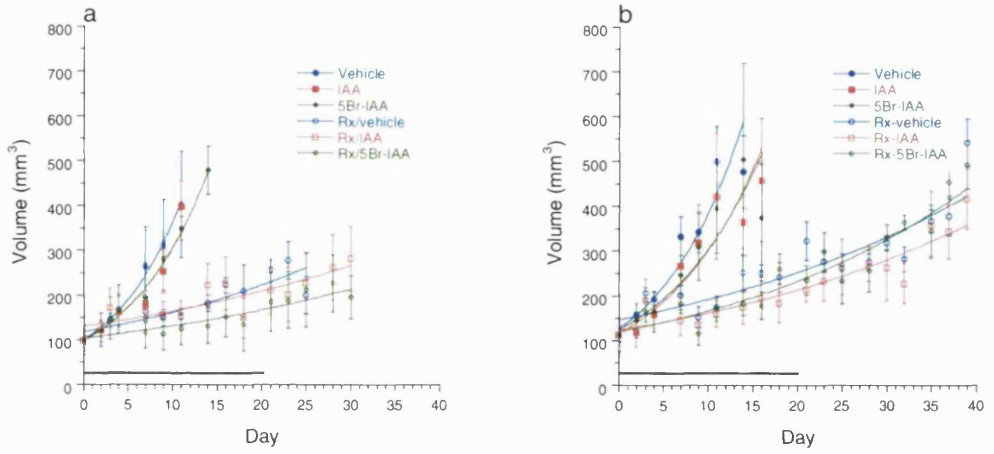


Figure 7.9. Growth of a) parental or b) HRP8 FaDu tumours. Animals received either prodrug, radiation or a combination. Data are mean ± s.e.m. 5 animals per group. Bar represents period of drug treatment.

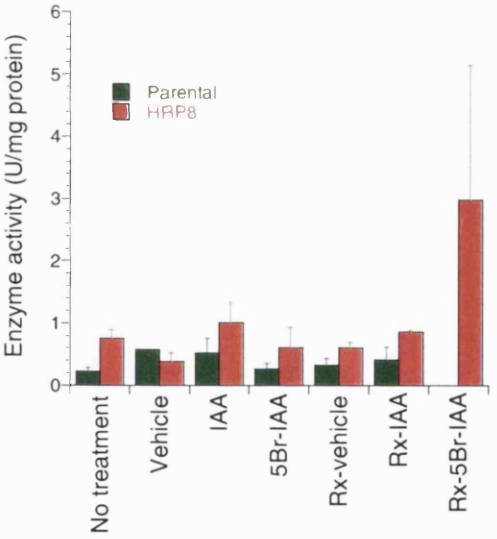


Figure 7.10. Results from TMB assay for tumours excised from animals in receipt of drug and/or radiation therapy when reaching approximately 500 mm^3. Data are mean ± s.e.m 2-4 tumours per group.

To determine whether HRP-producing cells were selectively killed by prodrug treatment, enzyme activity in treated tumours was assessed. With the exception of vehicle controls, there was a tendency for enzyme activity to be greater in HRP8

tumours than in parental, although this was not significant for any of the treatment groups (figure 7.10). Necrosis was also scored in tumours using haematoxylin and eosin stained frozen sections. There was no difference in score between treatments in the HRP8 tumours (data not shown).

7.4 Discussion

In this chapter the feasibility of using the HRP GDEPT strategy in whole animal models was assessed.

Ex-vivo clonogenic assays performed on FaDu tumours indicated that drug treatment of HRP8 tumours decreased cell survival, whilst treatment of GFP1 tumours appeared to increase cell survival. This increase in clonogenic ability was not seen in GFP1 cells *in vitro*, but IAA has been reported to increase the growth of mouse fibroblast cells, possibly through adenylate cyclase systems (Abu Sinna, 1983). IAA can also induce tumours in *Phaseolus vulvaris* L bean embryos (Sen & Stevenson, 1977).

However, the low plating efficiency of standard *ex vivo* clonogenic assays may make the results unreliable, as was the case with FaDu tumour cells plated *ex vivo*. In order to overcome this problem, a soft-agar clonogenic assay was used instead; the agar forms support for the cells, and discourages the growth of normal stromal cells (Courtenay & Mills, 1977; Sanders & Burford 1964). This approach did not improve plating efficiencies. It would be possible to further supplement the agar with rat red blood cells, however, that was not attempted in these experiments since it would require additional animal usage.

Treatment of tumours for seven days with either IAA (200 mg/kg/day in 10% ethanol) or 5Br-IAA (200 mg/kg/day in 10% ethanol) resulted in 1.5 and 2 day increases in doubling time, or 5 days growth delay, respectively. Differences in tumour volume began to appear at approximately day four and were pronounced on day six. Growth delay indicates a substantial level of cell kill. These early results were encouraging, in that they demonstrated, for the first time, an anti-tumour effect of the HRP GDEPT system *in vivo*. Further modifications in the treatment regime were subsequently carried out to improve the anti-tumour activity.

The lack of effect with 1Me-IAA however was surprising, since this prodrug produced similar levels of cell kill *in vitro* to IAA (figure 3.7). Pharmacokinetic analyses have not yet been performed using 1 Me-IAA, and it is possible that the compound did not reach the tumour tissue to the same degree as either IAA or 5Br-IAA. It is also possible that *in vivo* cell kill was insufficient to result in measurable growth delay. It is possible that as has been shown *in vitro*, (Abu Sinna, 1983), indoles are capable of increasing the growth rate of cells grown *in vivo*. Although the growth rate of GFP1 transfectants was increased by IAA, the rate did not reach the growth of parental cells or HRP8 cells grown *in vivo*. Also, the indoles did not appear to increase the growth of parental FaDu cells grown as subcutaneous tumours in the radiation studies (figure 7.9). It is unclear why this growth effect should be manifested in GFP1 tumours only. Because of the apparent lack of activity, no further *in vivo* studies were performed using the methylated indole.

In order to improve the modest levels in tumour response and to increase tumour exposure to prodrug, as well as to maintain an elevated concentration for longer, 5Br-IAA was administered (in 10% ethanol) b.i.d. for seven days at a range of doses. Although there appeared to be some decrease in growth rate it was not dose dependent. In addition, animals did not tolerate this schedule well, as indicated by a decrease in body weight. This also did not seem to be dose related. Interestingly, growth delay and weight loss were most pronounced at 50 mg/kg, the lowest dose used. Whether the two factors are related is unclear. Vehicle alone caused some decrease in body weight, and it may be that the animals were particularly sensitive to the ethanol.

Combination of GDEPT treatment with radiation is likely to be important in clinical protocols, and has been used experimentally with HSV-tk/GCV (Atkinson & Hall, 1999; Chhikara et al., 2001; Vlachaki et al., 2001), CD/5-FC (Hamstra et al., 1999; Hanna et al., 1997; Kievit et al., 2000; Stackhouse et al., 2000) and NADPH reductase/bioreductives, with at least an additive effect on tumour response. HRP/IAA treatment *in vitro* showed significant radiosensitisation at clinically relevant X-ray doses under both oxic and anoxic conditions (Greco et al., 2002c). The hypoxic targeting of this GDEPT strategy in combination with radiation is therefore a

promising therapeutic modality, since radiation is effective in causing growth delay and regression, but often results in sparing of hypoxic regions.

Unfortunately combination of the HRP GDEPT system with radiation *in vivo* failed to increase the growth delay seen with a fractionated radiotherapy schedule alone. No significant growth delay was seen in HRP8 tumours treated with prodrug. In this experiment DMSO was used in place of ethanol as the solvent, since our pharmacokinetic data had showed an increase in tumour half life and tumour AUC. A different prodrug solvent may alter the cytotoxicity of indoles, but treatment of monolayers with IAA in either 10% ethanol or 5% DMSO resulted in virtually identical survival curves (chapter 3). Cytotoxicity at lower doses of IAA was slightly decreased when DMSO was used, which may partly explain the lack of effect *in vivo*. However DMSO also affected the pharmacokinetics of IAA, with a longer time to peak tumour concentration (chapter 6).

In vitro, the interaction between the HRP/IAA combination was present regardless of whether IAA was present before, during or after irradiation (Greco et al., 2002c), therefore it is unlikely that the tumour effect *in vivo* was effected by the scheduling.

In our study female SCID mice were used throughout. SCID mice have been reported to show radiation hypersensitivity (Fulop & Phillips, 1990) due to a deficiency in the repair of double strand breaks (Biedermann et al., 1991; Hendrickson et al., 1991). Since the results were not being compared with animals not carrying the SCID mutation it is unlikely to adversely influence results. Previous studies within the Gray Cancer Institute have demonstrated that fractionated radiotherapy specifically to the tumour does not adversely affect the health of SCID mice (S.A. Hill, personal communication).

Animals used for the radiation therapy experiment had tumours implanted on the back to allow the tumour to protrude from the lead jig. However, in the initial growth delay experiment tumours were implanted on the flank. It is possible that differences in growth site of the tumour could result in changes affecting treatment outcome, due to differences in blood flow, supporting stroma and drug access.

In order to address the issue of prodrug solvent on tumour effect, growth delay experiments were repeated directly comparing ethanol and DMSO as vehicles with

IAA and 5Br-IAA prodrugs and a 7 day dosing schedule. Unfortunately in this study neither prodrug was able to decrease the growth of HRP8 or GFP1 tumours. Tumours were implanted in the flank, and it is therefore unlikely that the site of tumours was an influencing factor in the lack of growth delay in radiation experiments. Even though it is possible that there may have been cell kill in the HRP8 tumours, this was insufficient to result in either cytostasis or growth delay probably due to repopulation with viable cells.

In vitro the HRP/IAA system in direct comparison with the HSV-tk/GCV system, compares very well. However, the *in vivo* results appear to be disappointing in comparison with other gene therapy systems also using stable transfectants. Some of these combinations utilise prodrugs which alone can result in cell death (such as the mustard prodrugs), and the intratumoural presence of activating enzymes enhances the anti-tumour activity. Other systems utilise murine cell lines and immunocompetent mice, which may increase the bystander effect. It may be that the low level of expression in the stable transfectants, coupled with potential transgene silencing *in vivo* results in activities which do not exceed the minimal level of activity needed for efficient substrate catalysis. If the indoles have a growth promoting role in the GFP1 tumours it is conceivable that any cell death induced by the HRP/IAA system would have to counter the growth promoting effects of the indole alone in order to achieve any measureable growth delay. However, the lack of growth promoting ability in the parental FaDu cells complicates this, and it may be a peculiar feature of the GFP1 tumours.

Without a functional clonogenic assay it is difficult to determine whether the HRP/IAA combination is influencing cell survival. A possible alternative would be to use apoptosis markers to assess levels of apoptosis in tumour sections, such as the TUNEL assay. This would allow quantitation of this type of cell death.

In conclusion, it appears that the use of stable transfectants may not be a good model to test this GDEPT strategy. In the clinic, *in vivo* gene transfer would be used, and the mosaic pattern of high gene-expressing cells amongst non-expressors may be more effective in the HRP/IAA system than the low level distribution of HRP activity achieved in our system.

Chapter 8

Immunogenicity of GFP and HRP

8.1 Introduction

The presence of exogenous genetic material in cells in immunocompetent hosts may lead to an immune reaction in response to foreign proteins. This has been observed with non-therapeutic genes, such as the marker, GFP (Stripecke et al., 1999).

The presence of the suicide gene cytosine deaminase (CD) led to the spontaneous regression of tumours, which was accelerated by 5-fluorocytosine (5-FC) treatment (Haack et al., 2000; Pierrefite-Carle et al., 2002). Treatment of tumours expressing CD or herpes simplex virus-thymidine kinase (HSV-tk) genes with 5-FC or gancyclovir (GCV), respectively, has been reported to prevent growth upon rechallenge with the same cells, or cells from the parental cell line, but not cells of a different origin (Caruso et al., 1993; Haack et al., 2000; Kuriyama et al., 1999a; Kuriyama et al., 1999b; Vile et al., 1994b). In addition, mice bearing liver tumours from a non-modified cell line subsequently challenged s.c. with CD expressing cells of the same origin, and 5-FC treatment showed decreased growth of the existing liver tumour (Pierrefite-Carle et al., 2002).

The mechanism of this "distant bystander" effect is unclear. Groups have demonstrated increased infiltration of $CD4^+$ and $CD8^+$ cells (Caruso et al., 1993; Haack et al., 2000; Kuriyama et al., 1999a; Kuriyama et al., 1999b), natural killer (NK) cells (Pierrefite-Carle et al., 1999) and major histocompatability complex (MHC) restricted cytotoxic lymphocytes (Haack et al., 2000). The distant bystander effect may be important for the full therapeutic potential of suicide gene therapy to be realised. It also has the potential to result in therapeutic effects in metastases not targeted by the initial gene transfer.

This chapter describes the production of a murine cell line expressing HRP and the assessment of its immunogenicity in mice syngeneic with the original wild-type tumour cells.

8.2 Materials and methods

Cell culture, transfection, transgene assays and cytotoxicity testing were performed as described in chapter 2.

8.2.1 Restriction enzyme reactions

CaNT cells are intrinsically resistant to puromycin. Therefore, a new HRP plasmid was constructed with the neomycin resistance gene for selection in genetecin (G418), and used with the pEGFP-N1 plasmid (Invitrogen) as a control.

Plasmid pssHRP-puro was kindly provided by Dr O. Greco, Gray Cancer Institute. pCI-neo was obtained from Promega (Southampton, UK). DNA was digested with restriction endonucleases (NE Biolabs, Hitchen, UK), using the manufacturers' recommended reaction buffers and conditions. Multiple digests were carried out in compatible buffers. Digest reaction mixtures consisted of 5-20% DNA (1-4 μg), 1x reaction buffer, 1-5 units of enzyme (1 unit being the amount which will digest 1 μg DNA in one hour), and distilled water.

To produce pssHRP-neo (figure 7), pssHRP-puro and pCI-neo were digested with *Bsr*G1 and *Not* 1 to produce fragments with "sticky ends". The 2193 bp fragment from pssHRP-puro was ligated to the 4437 bp fragment of pCI-neo. The presence of HRP was confirmed by enzyme digests and transient transfection of FaDu cells followed by HRP enzyme activity assays.

8.2.2 Gel electrophoresis

For sizing and separation of DNA, a horizontal submerged agarose gel system was used. Samples were analysed on 1% agarose gels (Ultra Pure agarose, Life Technologies, UK), prepared by dissolving agarose in 1xTAE buffer (Tris-acetate-EDTA) containing 100 μg/ml ethidium bromide (Sigma, UK). Molten agarose was cast in a horizontal tray fitted with well forming combs, and was submerged in 1 x TAE buffer when set.

Prior to loading, one-sixth total volume tracking dye was added to DNA samples. The gel was then subjected to electrophoresis at 100 V for up to 2 h, with a DNA ladder as size marker (Life Technologies). DNA bands were visualised using a 254 nm transilluminator, and photographed using a Polaroid MP4 Camera and 667 film (Sigma).

Buffers and reagents

Tris-acetate EDTA (TAE) 50x:

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml
dH_2O	to 1 l

Gel tracking dye 6x:

Bromophenol blue	0.25 g
Sucrose	40 g
EDTA (0.5 M, pH 8.0)	4 ml
dH_2O	to 100 ml

8.2.3 Extraction of DNA from gels

Appropriate bands, visualised on a transilluminator, were excised from the gel with a sterile scalpel blade, and weighed in a 1.5 ml microfuge tube. DNA was isolated using the Concert rapid gel extraction system (Life Technologies). Briefly, 30 µl of Gel Solubilisation Buffer was added for every 10 mg of gel, and incubated at 50°C for approximately 15 min until the gel slice had completely dissolved. A further 5 min was then added to the incubation time. A cartridge was placed in a 2 ml wash tube and the dissolved gel mixture added, and centrifuged through the column at 12,000 g for 1 min followed by a wash in the same buffer. The cartridge was washed a second time with 700 µl Wash Buffer (which contains ethanol), incubated at room temperature for 5 min, centrifuged as before, and the flow-through discarded, and centrifuged again to remove any residual buffer. DNA was eluted with 50 µl warm (65°C) water added to the top of the cartridge, incubated for 1 min at room temperature, then centrifuged at 12,000 g for 2 min.

8.2.4 DNA ligation

Ligation was performed overnight at room temperature in a total volume of 20 µl, with 1 µl T4 DNA ligase (Life Technologies), and 2 µl 10x reaction buffer. Insert DNA concentration was approximately 4 times that of the concentration of the vector DNA.

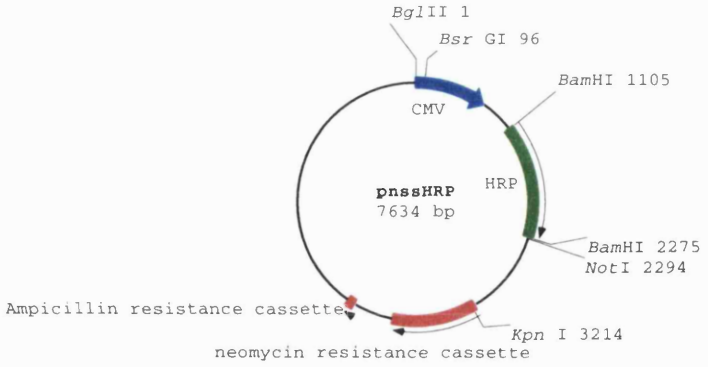


Figure 8.1. HRP plasmid constructed for use in CaNT cells.

8.2.5 Transformation of prokaryotic cells

Commercially available *Escherichia coli* Top 10 cells (Invitrogen, Paisley, UK) were transformed with the pssHRP-neo plasmid following the manufacturer's instructions.

Briefly, cells were thawed on ice, 5 µl DNA added to 20 µl cells, and incubated on ice for 30 min to allow DNA to be taken up. Cells were heat-shocked for 30 sec at 42°C, and then placed on ice for 2 min, in order to induce the enzymes involved in DNA repair and cellular processes associated with the recovery from transformation stress. 180 µl of warmed SOC medium was added and cells shaken at 37°C for 2h to allow expression of the plasmid.

100 µl of bacteria were spread on an agar plate containing ampicillin (100 µg/ml, Life Technologies). The remaining bacteria were diluted 1:10, and 100 µl spread on a second plate. Single colonies were isolated and grown on a small scale for confirmation of transformation, and plasmid integrity.

8.2.6 Immunogenicity of CaNT tumour cells

The immune response to HRP- and GFP-expressing tumours was assessed using 2 groups of animals, naive controls, and those which were pre-immunised with the appropriate tumour cell line, to stimulate the immune system. The immune response to the CaNT tumour line maintained *in vivo* was also assessed. This work was carried out in collaboration with Dr S. Hill and G. Lewis.

In experiments using cells from *in vitro* culture, pre-immunisation was carried

out by administration of an intraperitoneal (i.p.) injection of 3 x 10^6 cells (parental CaNT, CaNT GFP2 or CaNT HRP7) irradiated with 250 Gy ^{60}Co. 10-15 days later animals received a second identical immunisation. 10-15 days following the second immunisation, animals were injected with live cells subcutaneously (s.c.) in 4 sites on the back. The number of cells implanted ranged from $3x10^2$ to 10^6, 2 animals per group. The same schedule of live cell challenge was administered to control, non-immunised groups at this time.

This schedule was repeated for CaNT cells isolated from tumours as in 7.2.1. Cells were counted using trypan blue exclusion to ensure viable cells were implanted. 4 animals per group were used in this study.

Animals were monitored twice weekly for the appearance of tumours, and scored when tumours appeared. Visible tumour burden was not allowed to exceed 5% of total body weight.

The tumour immunogenicity was defined using an immunogenicity index:

$$\frac{TD_{50}\text{ immunised animals}}{TD_{50}\text{ control animals}}$$

Where TD_{50} was the number of cells required to produce tumour growth in 50% of implant sites.

8.3 Results

8.3.1 Creation of stable transfectants

CaNT cells were transfected with either the pEGFP-neo or pssHRP-neo plasmid. Transient efficiencies for pEGFP-neo were approximately 63% as assessed by FACS analysis. Stable clones were isolated, and named GFP2 (figure 8.2) that had expression of GFP in 92% of gated cells, although the population was not pure. The HRP clone was named HRP7 (figure 7.3) and had HRP activity equivalent to that of FaDu HRP8 clones.

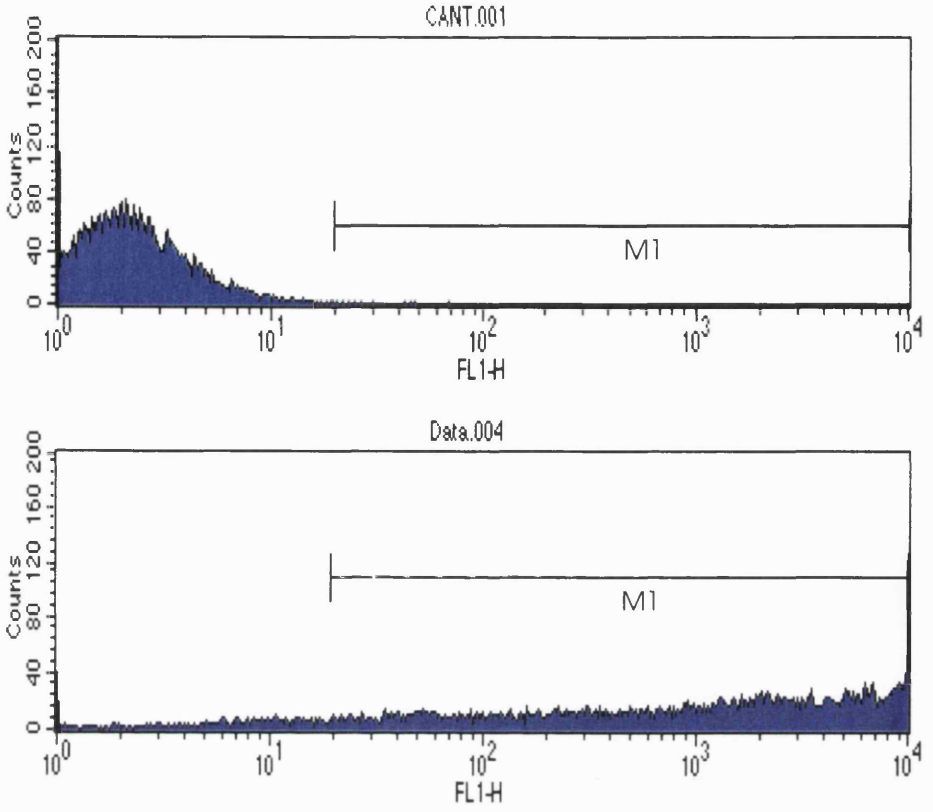


Figure 8.2. FACS results for untransfected CaNT cells (upper panel) and GFP2 clone (lower panel). Cells scored positive are marked using the M1 gate (untransfected, 0.1%, GFP2 92%).

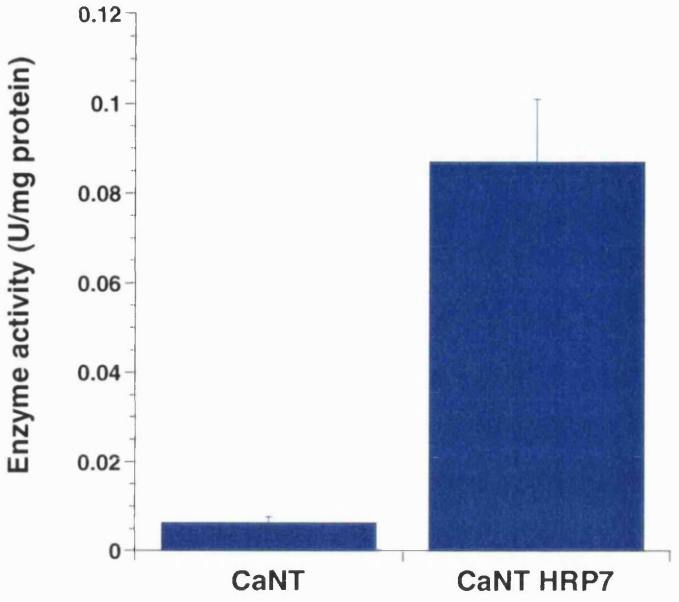


Figure 8.3. HRP activity in parental CaNT or cells stably transfected with the HRP gene. Data are mean ± s.e.m. minimum 3 samples.

8.3.2 Efficacy of HRP/indole GDEPT

Sensitivity of stable CaNT clones to IAA was assessed in clonogenic assays. When exposed to IAA for 4 h, HRP7 clones showed decreased survival compared with GFP transfected controls (figure 8.4). After 24 h exposure to IAA, HRP7 cells showed a similar response to that seen in FaDu HRP8 cells, with a plateau followed by a sharp decrease in survival at 5 mM. However, the GFP2 controls seemed to be very sensitive to IAA over the longer exposure period, and at concentrations of 3 mM and above, survival of GFP2 cells also decreased significantly.

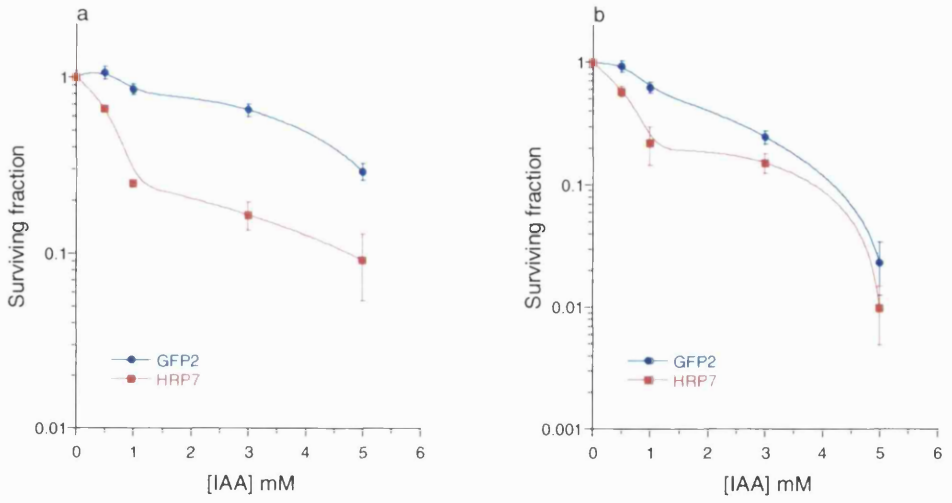


Figure 8.4. Survival of GFP2 and HRP7 cells following a) 4 h, or b) 24 h exposure to IAA. Data are mean ± s.e.m. 3 experiments, triplicate samples.

8.3.3 Immunogenicity of CaNT cells

Cells implanted s.c. in female CBA mice without prior immunistation of animals with heavily irradiated cells resulted in the formation and spontaneous regression of tumours (figures 8.5a, 8.6a, 8.7a).

Parental CaNT cells implanted into mice showed tumour development at 10^6 cells, which regressed after 5 days. Tumours in immunised mice then started to grow again, although after 50 days, less than 50% of implant sites showed any tumour (figure 8.5b).

CaNT cells expressing GFP (GFP2 cells) implanted into mice which had not been immunised with GFP2 cells at 10^6, $3x10^5$, 10^5, and $3x10^4$, formed tumours in 100%, 63%, 13% and 74% of sites respectively. All of these tumours subsequently regressed and did not reappear. In immunised mice, tumours were apparent in 50% and 75% of sites at 10^6 and $3x10^5$ cells respectively. These also regressed and did not regrow. Growth was monitored for 50 days.

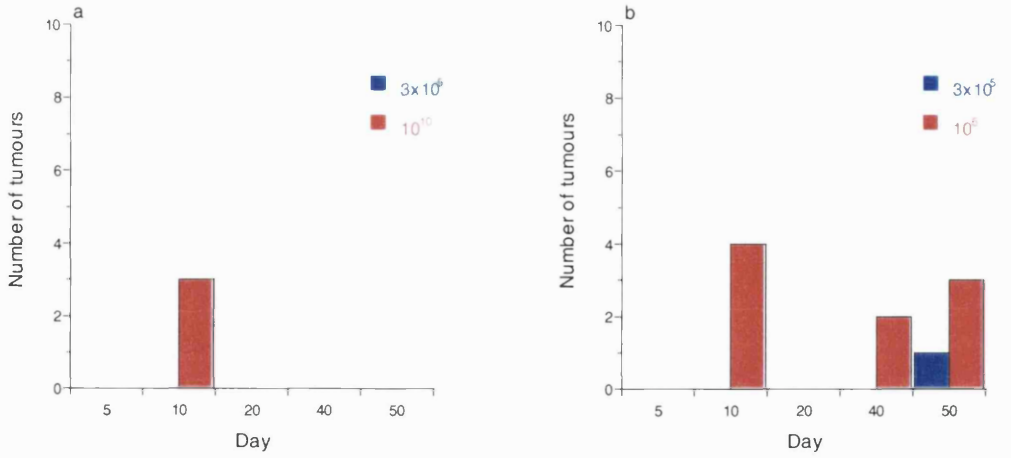


Figure 8.5. Appearance of visible tumours in CBA mice implanted with parental CaNT tumour cells s.c. at 4 sites. Data show the number of visible tumours at each time point after implantation, 2 mice per group, maximum of 8 potential tumours per cell implant number. a) Cells implanted in naïve mice b) implants in immunised mice.

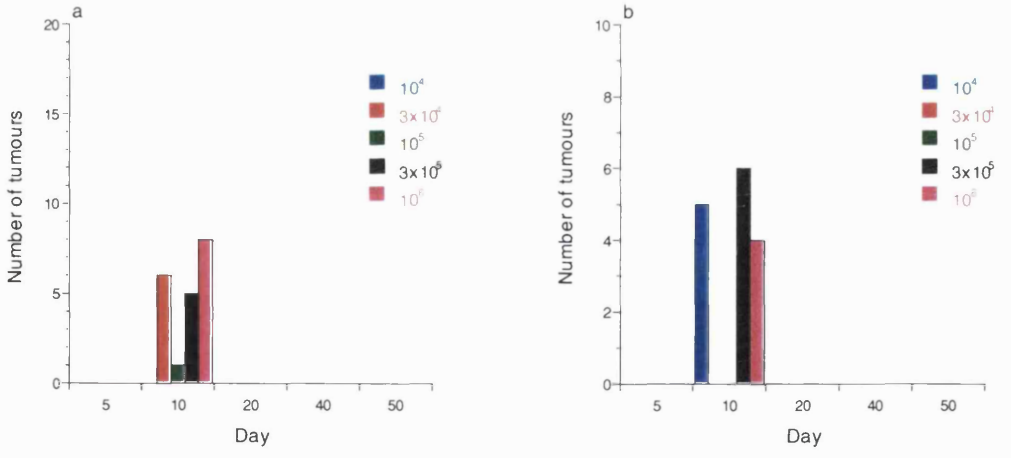


Figure 8.6. Appearance of visible tumours in CBA mice implanted with CaNT GFP2 tumour cells s.c. at 4 sites. Data show the number of visible tumours at each time point after implantation, 2 mice per group, maximum of 8 potential tumours per cell implant number. a) Cells implanted in naïve mice b) implants in immunised mice.

HRP7 cells implanted s.c. in naïve mice formed tumours after 7 days at the 2 highest implant numbers which subsequently regressed. Tumours were again apparent 10 days later. At the lower implant numbers of 10^5-10^4, tumours were visible after 30 days at one site out of 8, but further formation was not observed during the 50 day

period.

Immunisation of mice followed by challenge with HRP7 cells gave similar initial growth as naïve mice. However, tumours reappeared at 100% of sites when 10^6 cells were innoculated. 50% of sites also showed tumours when $3x10^4$ cells were implanted, and these persisted for 15 days, longer than initial tumours in other groups, which lasted at most, 6 days before numbers decreased.

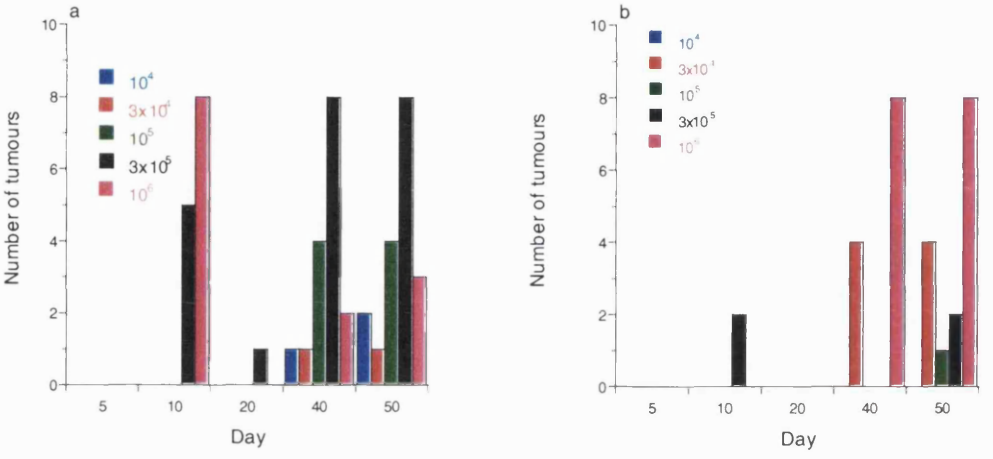


Figure 8.7. Appearance of visible tumours in CBA mice implanted with CaNT HRP7 tumour cells s.c. at 4 sites. Data show the number of visible tumours at each time point after implantation, 2 mice per group, maximum of 8 potential tumours per cell implant number. a) Cells implanted in naïve mice b) implants in immunised mice.

CaNT tumours are generally maintained by *in vivo* rather than *in vitro* transplantation. Immunisation and implantation of CaNT cells from *in vivo* tumours, rather than from cells cultured continuously *in vitro*, resulted in the formation of tumours that did not regress (figure 8.8). The TD_{50} value was below 10^3 cells, and there was no difference between immunised and control mice.

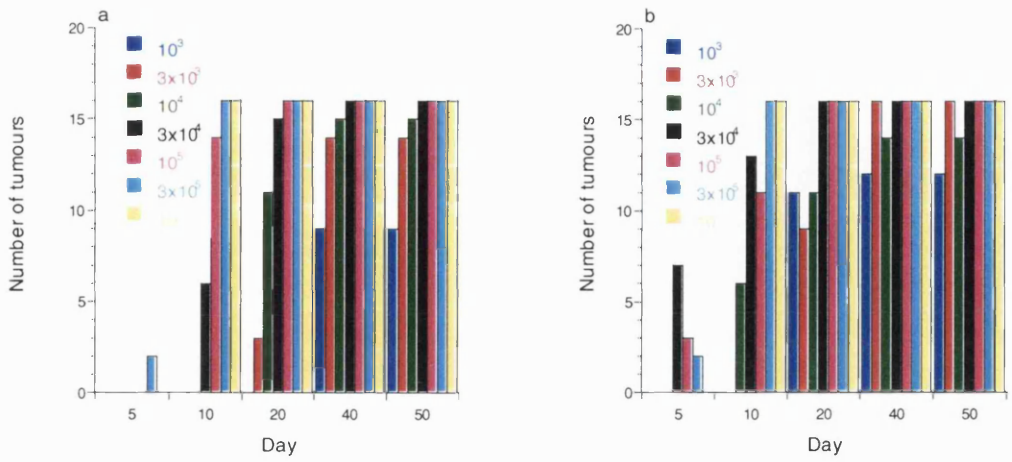


Figure 8.8. Appearance of visible tumours in CBA mice implanted with CaNT tumour cells s.c. at 4 sites. Data show the number of visible tumours at each time point after implantation, 4 mice per group, maximum of 16 potential tumours per cell implant number. a) Cells implanted in naïve mice b) implants in immunised mice.

8.4 Discussion

To determine whether the HRP protein itself was immunogenic, and would contribute to any cell killing by this GDEPT system in immunocompetent animals, experiments were conducted to determine the impact of the protein on tumour formation and growth.

Using a murine cell line (CaNT) stable clones were produced expressing either GFP or HRP (GFP2 and HRP7 respectively). These were tested for their response to treatment with IAA *in vitro*, and although short exposure times showed the expected sensitisation to IAA by HRP, there was little difference between the HRP and GFP clones following 24 h exposure to IAA. The reason for this discrepancy is not clear. CaNT cells may be intrinsically more sensitive to IAA, although perhaps greater cell kill in HRP7 cells would have been expected if this were the case, or at least a diminution of the plateau phase.

The GFP2 clone may have inserted the exogenous DNA within its genome at a sensitive point, which may have resulted in decreased ability to repair any damage produced by IAA itself on the cell, although this again would depend upon the CaNT cell line being more sensitive than others studied. The HMEC-1 endothelial cell line used in previous studies also showed intrinsic sensitivity to IAA in an MTS

proliferation assay (Greco et al., 2001) following 24 h exposure.

The immunogenicity of CaNT GFP cells *in vivo* was not unexpected, as GFP has previously been reported to elicit a strong immune response (Stripecke et al., 1999). Mice implanted with GFP2 tumours showed spontaneous regression and no regrowth, whereas parental CaNT and HRP7 tumours did show some re-emergence of tumours. However, the rejection of parental CaNT cells was unexpected. Rejection of syngeneic cells has been reported when tumours were induced by chemical means (Foley, 1953), and chemically induced tumours have therefore been described as a poor model of human cancer, since the tumour may represent an immunologically mutated tissue (Revesz, 1960). However, the CaNT tumour arose spontaneously in the Gray Cancer Institute animal colony (Hewitt et al., 1976).

A possibility is that the CaNT cell line and CBA mice are no longer syngeneic. "Venerable" tumours have been shown to be associated with immunogenicity lacking in spontaneous tumours of recent origin (Revesz, 1960). However, CaNT tumours are passaged *in vivo* to a maximum of 10, before returning to frozen stocks. The Gray Cancer Institute colony of CBA mice is highly inbred to greater than 50 generations. Therefore it is unlikely that there has been significant genetic drift over time, and we have shown that *in vivo* to *in vivo* transplantation of CaNT still results in the formation of tumours.

The most likely explanation is that passage *in vitro* is responsible for the immunogenicity observed. CaNT cells were isolated from tumour and passaged in tissue culture for approximately 5 months.

Passage of human tumour cells *in vitro* has led to groups observing the development of novel antigens apparently originating from bovine substances incorporated into the cell membrane from the foetal calf serum (FCS, (Irie et al., 1974), referred to as a heterologous membrane antigen (HM Ag). The amount of HM Ag present appeared to be dependent upon cell type rather than the length of time in culture or growth rate.

Decreased tumourigenicity of polycyclic hydrocarbon (PCH) induced carcinoma cells has been observed after as few as 5 passages *in vitro* (Jamasbi & Nettesheim, 1979). Immunisation of rats with *in vitro* cells led to resistance to tumour formation with cells passaged *in vivo*. However, *in vivo* cells were unable to provide

adequate immunisation against tumour challenge by *in vivo* cells. Passage of immunogenic cells from *in vitro* culture as ascites in one animal, was sufficient to restore tumourigenicity. It was suggested that *in vitro* growth of cells led to the unmasking of pre-existing antigens.

Growth of *N*-nitrosodiethylamine induced guinea pig hepatocarcinoma cells *in vitro* for 20 days in 10% foetal bovine serum (FBS) was sufficient to result in the spontaneous regression of intradermally implanted tumours (Correll et al., 1983). Animals were then resistant to challenge from fresh ascites cells. When cells from *in vitro* culture were injected i.p., ascites did form, and cells isolated from these were tumourigenic. Concentrations of FBS less than 10%, or the use of calf bovine serum or normal guinea pig serum were less effective at generating immunogenicity in cells. The authors postulated that the decreased growth rate seen when an alternative serum was utilised might have persisted *in vivo* allowing the host to respond. Indeed, a weak resistance may be masked by rapid proliferation of tumour cells. However, this was not the case with the PCH induced cell line used by Jamasbi and Nettlesheim. It was also suggested that heterologous serum activated T helper function. Serum alone is capable of increasing the activation of T cells from the spleen, and only an extremely small amount is necessary, since cells washed 3 times and implanted in salt solution retained their ability to sensitise animals (Lord, 1987).

The emergence of immunogenicity due to *in vitro* culturing appears to be widespread. In the follow-up results of a clinical trial for gene therapy of adenosine deficiency disorder, two patients were demonstrated to have antibodies against FCS (Tuschong et al., 2002). One patient developed increased IgG responses to FCS. In this trial autologous T cells had been grown in FCS containing media prior to transduction with a retrovirus. This also appeared to correlate with decreased vector DNA present in the patients' T cells.

In contrast to the former studies, (Chiba et al., 1987) showed that the immunogenicity of cells from methylcholanthrene (MCA) induced fibrosarcomas, was greater when grown in 1% FCS rather than in 10%. They suggested the presence of a new glycoprotein antigen which could be revealed in cells grown in 10% FCS by the removal of surface sialic acid.

Hence it is possible that the growth of CaNT cells *in vitro* in the presence of 10% FCS increased the immunogenicity either by unmasking of hidden antigens, or by incorporation of FCS antigens by the cells. Alternatively, *in vitro* passaging of the

CaNT cells may have selected a spontaneous mutant clone with increased antigenicity. This is, however, unlikely, since mixed cultures were passaged rather than single clones during the isolation of CaNT cells from the original tumour.

However, the immunogenicity testing carried out by Jamasbi, Correll and Chiba utilised chemically induced tumour lines, which may already represent antigenically mutated tissue, which are more likely to carry foreign antigens than the spontaneous CaNT line. In this case, it is more likely that the immunogenicity was a result of growth in heterologous serum.

Stable GFP and HRP cells were grown in the presence of G418 antibiotic. Although this was washed from the cells before implantation, it is possible that it may have altered the cells in some way to increase their antigenicity. One method to circumvent this would be to culture cells for implantation in the absence of the selection pressure (in which case it would be prudent to ensure transgene expression). Parental CaNT cells however, were not grown with any antibiotics and this therefore, would not have been an influencing factor when implanted into mice.

It may also be possible to reduce immunogenicity of new antigens from *in vitro* culture by passage of cells in immunocompromised hosts, such as SCID or nude mice, prior to implantation in immunocompetent animals. Previous studies have shown that this can result in reversal of immunogenicity and so, tumours will form, and this may allow for our experiments to be repeated. However, it may result in decreased levels of transgene expression due to the prolonged lack of selection pressure. Direct delivery of the HRP gene to the tumour would clearly be a better method to assess the efficacy of the HRP/IAA system *in vivo*.

In conclusion the results presented here demonstrate that growth of CaNT cells *in vitro* results in development of antigenicity in the syngeneic CBA mouse stock. This is likely to be due to the presence of FCS within the growth medium. In animals implanted with cells isolated directly from tumours there was no apparent immunogenicity, although FCS was present in the inoculating medium.

Chapter 9

Non-viral *in vivo* gene delivery

9.1 Introduction

Delivery of genes to tumour cells is the initial and critical stage for any gene therapy strategy. The use of bacteria (Pawelek et al., 1997) or macrophages (Griffiths et al., 2000) to deliver genetically modified cells to the site of interest, allows for *ex vivo* modification of carrier cells. These strategies will therefore rely totally on the bystander effect (see section 1.3.1), since only the introduced cells will be capable of producing the therapeutic gene. However, research into *in situ* gene transfer is a vast area of interest and is constantly producing improved results.

The majority of clinical trial protocols utilise viral delivery of genes (70%, www.wiley.co.uk, J. Gene Med 2002), since viruses are designed to deliver their own genetic material to cells upon infection. Concerns over safety, and limitations due to immune responses, have led to increasingly novel methods of delivery avoiding viruses. There are two general methods of non-viral delivery; naked DNA delivery with a physical method of enhancement, or delivery mediated by a chemical carrier.

Electroporation is one of the methods of enhancing delivery of naked DNA (Neumann et al., 1982), and is now widely used to deliver genes to prokaryotic and eukaryotic cells in culture. The lipid bilayer of cells acts as a capacitor under the application of an electric field, and the membrane potential increases until the dielectric strength is exceeded, resulting in a permeation event, and an increase in transmembrane electrical conductivity. This is thought to result in the transient formation of inverted hydrophilic pores. The poration produced by an electric field is influenced by a variety of factors, including the functional size of the cell (the size of an individual cell may be increased through the presence of electrical contact with neighbouring cells), architecture, membrane dielectric strength and duration of exposure to the field (for review see (Gehl, 2003).

The formation of pores in the plasma membrane is reliant on the threshold voltage being exceeded. If the applied voltage is too low, alteration of the plasma membrane will be insufficient to allow transfer of introduced molecules. However,

high voltages can cause irreversible dielectric breakdown, facilitating massive transport of the cell contents out of the cell, and irreversible damage.

Once pores have been formed, DNA transfer requires surface adsorption, insertion into the pore, and finally, travel, which is electrophoretically mediated. Electroporation has been used to deliver exogenous genetic material to a variety of tissue types and also to increase the delivery of drugs which are unable to cross the plasma membrane (Hyacinthe et al., 1999).

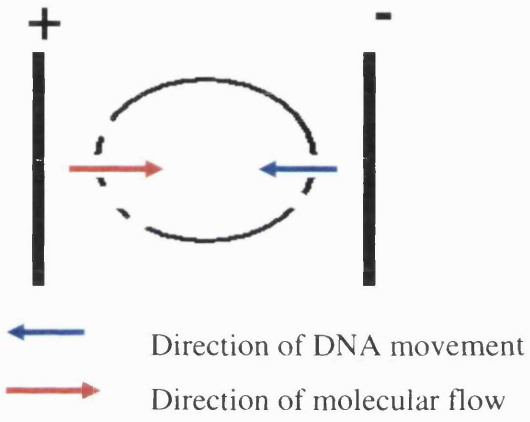


Figure 9.1. Electroporation of cells. Pores form at the poles of the cells, and predominantly at the anode.

Enhanced gene delivery using chemical carriers is another method under investigation. Cationic liposomes are non-infectious particles able to package DNA. The surface of the liposomes is positively charged and is electrostatically attracted to the negatively charged phosphate backbone of DNA and cell membranes (figure 9.2). The liposomes attach and fuse to the plasma membrane, and are then internalised. The DNA has to leave the vesicle, in order to evade enzymatic degradation, before entering the nucleus through pores, or during the nuclear breakdown that occurs in mitosis.

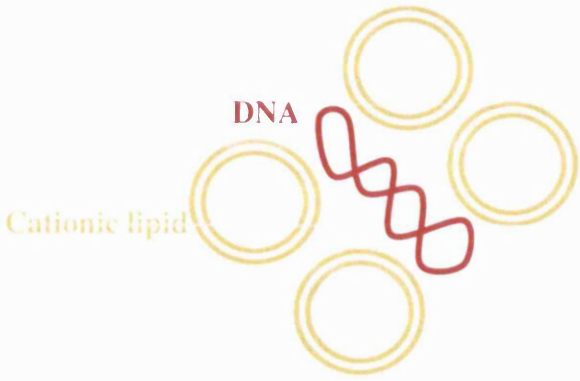


Figure 9.2. DNA-cationic lipid complexes. Approximately 2-4 liposomes are thought to associate with a single 5 kb plasmid (www.invitrogen.com, (Felgner et al., 1987)).

This chapter describes the delivery of genes directly to tumour tissue using non-viral methods.

9.2 Materials and methods

9.2.1 *In vivo* transfection using electroporation

When tumours reached 7 mm in diameter they were electoporated. Electric pulses were delivered using custom made (Gray Cancer Institute) flat parallel stainless steel electrodes 7 mm apart, which were placed percutaneously at the opposite margins of the tumour. A conductive gel (Parker Laboratories Inc, New York, USA) was used to ensure good electrical contact between the electrodes and the overlying skin. Eight square wave pulses were administered in 2 directions perpendicular to each other, with a 1 Hz frequency generated by a Jouan GHT 1287 electroporator (Jouan, St. Herblain, France). The amplitude and pulse duration were varied, and were either 600 V/cm for 5 ms (EP1) or 1300 V/cm 100 μs (EP2). 24 h after injection, tumours were excised and frozen in OCT.

9.2.2 *In vivo* transfection using lipofectin

Mice bearing FaDu tumours received a single intratumoural (i.t.) injection of water, 50 μg pEGFP-N1 (Clontech, UK), or DNA + lipofectin (DNA-lipid complexes were formed by combining 14.5 μl lipofectin, 10 μg DNA and 36.5 μl PBS, which were allowed to stand for 2 h prior to injection) in a total injection volume of 50 μl. In some cases i.t. injection was followed by the application of electric pulses across the

tumour. Gene transfer was assessed on frozen sections using fluorescent microscopy.

9.2.3 *In vivo* transfection using Trans IT

For gene transfer using the therapeutic transgene, pRK34-HRP or pCI-neo plasmid DNA was used. Trans IT is a polymer-lipid delivery system developed by Mirus technologies. Animals received a single i.t. injection of either 25 µg DNA, or 25 µg DNA in a complex with the Mirus Trans IT *in vivo* gene delivery system (Fischer Scientific, UK), with or without electroporation using EP1 parameters. A 100 µl volume was injected. Trans IT solutions were prepared as described by (Worthington et al., 2000) using a volume ratio of 1.6:1:1, water:DNA:polymer solution. This was allowed to stand for 10 min at room temperature before the addition of delivery solution to give 100 µl.

24 h after injection, tumours were excised, and either prepared for the TMB assay as in section 2.5, or cross-scalpeled in lysis buffer. Samples were centrifuged at 1,500 rpm and the supernatant stored at –20°C prior to western blot analysis with Biogenesis rabbit anti-HRP diluted 1:200 (Biogenesis, Poole, UK).

Buffers and reagents

Lysis buffer:

1M Tris HCl pH 6.8	5 ml
3 M NaCl	3.3 ml
500 mM EDTA	1 ml
dH_2O	to 100 ml

Store at 4°C

Prior to use add 10 µl/ml protease inhibitor cocktail (Sigma).

9.2.4 GFP quantitation

Sections of 10 µm thickness were cut throughout the tumour using a cryostat, with approximately 40 sections per tumour. An epi-fluorescence microscope (Nikon TE200) with a narrow band filter (500-510 nm, Glen Spectra Ltd, Stanmore, UK) and a custom imaging system were used to visualise and quantify GFP fluorescence as a percentage of the total area.

9.3 Results

9.3.1 Transfection using the marker GFP

Tumours subjected to electroporation retained their shape and structure, with no obvious gross morphological differences between treated and untreated controls. Regions of GFP expressing cells were relatively easy to identify on serial sections through the FaDu tumours under fluorescent microscopy. Transfected areas were predominantly seen surrounding the needle tract and periphery of the tumour irrespective of delivery method (figure 9.3).

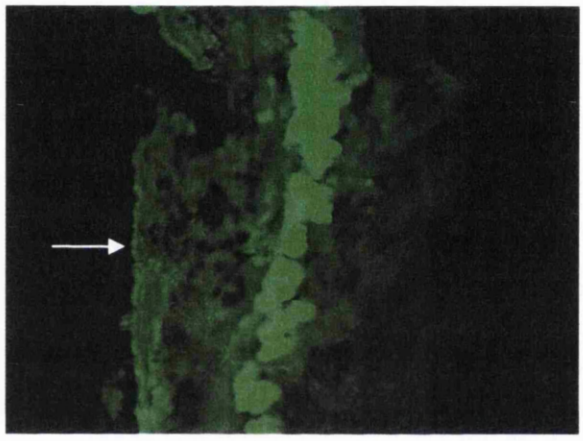


Figure 9.3. GFP expression in FaDu tumour 24 h following intratumoural injection of pEGFP-N1 and electroporation at 600 V/cm (EP1). Arrow marks tumour boundary.

Transfection efficiencies using either naked DNA or DNA lipofectin complexes with or without electroporation ranged from 0.002-0.042% (figure 9.4). Use of DNA and liposome complexes doubled the mean transfection efficiency compared with naked DNA, although this was not statistically significant. However, electroporation with either EP1 or EP2 did increase GFP expression, with EP1 being more effective (40-fold increase in transfected area compared to DNA alone). EP1 in conjunction with the lipid complex resulted in higher levels of GFP expression compared with DNA alone (20 times greater), but levels were still lower than when EP1 was performed after injection of naked DNA.

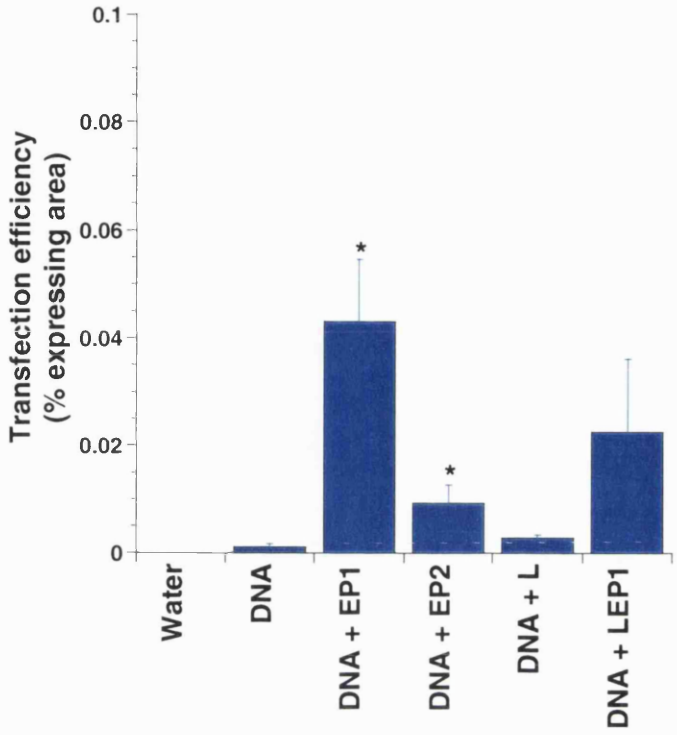


Figure 9.4. Transfection efficiencies for the pEFGP-N1 plasmid in FaDu tumours. EP1, 600 V/cm 5 ms; EP2, 1300 V/cm 100 μs; L, lipofectin-DNA complexes. Data are mean ± s.e.m. 3-7 animals per group. *$p<0.01$ compared with naked DNA injection.

9.3.2 Transfection using the HRP gene

Mouse carcinoma CaNT tumours were injected i.t. with either DNA, DNA in a lipid-polymer complex, with or without electroporation using the pRK34-HRP plasmid or pCI-neo control plasmid. Transfection efficiencies in whole tumours were assessed using peroxidase activity measured using the TMB assay (figure 9.5).

Tumours which did not receive any treatment showed low background levels of peroxidase activity. Injection of Trans IT and control DNA did not alter the peroxidase activity. The injection of control DNA plus electroporation at 600 V/cm 5 ms duration significantly increased peroxidase activity in CaNT tumours. Delivery of the HRP gene to tumours using the Trans IT system increased peroxidase activity relative to both control and vector Trans IT injected tumours, but the increase was not statistically significant. Treatment of tumours with pRK34-HRP DNA followed by electroporation did increase peroxidase activity significantly, but to a lesser degree than electroporation of tumours injected with pCI-neo.

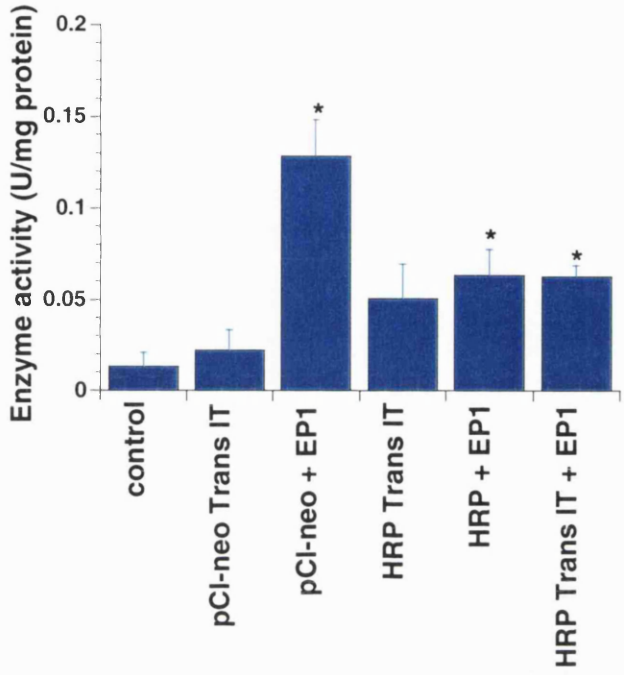


Figure 9.5. Peroxidase activities of CaNT tumours following administration of pCI-neo or pRK34-HRP plasmid DNA in a complex with Trans IT, with electroporation or a combination of the 2. EP1 has the same parameters as in 8.3.1. Data are mean ± s.e.m. 5 tumours per group. *$p<0.01$ compared to control tumours.

Western blotting was performed on tumour lysates to detect the presence of HRP protein after *in vivo* transfection (figure 9.6b). An immunoreactive band of 52 kDa was expected, as clearly seen in the *in vitro* cultures of stable HRP8 cells (figure 9.6.a). The purified HRP protein loaded as a control adjacent to the tumour samples has a slightly smaller size than that expected from pRK34-HRP, as it lacks the C- and N- terminal extensions of the recombinant protein. Unfortunately highly reactive non-specific immunoreactive background staining could not be removed and this masked the potential HRP band.

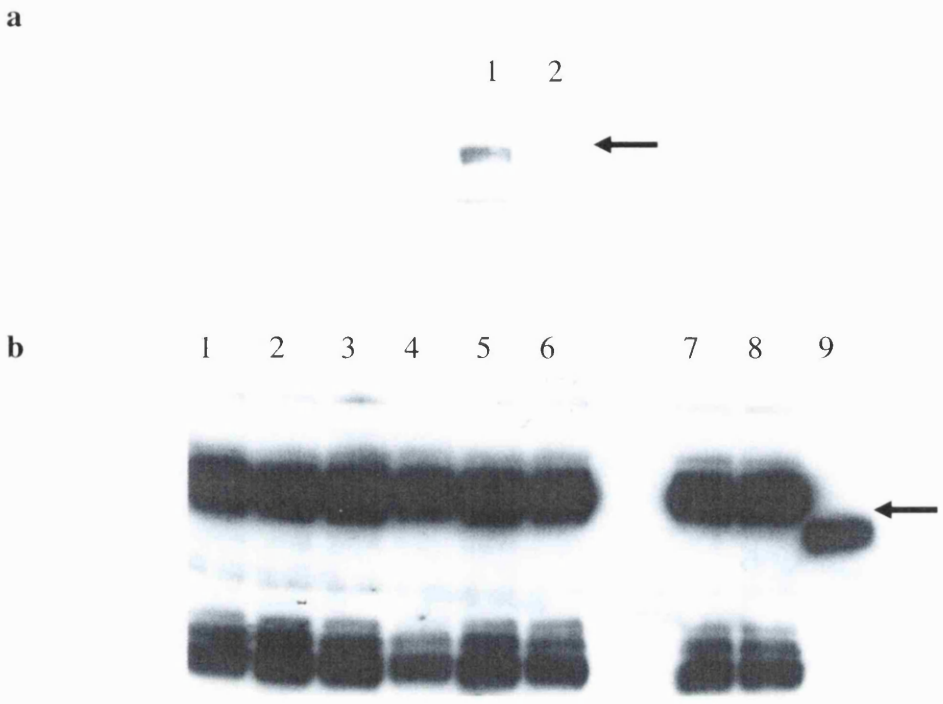


Figure 9.6. Western blot for the presence of HRP. Arrow indicates size of expected synthetic HRP band. Panel a) samples from *in vitro* cell culture: Lane 1, HRP8; 2, GFP1. Panel b) samples from tumours: Lane 1, CaNT tumour; 2-3, pCI-neo Trans IT; 4-5, pRK34-HRP Trans IT; 6, pRK34-HRP EP1; 7-8, pRK34-HRP Trans IT EP1; 9 purified recombinant HRP control (Sigma).

9.4 Discussion

Clinical use of gene therapy to treat cancer requires delivery of the therapeutic genes to the tumour tissue. As an alternative to viral delivery, which can be expensive and raises safety concerns, liposomes and electroporation were analysed in a xenograft and also in a murine carcinoma model.

There appears to be a range of optimal electroporation parameters which depend upon the tissue and cell type involved. In the FaDu tumour, expression of the marker GFP was increased when DNA injection was combined with electroporation, with 600 V/cm, 5 ms (EP1) achieving the greatest expression of the two electroporation parameters studied. This is consistent with previous results (Cemazar et al., 2002) in a range of solid tumours.

Square wave rather than exponentially decaying pulses were used, as it is the initial voltage which is though to lead to poration, whilst the trailing decay does not contribute. It does however produce heat which can lead to cell and tissue damage.

Successful electroporation of muscle has been reported and many studies have demonstrated that muscle can be porated to a high degree with low voltage pulses, which resulted in extended periods of gene expression (Rizzuto et al., 1999). The low voltages required may be a result of the fairly large *functional* size of muscle cells as a result of electrical coupling. In contrast, poration of tumour cells usually leads to expression for less than one week.

Previous studies have reported a threshold voltage which needs to be exceeded *in vivo* in order to achieve easily detectable levels of gene expression. Generally for tumours this is around 400-600 V/cm (Cemazar et al., 2002; Mikata et al., 2002; Wells et al., 2000). However, high voltages will lead to cell death and decreased transfection. In this study, this appeared to be the case when 1300 V/cm was used. The parameters at which cell death occurs may be dependent on both the voltage and the number of administered pulses across the skin. Resistance of the skin was observed to decrease following the initial pulses in a series of eight (Wells et al., 2000). This decreased resistance led to an increase in current and could potentially cause a subsequent increased cell mortality. The optimal electroporation parameters will therefore be a balance between maximal expression and minimal cell death. Cell death in tumours will ultimately be the goal, but death by the delivery method negates the use of gene transfer.

Electroporation was performed after DNA injection. (Tekle et al., 1994) demonstrated that *in vitro* transfer of molecules is not affected whether electroporation is performed before or after administration of exogenous material. Indeed, electroporation followed by DNA injection with a plasmid encoding the p53 gene has demonstrated some growth delay (Mikata et al., 2002). In muscle it was shown that DNA will persist for an hour before being cleared or degraded (Cappelletti et al., 2003). However, the majority of *in vivo* protocols apply electric pulses to tissues after DNA delivery.

It is difficult to correlate the transfection efficiencies reported here with levels obtained in published reports in a range of tumours (Heller & Coppola, 2002; Nishi et al., 1996; Wells et al., 2000) when luciferase is used as the reporter gene. Luciferase is an enzyme which is able to convert many substrate molecules and hence results in

an amplification effect. Assessment of transfection efficiency using GFP relies on a one to one relationship between transfected and detected cells. The use of luciferase means that the level of expression is measured, whereas in this study, the fraction of expressing cells is assessed, and not the fluorescence intensity.

The actual quantity of DNA administered with lipofectin was 5 times lower than with naked DNA alone due to volume limitations. This may contribute to the low level of transfection seen with this combination alone and in combination with EP1. Several studies have reported that greater transfection was seen when greater quantities of DNA were administered (Cemazar et al., 2002; Dean et al., 2003; Heller et al., 2000).

Combination of electroporation with liposomes has been reported to decrease transfection efficiencies compared with electroporation alone (Cemazar et al., 2002; Wells et al., 2000), and it was suggested that the larger lipoplex required a more critical level of membrane disruption for efficient transfer (Wells et al., 2000).

The lower levels of gene transfer in the FaDu tumours compared to other types may be related to the cell density. (Cemazar et al., 2002) showed that efficient transfection using electroporation appeared to be associated with a lower cell density. On the other hand, it has also been reported that tumours which appeared to be less firm and more scarred (indicating some degree of necrosis) transfected less well with electroporation than firm solid tumours (Wells et al., 2000), which would appear to be contradictory, and cell origin may again be important.

It is possible that the level of transfection was underestimated due to regions of hypoxia. GFP fluorescence requires molecular oxygen (Heim et al., 1994), and we have shown that FaDu tumours contain regions of hypoxia (chapter 6), Additionally, intratumoural injection (Olive et al., 2002) has been shown to increase local hypoxia and electroporation above 640 V was shown to decrease blood flow in tumours for 24 hours (Sersa et al., 1999). Electroporation was also demonstrated to increase the activity of bioreductive drugs (Cemazar et al., 2001).

Due to low transfection efficiencies in FaDu tumours, the CaNT tumour model was used to analyse transfer of therapeutic DNA. In order to deliver the potentially therapeutic HRP gene to solid tumours, electroporation was again used at the optimal electroporation parameter (EP1) as determined from the GFP study, in combination with a commercially available delivery system. The Trans IT system is designed for

i.v. delivery of genetic material. However, as this targets the liver, a modified protocol was used. This protocol had previously been shown to effectively deliver the inducible nitric oxide synthase (iNOS) gene under the control of a radiation inducible promoter to tumours, resulting in therapeutic effects in combination with radiation (Worthington et al., 2002).

In our study to deliver the HRP gene, results were inconclusive. The TMB enzyme assay appeared to show higher peroxidase activity in tumours injected with the vector plasmid followed by electroporation than tumours receiving the HRP containing plasmid. The levels achieved using the HRP containing plasmid were approximately 0.075 U/mg protein (figure 9.5). This is less than half that seen in stable transfectants (0.2 U/mg protein, chapter 6).

The high levels seen in the vector electroporated tumours may be a result of high numbers of infiltrating lymphocytes and myeloperoxidase activity, although this was not analysed. It is also possible that the injection and electric pulses resulted in damage to blood vessels and haemorrhage, since the EP1 treated pCI-neo tumours appeared to more bloody than others. Haemoglobin in samples will increase the apparent peroxidase activity. Although electroporation has been reported to decrease blood flow, all reports suggest that electric pulses themselves do not lead to histological changes (Cemazar et al., 2002; Dean et al., 2003; Yamashita et al., 2001). In future it would be advisable to correct for haemoglobin content.

Due to the difficulties encountered with the TMB assay, western blot analysis was used. Unfortunately non-specific immunoreactive bands prevented the quantitation of HRP protein by western blot. It is not clear why this is, since a rabbit antibody was used, and washing stages were introduced. A possible alternative to overcome the non-specific antibody binding would be to use solely the microsomal fraction of the tissue homogenate, since the synthetic HRP gene contains a KDEL sequence which tethers it to the reticular endothelium (Connolly et al., 1994). Primers also exist for the HRP gene, and it may be possible to look at gene delivery using semi-quantitative RT-PCR.

Although electroporation could increase delivery of the GFP gene to FaDu tumours, its ability to affect HRP gene delivery was difficult to assess. Levels of expression are likely to be well below those required to achieve significant therapeutic effect.

Chapter 10

General discussion and future directions

Cancer therapies should show both efficacy and specificity in order to effectively target the disease. On this premise, gene directed enzyme prodrug therapy (GDEPT) was developed. The results in this thesis demonstrate the steps taken to develop and assess the horseradish peroxidase-directed GDEPT system from simple tissue culture, through complex *in vitro*, to whole animal studies.

To facilitate the use of relevant tumour models, cells stably expressing the HRP gene were produced, and confirmed to be more sensitive to treatment with indoles, than GFP expressing controls (chapter 3). These cells were then used to develop a 3-dimensional *in vitro* model of the tumour microenvironment (chapter 4). All three of the indole prodrugs tested in this model showed selective cell kill of HRP spheroids. In order to determine the relative sensitivity of the different subpopulations within the spheroid, further experiments could be conducted to determine the location of prodrug activation. Instead of creating a single cell suspension from the entire spheroid, spheroids could be subjected to sequential trypsinisation. This would liberate cells from the spheroid in layers. These cell fractions could then be plated for clonogenic assay. This technique may be of particular interest for cells derived from large spheroids exposed to 5Br-IAA for short periods. Previously, 5Br-IAA was shown to be selectively toxic to monolayers under anoxia after short incubation periods (Greco et al., 2001). If, as suggested, it is specifically activated under hypoxic conditions, survival would be predicted to be lower in spheroid-derived cells close to the necrotic core.

The spheroid model could also be used to study the diffusion of the parent prodrugs. Regions of chronic hypoxia in tumours are distant from functional blood vessels. Therefore, any therapeutic agent, which aims to target this population of cells, needs to be capable of diffusion through metabolising cell layers. Spheroids derived from parental cells could be used to measure diffusion through cell layers, possibly using radiolabelled IAA, and compared to HRP expressing spheroids which specifically metabolise the prodrugs. Alternatively, models composed of cell multilayers could be used. These are composed of cells grown to varying thickness between two chambers, and have been used to model the diffusion of the bioreductive

agent tirapazamine (Hicks et al., 1998).

Spheroids could also be used to model the interaction between the HRP-GDEPT system and radiation. At present this system has been shown to potentiate the effects of radiation treatment both under oxic and anoxic conditions (Greco et al., 2002c). In the spheroid model both oxia and hypoxia are present, and cells are asynchronous with regards to their cell cycles. The survival of cells from spheroids exposed to radiation can be dependent upon size and hypoxic fraction (Rofstad & Sutherland, 1989). It would be interesting to determine whether the HRP/IAA combination could potentiate the effects of radiation under these conditions, either by killing the hypoxic cells itself whilst radiation killed the oxic fraction, or by increasing the radiation susceptibility of hypoxic cells.

In addition to the reported indole prodrugs, this study demonstrated that the analgesic paracetamol could be activated to a cytotoxic compound intracellularly by the HRP enzyme (chapter 5). The reaction between HRP and paracetamol had previously been shown using purified enzyme (Potter & Hinson, 1987), but this is the first demonstration of an intracellularly produced cytotoxin for gene therapy. As future work the presence of *N*-acetyl-*p*-benzoquinoneimine (NAPQI) and its conjugates could be determined in cell extracts by HPLC.

Further experiments are required both to determine the method of cell death. Our study, using propidium iodide staining, indicated apoptosis as one mode of cell death. However, DNA staining alone is not confirmatory of apoptosis, and changes in a variety of signalling cascades, including the caspases and members of the Bax family, should be analysed. Further experiments including analysis of micronucleus formation, annexin V binding, and PARP cleavage would help to elucidate potential apoptotic routes. In addition, since liver necrosis is an important observation *in vivo* in cases of paracetamol overdose, trypan blue exclusion could be used to measure this *in vitro*. Trypan blue is generally excluded from viable and apoptotic cells. The possibility of NAPQI forming double strand breaks in DNA has been proposed, and this could be determined using the comet assay, whereby cells are embedded within agarose gel, the cell digested and DNA allowed to unwind. The gel is then subjected to an electric current and any double strand breaks will migrate away from the nucleus resulting in a "comet tail". Immunocytochemistry for the γH2AX protein, which is translocated to the site of DNA damage to aid in the repair of DNA double strand

breaks (MacPhail et al., 2003) would also be possible.

Our study showed that incubation of purified HRP enzyme with furafylline, a potent cytochrome P450 (CYP) 1A2 inhibitor did not result in a decrease in activity by half, to the limit of the inhibitor's solubility. Further experiments are required to assess the feasibility of using furafylline or glutathione donors to limit potential side effects of the HRP/paracetamol combination. The efficacy of methionine or *N*-acetyl cysteine to prevent cell death in response to paracetamol treatment could be determined by analysing cells that retain expression of CYP enzymes in culture (in particular 1A2 and 2E1), such as hepatocytes, or tumour cell lines transfected with the CYP genes. The FaDu transfectants could be used with greater concentrations of NAC, or with paracetamol in combination with furafylline to determine whether the enzyme/prodrug combination retains activity towards HRP8 cells. These results should give some indication of whether adequate concentrations of paracetamol could be administered *in vivo* to give therapeutic levels within the tumour without resulting in severe hepatic damage. Upon confirmation, *in vivo* experiments using the HRP/paracetamol combination with or without furafylline could be started.

Stable transfectants were used to form solid tumours in SCID mice, in order to test the *in vivo* efficacy of the HRP/IAA system (chapters 6 and 7). The FaDu tumour cell line appeared to be an appropriate model for hypoxia targeting of the HRP gene based upon pimonidazole staining (chapter 6). Unfortunately the initial results showing a modest delay in tumour growth could not be reproduced. Several reasons can be considered to explain the lack of *in vivo* efficacy, including drug accessibility and enzyme activity.

Firstly, the prodrug concentration at the site may not have reached therapeutic levels. It was not possible to increase the dose of 5Br-IAA administered using fractionated dosing schedules, due to weight loss and non-specific toxicities. It may be possible to increase the tumour exposure to prodrugs, and thus increase the potential cell kill, by modification of prodrugs. One way could be the use of polymer conjugation to allow for increased dosage with decreased normal tissue toxicities. This would alter the pharmacokinetic properties of the prodrugs, as the increased size of the molecule hinders leakage from the blood vessels. However, the tortuous, irregular and often immature vessels within tumours can display increased permeability, resulting in the phenomenon of "enhanced permeability and retention"

of large molecules (Matsumura & Maeda, 1986). Additionally, immungenicity of compounds is usually reduced, whilst increasing circulation times (Delgado et al., 1992). Toxicity to the reticuloendothelial system would need to be monitored, due to the scavenging of large molecules (Seymour et al., 1987).

Cleavage of the polymer-drug linkage can be targeted to require specific enzymes, and so further reduce potential side effects (although this is probably a more useful feature for direct cytotoxic agents). For example, linkage could be through the use of a bioreductively cleaved bond, so releasing the prodrug under conditions of hypoxia.

Secondly, the HRP gene expression, and hence activity, may have been suboptimal. Already the effectiveness of the HRP/IAA system in stable transfectants (chapter 3) was less than in transient transfectants of the same cell line (Greco et al., 2001). Hence, the use of stable transfectants *in vivo* may be a potential contributing factor in the poor growth delay results. Additionally, HRP gene expression may have been reduced or lost during *in vivo* growth. It may therefore be of benefit to utilise *in vivo* gene delivery to attempt to increase the therapeutic efficacy in tumour models.

The success of any suicide gene therapy system is dependent upon the bystander effect. In addition to the transfer of toxic metabolites, infiltration of tumours by cells of the immune system has been shown in both the HSV-tk/GCV and CD/5-FC systems. This cannot be tested in a xenograft model as the host is immunocompromised, and so a syngeneic mouse cell line was transfected with the HRP gene for use as a syngeneic tumour model in immunocompetent mice (chapter 8). It would be expected that tumour growth delay would be more pronounced in these animals if the immuno-bystander effect was active. In order to determine whether the HRP protein alone was sufficiently immunogenic to result in growth delay, as is the case with the CD enzyme (Haack et al., 2000; Pierrefite-Carle et al., 2002), TD_{50} experiments were conducted. The results showed that the murine cell line itself when grown *in vitro* was immunogenic, but not when passaged *in vivo*. This was most likely to be due to antigens incorporated into the cells from the FCS. Passage of cells from *in vitro* culture in immunocompromised hosts, such as SCID or nude mice, prior to implantation in CBA animals may reduce or eliminate this antigenicity. However, it may also result in decreased levels of transgene expression, due to prolonged passage in the absence of selection pressure, which is not desirable. An

alternative would be to grow cells in isogenic serum, although this is not particularly practical. Direct delivery of the HRP gene to the established CaNT tumour would be an alternative. The immune response to any delivery method and DNA sequences would need to be assessed.

Non-viral gene delivery circumvents the safety concerns surrounding viral delivery methods, and is often simpler and cheaper to use. The data in chapter 9 show that the electroporation of subcutaneous FaDu tumours could significantly increase expression of intratumourally injected DNA. However, the total expression was still below 1% of cells. Previous studies have shown that some tissues and tumour types are more amenable to electroporation than others. The B16 tumour line showed high levels of GFP expression following electroporation with the EP1 parameters used in this study (Cemazar et al., 2002). A circular needle array and low voltage long duration pulses also resulted in high levels of gene expression in the B16 tumour (Kishida et al., 2001). It may be that the B16 tumour is more easily porated, and could be used to study the HRP/IAA system *in vivo* with greater success. Delivery of the HRP gene could be achieved using viral means. Currently, gene delivery *in vivo* is most successful following the use of viruses, and this may result in therapeutically relevant levels of enzyme activity being achieved.

In conclusion the results show the continued effectiveness of the HRP-GDEPT system in *in vitro* models, and in particular that the targeting of hypoxic cells is feasible. The indole prodrugs show reasonable pharmacokinetic properties, and fairly high levels of prodrug were reached within the tumour tissue, especially of the halogenated indole 5Br-IAA. Unfortunately *in vivo* experiments thus far were disappointing and did not meet the *in vitro* expectations. This could potentially be improved through the use of syngeneic models with successful *in vivo* gene delivery.

References

Abu Sinna, G. (1983). The effect of the plant hormone indole-3-acetic acid and chemically related compounds on the growth of mouse fibroblast 3T3 cells. *Comp Biochem Physiol C*, **74,** 433-6.

Adachi, Y., Tamiya, T., Ichikawa, T., Terada, K., Ono, Y., Matsumoto, K., Furuta, T., Hamada, H. & Ohmoto, T. (2000). Experimental gene therapy for brain tumors using adenovirus-mediated transfer of cytosine deaminase gene and uracil phosphoribosyltransferase gene with 5-fluorocytosine. *Hum Gene Ther*, **11,** 77-89.

Aebi, S. & Lauterburg, B.H. (1992). Divergent effects of intravenous GSH and cysteine on renal and hepatic GSH. *Am J Physiol*, **263,** R348-52.

Aghi, M., Kramm, C.M., Chou, T.C., Breakefield, X.O. & Chiocca, E.A. (1998). Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-fluorocytosine/cytosine deaminase gene therapies. *J Natl Cancer Inst*, **90,** 370-80.

Alvarez, R.D., Gomez-Navarro, J., Wang, M., Barnes, M.N., Strong, T.V., Arani, R.B., Arafat, W., Hughes, J.V., Siegal, G.P. & Curiel, D.T. (2000). Adenoviral-mediated suicide gene therapy for ovarian cancer. *Mol Ther*, **2,** 524-30.

Anderson, W.F., Blaese, R.M. & Culver, K. (1990). The ADA human gene therapy clinical protocol: Points to Consider response with clinical protocol, July 6, 1990. *Hum Gene Ther*, **1,** 331-62.

Andrade-Rozental, A.F., Rozental, R., Hopperstad, M.G., Wu, J.K., Vrionis, F.D. & Spray, D.C. (2000). Gap junctions: the "kiss of death" and the "kiss of life". *Brain Res Brain Res Rev*, **32,** 308-15.

Andreesen, R., Hennemann, B. & Krause, S.W. (1998). Adoptive immunotherapy of cancer using monocyte-derived macrophages: rationale, current status, and perspectives. *J Leukoc Biol*, **64,** 419-26.

Anlezark, G.M., Melton, R.G., Sherwood, R.F., Coles, B., Friedlos, F. & Knox, R.J. (1992). The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)-- I. Purification and properties of a nitroreductase enzyme from Escherichia coli--a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). *Biochem Pharmacol*, **44,** 2289-95.

Antoniou, M., Harland, L., Mustoe, T., Williams, S., Holdstock, J., Yague, E., Mulcahy, T., Griffiths, M., Edwards, S., Ioannou, P.A., Mountain, A. & Crombie, R. (2003). Transgenes encompassing dual-promoter CpG islands from the human TBP and HNRPA2B1 loci are resistant to heterochromatin-mediated silencing. *Genomics*, **82,** 269-79.

Arafat, W.O., Casado, E., Wang, M., Alvarez, R.D., Siegal, G.P., Glorioso, J.C., Curiel, D.T. & Gomez-Navarro, J. (2000). Genetically modified CD34+ cells exert a cytotoxic bystander effect on human endothelial and cancer cells. *Clin Cancer Res*, **6,** 4442-8.

Atkinson, G. & Hall, S.J. (1999). Prodrug activation gene therapy and external beam irradiation in the treatment of prostate cancer. *Urology*, **54,** 1098-104.

Augusto, O. (1993). Alkylation and cleavage of DNA by carbon-centered radical metabolites. *Free Radic Biol Med*, **15,** 329-36.

Bachtiary, B., Schindl, M., Potter, R., Dreier, B., Knocke, T.H., Hainfellner, J.A., Horvat, R. & Birner, P. (2003). Overexpression of hypoxia-inducible factor 1alpha indicates diminished response to radiotherapy and unfavorable prognosis in patients receiving radical radiotherapy for cervical cancer. *Clin Cancer Res*, **9,** 2234-40.

Bae, M.A., Pie, J.E. & Song, B.J. (2001). Acetaminophen induces apoptosis of C6 glioma cells by activating the c-Jun NH(2)-terminal protein kinase-related cell death pathway. *Mol Pharmacol*, **60,** 847-56.

Bai, S., Du, L., Liu, W., Whittle, I.R. & He, L. (1999). Tentative novel mechanism of the bystander effect in glioma gene therapy with HSV-TK/GCV system. *Biochem Biophys Res Commun*, **259,** 455-9.

Balfour, H.H., Jr. (1990). Management of cytomegalovirus disease with antiviral drugs. *Rev Infect Dis*, **12 Suppl 7,** S849-60.

Baumann, M., Appold, S., Zimmer, J., Scharf, M., Beuthien-Baumann, B., Dubben, H.H., Enghardt, W., Schreiber, A., Eicheler, W. & Petersen, C. (2001). Radiobiological hypoxia, oxygen tension, interstitial fluid pressure and relative viable tumour area in two human squamous cell carcinomas in nude mice during fractionated radiotherapy. *Acta Oncol*, **40,** 519-28.

Beck, I., Ramirez, S., Weinmann, R. & Caro, J. (1991). Enhancer element at the 3'-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. *J Biol Chem*, **266,** 15563-6.

Beltinger, C., Fulda, S., Kammertoens, T., Meyer, E., Uckert, W. & Debatin, K.M. (1999). Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. *Proc Natl Acad Sci U S A*, **96,** 8699-704.

Beltinger, C., Fulda, S., Kammertoens, T., Uckert, W. & Debatin, K.M. (2000). Mitochondrial amplification of death signals determines thymidine kinase/ganciclovir-triggered activation of apoptosis. *Cancer Res*, **60,** 3212-7.

Benouchan, M., Do Nascimento, F., Sebbah-Louriki, M., Crepin, M., Perret, G.Y. & Colombo, B.M. (2003). Bystander cell killing spreading from endolthelial to tumour cells in a three-dimensional multicellular nodule model after Escherichia coli nitroreductase gene delivery. *Biochem Biophys Res Commun*, **311,** 822-828.

Berenstein, M., Adris, S., Ledda, F., Wolfmann, C., Medina, J., Bravo, A., Mordoh, J., Chernajovsky, Y. & Podhajcer, O.L. (1998). [Antitumor gene therapy using suicide genes]. *Medicina*, **58,** 185-8.

Bernier, J., Denekamp, J., Rojas, A., Minatel, E., Horiot, J., Hamers, H., Antognoni, P., Dahl, O., Richaud, P., van Glabbeke, M. & Pi inverted question markerart, M. (2000). ARCON: accelerated radiotherapy with carbogen and nicotinamide in head and neck squamous cell carcinomas. The experience of the Co-operative group of radiotherapy of the european organization for research and treatment of cancer (EORTC). *Radiother Oncol*, **55,** 111-9.

Biaglow, J.E., Varnes, M.E., Epp, E.R., Clark, E.P., Tuttle, S.W. & Held, K.D. (1989). Role of glutathione and other thiols in cellular response to radiation and drugs. *Drug Metab Rev*, **20,** 1-12.

Biedermann, K.A., Sun, J.R., Giaccia, A.J., Tosto, L.M. & Brown, J.M. (1991). scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. *Proc Natl Acad Sci U S A*, **88,** 1394-7.

Bos, R., van der Groep, P., Greijer, A.E., Shvarts, A., Meijer, S., Pinedo, H.M., Semenza, G.L., van Diest, P.J. & van der Wall, E. (2003). Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, **97,** 1573-81.

Bos, R., Zhong, H., Hanrahan, C.F., Mommers, E.C., Semenza, G.L., Pinedo, H.M., Abeloff, M.D., Simons, J.W., van Diest, P.J. & van der Wall, E. (2001). Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst*, **93,** 309-14.

Boucher, P.D., Ruch, R.J. & Shewach, D.S. (1998). Differential ganciclovir-mediated cytotoxicity and bystander killing in human colon carcinoma cell lines expressing herpes simplex virus thymidine kinase. *Hum Gene Ther*, **9,** 801-14.

Braiden, V., Ohtsuru, A., Kawashita, Y., Miki, F., Sawada, T., Ito, M., Cao, Y., Kaneda, Y., Koji, T. & Yamashita, S. (2000). Eradication of breast cancer xenografts by hyperthermic suicide gene therapy under the control of the heat shock protein promoter. *Hum Gene Ther*, **11,** 2453-63.

Bridgewater, J.A., Knox, R.J., Pitts, J.D., Collins, M.K. & Springer, C.J. (1997). The bystander effect of the nitroreductase/CB1954 enzyme/prodrug system is due to a cell-permeable metabolite. *Hum Gene Ther*, **8,** 709-17.

Bridgewater, J.A., Springer, C.J., Knox, R.J., Minton, N.P., Michael, N.P. & Collins, M.K. (1995). Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. *Eur J Cancer*, **31A,** 2362-70.

Brizel, D.M., Dodge, R.K., Clough, R.W. & Dewhirst, M.W. (1999). Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. *Radiother Oncol*, **53,** 113-7.

Brizel, D.M., Rosner, G.L., Harrelson, J., Prosnitz, L.R. & Dewhirst, M.W. (1994). Pretreatment oxygenation profiles of human soft tissue sarcomas. *Int J Radiat Oncol Biol Phys*, **30,** 635-42.

Brizel, D.M., Rosner, G.L., Prosnitz, L.R. & Dewhirst, M.W. (1995). Patterns and variability of tumor oxygenation in human soft tissue sarcomas, cervical carcinomas, and lymph node metastases. *Int J Radiat Oncol Biol Phys*, **32,** 1121-5.

Broday, L., Lee, Y.W. & Costa, M. (1999). 5-azacytidine induces transgene silencing by DNA methylation in Chinese hamster cells. *Mol Cell Biol*, **19,** 3198-204.

Browder, T., Butterfield, C.E., Kraling, B.M., Shi, B., Marshall, B., O'Reilly, M.S. & Folkman, J. (2000). Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res*, **60,** 1878-86.

Brown, J.M. & Giaccia, A.J. (1998). The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res*, **58,** 1408-16.

Brown, J.M. & Lemmon, M.J. (1991). Tumor hypoxia can be exploited to preferentially sensitize tumors to fractionated irradiation. *Int J Radiat Oncol Biol Phys*, **20,** 457-61.

Brown, J.M. & Wang, L.H. (1998). Tirapazamine: laboratory data relevant to clinical activity. *Anticancer Drug Des*, **13,** 529-39.

Buchschacher, G.L., Jr. & Wong-Staal, F. (2001). Approaches to gene therapy for human immunodeficiency virus infection. *Hum Gene Ther*, **12,** 1013-9.

Buffa, F.M., West, C., Byrne, K., Moore, J.V. & Nahum, A.E. (2001). Radiation response and cure rate of human colon adenocarcinoma spheroids of different size: the significance of hypoxia on tumor control modelling. *Int J Radiat Oncol Biol Phys*, **49,** 1109-18.

Burke, B., Sumner, S., Maitland, N. & Lewis, C.E. (2002a). Macrophages in gene therapy: cellular delivery vehicles and in vivo targets. *J Leukoc Biol*, **72,** 417-28.

Burke, B., Tang, N., Corke, K.P., Tazzyman, D., Ameri, K., Wells, M. & Lewis, C.E. (2002b). Expression of HIF-1alpha by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. *J Pathol*, **196,** 204-12.

Burri, P., Djonov, V., Aebersold, D.M., Lindel, K., Studer, U., Altermatt, H.J., Mazzucchelli, L., Greiner, R.H. & Gruber, G. (2003). Significant correlation of hypoxia-inducible factor-1alpha with treatment outcome in cervical cancer treated with radical radiotherapy. *Int J Radiat Oncol Biol Phys*, **56,** 494-501.

Candeias, L.P., Folkes, L.K., Porssa, M., Parrick, J. & Wardman, P. (1996). Rates of reaction of indoleacetic acids with horseradish peroxidase compound I and their dependence on the redox potentials. *Biochemistry*, **35,** 102-8.

Cao, G., Kuriyama, S., Cui, L., Nagao, S., Pan, X., Toyokawa, Y., Zhang, X., Nishiwaki, I. & Qi, Z. (1999). Analysis of the human carcinoembryonic antigen promoter core region in colorectal carcinoma-selective cytosine deaminase gene therapy. *Cancer Gene Ther*, **6,** 572-80.

Cappelletti, M., Zampaglione, I., Rizzuto, G., Ciliberto, G., Monica, N.L. & Fattori, E. (2003). Gene electro-transfer improves transduction by modifying the fate of intramuscular DNA. *J Gene Med*, **5,** 324-32.

Caruso, M., Panis, Y., Gagandeep, S., Houssin, D., Salzmann, J.L. & Klatzmann, D. (1993). Regression of established macroscopic liver metastases after in situ transduction of a suicide gene. *Proc Natl Acad Sci U S A*, **90,** 7024-8.

Cemazar, M., Parkins, C.S., Holder, A.L., Kranjc, S., Chaplin, D.J. & Sersa, G. (2001). Cytotoxicity of bioreductive drug tirapazamine is increased by application of electric pulses in SA-1 tumours in mice. *Anticancer Res*, **21,** 1151-6.

Cemazar, M., Sersa, G., Wilson, J., Tozer, G.M., Hart, S.L., Grosel, A. & Dachs, G.U. (2002). Effective gene transfer to solid tumors using different nonviral gene delivery techniques: electroporation, liposomes, and integrin-targeted vector. *Cancer Gene Ther*, **9,** 399-406.

Chang, T.K. & Waxman, D.J. (1993). Cyclophosphamide modulates rat hepatic cytochrome P450 2C11 and steroid 5 alpha-reductase activity and messenger RNA levels through the combined action of acrolein and phosphoramide mustard. *Cancer Res*, **53,** 2490-7.

Chang, T.K., Weber, G.F., Crespi, C.L. & Waxman, D.J. (1993). Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes. *Cancer Res*, **53,** 5629-37.

Chaplin, D.J., Acker, B. & Olive, P.L. (1989). Potentiation of the tumor cytotoxicity of melphalan by vasodilating drugs. *Int J Radiat Oncol Biol Phys*, **16,** 1131-5.

Chaplin, D.J. & Hill, S.A. (1995). Temporal heterogeneity in microregional erythrocyte flux in experimental solid tumours. *Br J Cancer*, **71,** 1210-3.

Chaplin, D.J., Horsman, M.R. & Trotter, M.J. (1990). Effect of nicotinamide on the microregional heterogeneity of oxygen delivery within a murine tumor. *J Natl Cancer Inst*, **82,** 672-6.

Chen, L. & Waxman, D.J. (1995). Intratumoral activation and enhanced chemotherapeutic effect of oxazaphosphorines following cytochrome P-450 gene transfer: development of a combined chemotherapy/cancer gene therapy strategy. *Cancer Res*, **55,** 581-9.

Chen, L., Waxman, D.J., Chen, D. & Kufe, D.W. (1996). Sensitization of human breast cancer cells to cyclophosphamide and ifosfamide by transfer of a liver cytochrome P450 gene. *Cancer Res*, **56,** 1331-40.

Chhikara, M., Huang, H., Vlachaki, M.T., Zhu, X., Teh, B., Chiu, K.J., Woo, S., Berner, B., Smith, E.O., Oberg, K.C., Aguilar, L.K., Thompson, T.C., Butler, E.B. & Aguilar-Cordova, E. (2001). Enhanced therapeutic effect of HSV-tk+GCV gene therapy and ionizing radiation for prostate cancer. *Mol Ther*, **3,** 536-42.

Chiba, I., Oikawa, T., Naiki, M., Takimoto, M., Miyoshi, I., Mizuno, S., Yamashina, K., Yamagiwa, S. & Kobayashi, H. (1987). Enhanced immunogenicity of the cultured rat fibrosarcoma KMT-17 by cultivation in a low concentration of fetal calf serum. *Cancer Res*, **47,** 1815-9.

Chung-Faye, G., Palmer, D., Anderson, D., Clark, J., Downes, M., Baddeley, J., Hussain, S., Murray, P.I., Searle, P., Seymour, L., Harris, P.A., Ferry, D. & Kerr, D.J. (2001). Virus-directed, enzyme prodrug therapy with nitroimidazole reductase: a phase I and pharmacokinetic study of its prodrug, CB1954. *Clin Cancer Res*, **7,** 2662-8.

Churchill-Davidson, I. (1968). Hyperbaric oxygen and radiotherapy: clinical experiences. *Nunt Radiol*, **34,** 215-25.

Clark, A.J., Iwobi, M., Cui, W., Crompton, M., Harold, G., Hobbs, S., Kamalati, T., Knox, R., Neil, C., Yull, F. & Gusterson, B. (1997). Selective cell ablation in transgenic mice expression E. coli nitroreductase. *Gene Ther*, **4,** 101-10.

Clarke, L. & Waxman, D.J. (1989). Oxidative metabolism of cyclophosphamide: identification of the hepatic monooxygenase catalysts of drug activation. *Cancer Res*, **49,** 2344-50.

Connolly, C.N., Futter, C.E., Gibson, A., Hopkins, C.R. & Cutler, D.F. (1994). Transport into and out of the Golgi complex studied by transfecting cells with cDNAs encoding horseradish peroxidase. *J Cell Biol*, **127,** 641-52.

Consalvo, M., Mullen, C.A., Modesti, A., Musiani, P., Allione, A., Cavallo, F., Giovarelli, M. & Forni, G. (1995). 5-Fluorocytosine-induced eradication of murine adenocarcinomas engineered to express the cytosine deaminase suicide gene requires host immune competence and leaves an efficient memory. *J Immunol*, **154,** 5302-12.

Coralli, C., Cemazar, M., Kanthou, C., Tozer, G.M. & Dachs, G.U. (2001). Limitations of the reporter green fluorescent protein under simulated tumor conditions. *Cancer Res*, **61,** 4784-90.

Correll, L.L., Nielsen, L.D., Kelleher, P.J., Harbell, J.W. & Minden, P. (1983). Enhanced immunogenicity of line 10 guinea pig hepatocarcinoma cells after culture. *J Natl Cancer Inst*, **71,** 1343-6.

Craighead, P.S., Pearcey, R. & Stuart, G. (2000). A phase I/II evaluation of tirapazamine administered intravenously concurrent with cisplatin and radiotherapy in women with locally advanced cervical cancer. *Int J Radiat Oncol Biol Phys*, **48,** 791-5.

Crystal, R.G. (1995). Transfer of genes to humans: early lessons and obstacles to success. *Science*, **270,** 404-10.

Crystal, R.G., Hirschowitz, E., Lieberman, M., Daly, J., Kazam, E., Henschke, C., Yankelevitz, D., Kemeny, N., Silverstein, R., Ohwada, A., Russi, T., Mastrangeli, A., Sanders, A., Cooke, J. & Harvey, B.G. (1997). Phase I study of direct administration of a replication deficient adenovirus vector containing the E. coli cytosine deaminase gene to metastatic colon carcinoma of the liver

in association with the oral administration of the pro-drug 5-fluorocytosine. *Hum Gene Ther*, **8,** 985-1001.

Cui, W., Gusterson, B. & Clark, A.J. (1999). Nitroreductase-mediated cell ablation is very rapid and mediated by a p53-independent apoptotic pathway. *Gene Ther*, **6,** 764-70.

Cunningham, C. & Nemunaitis, J. (2001). A phase I trial of genetically modified Salmonella typhimurium expressing cytosine deaminase (TAPET-CD, VNP20029) administered by intratumoral injection in combination with 5-fluorocytosine for patients with advanced or metastatic cancer. Protocol no: CL-017. Version: April 9, 2001. *Hum Gene Ther*, **12,** 1594-6.

Cunningham, S., Meng, Q.H., Klein, N., McAnulty, R.J. & Hart, S.L. (2002). Evaluation of a porcine model for pulmonary gene transfer using a novel synthetic vector. *J Gene Med*, **4,** 438-46.

Curiel, D. (1999). Strategies to adapt adenoviral vectors for targeted delivery. *Ann NY Acad Sci*, **886,** 158-171.

Cutler, R.E., Blair, A.D. & Kelly, M.R. (1978). Flucytosine kinetics in subjects with normal and impaired renal function. *Clin Pharmacol Ther*, **24,** 333-42.

Dachs, G.U., Patterson, A.V., Firth, J.D., Ratcliffe, P.J., Townsend, K.M., Stratford, I.J. & Harris, A.L. (1997). Targeting gene expression to hypoxic tumor cells. *Nat Med*, **3,** 515-20.

Dachs, G.U. & Tozer, G.M. (2000). Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation. *Eur J Cancer*, **36,** 1649-60.

Daneshmend, T.K. & Warnock, D.W. (1983). Clinical Pharmacokinetics of systemic antifungal drugs. *Clin Pharmacokinet*, **8,** 17-42.

Daniels, J.S., Gates, K.S., Tronche, C. & Greenberg, M.M. (1998). Direct evidence for bimodal DNA damage induced by tirapazamine. *Chem Res Toxicol*, **11,** 1254-7.

Danos, O. & Mulligan, R.C. (1988). Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci U S A*, **85,** 6460-4.

Datta, R., Rubin, E., Sukhatme, V., Qureshi, S., Hallahan, D., Weichselbaum, R.R. & Kufe, D.W. (1992). Ionizing radiation activates transcription of the EGR1 gene via CArG elements. *Proc Natl Acad Sci U S A*, **89,** 10149-53.

Datta, R., Taneja, N., Sukhatme, V.P., Qureshi, S.A., Weichselbaum, R. & Kufe, D.W. (1993). Reactive oxygen intermediates target CC(A/T)6GG sequences to mediate activation of the early growth response 1 transcription factor gene by ionizing radiation. *Proc Natl Acad Sci U S A*, **90,** 2419-22.

Davenport J, N.G., Goorha R. (2000). Identification of genes potentially involved in LMO2-induced leukemogenesis. *Leukemia*, **14,** 1986-1996.

De Jaeger, K., Kavanagh, M.C. & Hill, R.P. (2001). Relationship of hypoxia to metastatic ability in rodent tumours. *Br J Cancer*, **84,** 1280-5.

Dean, D.A., Machado-Aranda, D., Blair-Parks, K., Yeldandi, A.V. & Young, J.L. (2003). Electroporation as a method for high-level nonviral gene transfer to the lung. *Gene Ther*, **10,** 1608-15.

Delahoussaye, Y.M., Hay, M.P., Pruijn, F.B., Denny, W.A. & Brown, J.M. (2003). Improved potency of the hypoxic cytotoxin tirapazamine by DNA-targeting. *Biochem Pharmacol*, **65,** 1807-15.

Delgado, C., Francis, G.E. & Fisher, D. (1992). The uses and properties of PEG-linked proteins. *Crit Rev Ther Drug Carrier Syst*, **9,** 249-304.

deMiranda, P. & Blum, M. (1983). Pharmacokintetics of acyclovir after intravenous and oral administration. *J Antimicrob Chemother*, **12,** 29-37.

Deshane, J., Cabrera, G., Grim, J.E., Siegal, G.P., Pike, J., Alvarez, R.D. & Curiel, D.T. (1995). Targeted eradication of ovarian cancer mediated by intracellular expression of anti-erbB-2 single-chain antibody. *Gynecol Oncol*, **59,** 8-14.

Dilber, M.S., Abedi, M.R., Christensson, B., Bjorkstrand, B., Kidder, G.M., Naus, C.C., Gahrton, G. & Smith, C.I. (1997). Gap junctions promote the bystander effect of herpes simplex virus thymidine kinase in vivo. *Cancer Res*, **57,** 1523-8.

Dilber, M.S., Phelan, A., Aints, A., Mohamed, A.J., Elliott, G., Smith, C.I. & O'Hare, P. (1999). Intercellular delivery of thymidine kinase prodrug activating enzyme by the herpes simplex virus protein, VP22. *Gene Ther*, **6,** 12-21.

DiMaio, J.M., Clary, B.M., Via, D.F., Coveney, E., Pappas, T.N. & Lyerly, H.K. (1994). Directed enzyme pro-drug gene therapy for pancreatic cancer in vivo. *Surgery*, **116,** 205-13.

Dirix, L.Y., Tonnesen, F., Cassidy, J., Epelbaum, R., ten Bokkel Huinink, W.W., Pavlidis, N., Sorio, R., Gamucci, T., Wolff, I., Te Velde, A., Lan, J. & Verweij, J. (1996). EO9 phase II study in advanced breast, gastric, pancreatic and colorectal carcinoma by the EORTC Early Clinical Studies Group. *Eur J Cancer*, **32A,** 2019-22.

Dische, S. (1985). Chemical sensitizers for hypoxic cells: a decade of experience in clinical radiotherapy. *Radiother Oncol*, **3,** 97-115.

Djordjevic, S.P., Hayward, N.K. & Lavin, M.F. (1986). Effect of N-hydroxyparacetamol on cell cycle progression. *Biochem Pharmacol*, **35,** 3511-6.

Domin, B.A., Mahony, W.B. & Zimmerman, T.P. (1993). Transport of 5-fluorouracil and uracil into human erythrocytes. *Biochem Pharmacol*, **46,** 503-10.

Douglas, J.T., Rogers, B.E., Rosenfeld, M.E., Michael, S.I., Feng, M. & Curiel, D.T. (1996). Targeted gene delivery by tropism-modified adenoviral vectors. *Nat Biotechnol*, **14,** 1574-8.

Drabek, D., Guy, J., Craig, R. & Grosveld, F. (1997). The expression of bacterial nitroreductase in transgenic mice results in specific cell killing by the prodrug CB1954. *Gene Ther*, **4,** 93-100.

Drake, R.R., Pitlyk, K., McMasters, R.A., Mercer, K.E., Young, H. & Moyer, M.P. (2000). Connexin-independent ganciclovir-mediated killing conferred on bystander effect-resistant cell lines by a herpes simplex virus-thymidine kinase-expressing colon cell line. *Mol Ther*, **2,** 515-23.

Elion, G.B. (1983). The biochemistry and mechanism of action of acyclovir. *J Antimicrob Chemother*, **12 Suppl B,** 9-17.

Elliott, G. & O'Hare, P. (1997). Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell*, **88,** 223-33.

Elshami, A.A., Saavedra, A., Zhang, H., Kucharczuk, J.C., Spray, D.C., Fishman, G.I., Amin, K.M., Kaiser, L.R. & Albelda, S.M. (1996). Gap junctions play a role in the 'bystander effect' of the herpes simplex virus thymidine kinase/ganciclovir system in vitro. *Gene Ther*, **3,** 85-92.

Enger, P.O., Thorsen, F., Lonning, P.E., Bjerkvig, R. & Hoover, F. (2002). Adeno-associated viral vectors penetrate human solid tumor tissue in vivo more effectively than adenoviral vectors. *Hum Gene Ther*, **13,** 1115-25.

Erbs, P., Regulier, E., Kintz, J., Leroy, P., Poitevin, Y., Exinger, F., Jund, R. & Mehtali, M. (2000). In vivo cancer gene therapy by adenovirus-mediated

transfer of a bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion gene. *Cancer Res*, **60,** 3813-22.

Fass, R.J. & Perkins, R.L. (1971). 5-fluorocytosine in the treatment of cryptococcal and candida mycoses. *Annals of Int Med*, **74,** 535-539.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. & Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A*, **84,** 7413-7.

Fidler, I.J. (1974). Inhibition of pulmonary metastasis by intravenous injection of specifically activated macrophages. *Cancer Res*, **34,** 1074-8.

Firth, J.D., Ebert, B.L., Pugh, C.W. & Ratcliffe, P.J. (1994). Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. *Proc Natl Acad Sci U S A*, **91,** 6496-500.

Fisher, K.D., Stallwood, Y., Green, N.K., Ulbrich, K., Mautner, V. & Seymour, L.W. (2001). Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther*, **8,** 341-8.

Fleckenstein, W., R., J.J. & Suckfull, M. (1990). Distribution of oxygen pressure in the peripheral and centre of malignant head and neck tumours. In *Clinical oxygen pressure measurement II.*, Ehrly, A.M. (ed) pp. 81-90. Blackwell: Berlin.

Fletcher, C., Sawchuck, R., Chinnock, B., De Miranda, P. & Balfour, H.H., Jr. (1981). Human pharmacokinetics of the antiviral drug DHPG. *Clinical pharmacology and therapeutics*, **40,** 281-286.

Floeth, F.W., Shand, N., Bojar, H., Prisack, H.B., Felsberg, J., Neuen-Jacob, E., Aulich, A., Burger, K.J., Bock, W.J. & Weber, F. (2001). Local inflammation and devascularization--in vivo mechanisms of the "bystander effect" in VPC-mediated HSV-Tk/GCV gene therapy for human malignant glioma. *Cancer Gene Ther*, **8,** 843-51.

Flotte, T.R. & Laube, B.L. (2001). Gene therapy in cystic fibrosis. *Chest*, **120,** 124S-131S.

Foley, E.J. (1953). Antigenic properties of methylcholanthrene-induced tumours in mice of the strain of origin. *Cancer Res*, **13,** 835-837.

Folkes, L.K., Candeias, L.P. & Wardman, P. (1998). Toward targeted "oxidation therapy" of cancer: peroxidase-catalysed cytotoxicity of indole-3-acetic acids. *Int J Radiat Oncol Biol Phys*, **42,** 917-20.

Folkes, L.K., Dennis, M.F., Stratford, M.R., Candeias, L.P. & Wardman, P. (1999). Peroxidase-catalyzed effects of indole-3-acetic acid and analogues on lipid membranes, DNA, and mammalian cells in vitro. *Biochem Pharmacol*, **57,** 375-82.

Folkes, L.K., Greco, O., Dachs, G.U., Stratford, M.R. & Wardman, P. (2002). 5-Fluoroindole-3-acetic acid: a prodrug activated by a peroxidase with potential for use in targeted cancer therapy. *Biochem Pharmacol*, **63,** 265-72.

Folkes, L.K. & Wardman, P. (2001). Oxidative activation of indole-3-acetic acids to cytotoxic species- a potential new role for plant auxins in cancer therapy. *Biochem Pharmacol*, **61,** 129-36.

Folkman, J. (1990). Endothelial cells and angiogenic growth factors in cancer growth and metastasis. Introduction. *Cancer Metastasis Rev*, **9,** 171-4.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D. & Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*, **16,** 4604-13.

Freeman, S.M., Abboud, C.N., Whartenby, K.A., Packman, C.H., Koeplin, D.S., Moolten, F.L. & Abraham, G.N. (1993). The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res*, **53,** 5274-83.

Freytag, S.O., Khil, M., Stricker, H., Peabody, J., Menon, M., DePeralta-Venturina, M., Nafziger, D., Pegg, J., Paielli, D., Brown, S., Barton, K., Lu, M., Aguilar-Cordova, E. & Kim, J.H. (2002). Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res*, **62,** 4968-76.

Freytag, S.O., Stricker, H., Pegg, J., Paielli, D., Pradhan, D.G., Peabody, J., DePeralta-Venturina, M., Xia, X., Brown, S., Lu, M. & Kim, J.H. (2003). Phase I study of replication -competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. *Cancer Res*, **63,** 7497-7506.

Friedlos, F., Court, S., Ford, M., Denny, W.A. & Springer, C. (1998). Gene-directed enzyme prodrug therapy: quantitative bystander cytotoxicity and DNA damage induced by CB1954 in cells expressing bacterial nitroreductase. *Gene Ther*, **5,** 105-12.

Friery, O.P., Gallagher, R., Murray, M.M., Hughes, C.M., Galligan, E.S., McIntyre, I.A., Patterson, L.H., Hirst, D.G. & McKeown, S.R. (2000). Enhancement of the anti-tumour effect of cyclophosphamide by the bioreductive drugs AQ4N and tirapazamine. *Br J Cancer*, **82,** 1469-73.

Fuller, R.W., Lacefield, W.B., Kattau, R.W., Nickander, R.C. & Snoddy, H.D. (1971). Myotonia produced by indoleacetic acid. Studies with related compounds and correlation with drug levels in tissues. *Arch Int Pharmacodyn Ther*, **193,** 48-60.

Fulop, G.M. & Phillips, R.A. (1990). The scid mutation in mice causes a general defect in DNA repair. *Nature*, **347,** 479-82.

Fyles, A.W., Milosevic, M., Pintilie, M., Syed, A. & Hill, R.P. (2000). Anemia, hypoxia and transfusion in patients with cervix cancer: a review. *Radiother Oncol*, **57,** 13-9.

Gagandeep, S., Brew, R., Green, B., Christmas, S.E., Klatzmann, D., Poston, G.J. & Kinsella, A.R. (1996). Prodrug-activated gene therapy: involvement of an immunological component in the "bystander effect". *Cancer Gene Ther*, **3,** 83-8.

Gazzard, B.G., Ford-Hutchinson, A.W., Smith, M.J. & Williams, R. (1973). The binding of paracetamol to plasma proteins of man and pig. *J Pharm Pharmacol*, **25,** 964-7.

Ge, K., Xu, L., Zheng, Z., Xu, D., Sun, L. & Liu, X. (1997). Transduction of cytosine deaminase gene makes rat glioma cells highly sensitive to 5-fluorocytosine. *Int J Cancer*, **71,** 675-9.

Gehl, J. (2003). Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol Scand*, **177,** 437-47.

Goto, T., Nishi, T., Tamura, T., Dev, S.B., Takeshima, H., Kochi, M., Yoshizato, K., Kuratsu, J., Sakata, T., Hofmann, G.A. & Ushio, Y. (2000). Highly efficient electro-gene therapy of solid tumor by using an expression plasmid for the

herpes simplex virus thymidine kinase gene. *Proc Natl Acad Sci U S A*, **97,** 354-9.

Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W. & Giaccia, A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*, **379,** 88-91.

Graham, M.A., Senan, S., Robin, H., Jr., Eckhardt, N., Lendrem, D., Hincks, J., Greenslade, D., Rampling, R., Kaye, S.B., von Roemeling, R. & Workman, P. (1997). Pharmacokinetics of the hypoxic cell cytotoxic agent tirapazamine and its major bioreductive metabolites in mice and humans: retrospective analysis of a pharmacokinetically guided dose-escalation strategy in a phase I trial. *Cancer Chemother Pharmacol*, **40,** 1-10.

Grau, C. & Overgaard, J. (1988). Effect of cancer chemotherapy on the hypoxic fraction of a solid tumor measured using a local tumor control assay. *Radiother Oncol*, **13,** 301-9.

Graven, K.K., Yu, Q., Pan, D., Roncarati, J.S. & Farber, H.W. (1999). Identification of an oxygen responsive enhancer element in the glyceraldehyde-3-phosphate dehydrogenase gene. *Biochim Biophys Acta*, **1447,** 208-18.

Gray, L.H., Conger, A.D. & Ebert, M. (1953). The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol*, **26,** 638-648.

Greco, O., Dachs, G.U., Tozer, G.M. & Kanthou, C. (2002a). Mechanisms of cytotoxicity induced by horseradish peroxidase/indole-3-acetic acid gene therapy. *J Cell Biochem*, **87,** 221-32.

Greco, O., Folkes, L.K., Wardman, P., Tozer, G.M. & Dachs, G.U. (2000). Development of a novel enzyme/prodrug combination for gene therapy of cancer: horseradish peroxidase/indole-3-acetic acid. *Cancer Gene Ther*, **7,** 1414-20.

Greco, O., Marples, B., Dachs, G.U., Williams, K.J., Patterson, A.V. & Scott, S.D. (2002b). Novel chimeric gene promoters responsive to hypoxia and ionizing radiation. *Gene Ther*, **9,** 1403-11.

Greco, O., Rossiter, S., Kanthou, C., Folkes, L.K., Wardman, P., Tozer, G.M. & Dachs, G.U. (2001). Horseradish peroxidase-mediated gene therapy: Choice of prodrugs in oxic and anoxic tumour conditions. *Molecular Cancer Therapeutics*, **1,** 1-10.

Greco, O., Tozer, G.M. & Dachs, G.U. (2002c). Oxic and anoxic enhancement of radiation-mediated toxicity by horseradish peroxidase/indole-3-acetic acid gene therapy. *Int J Radiat Biol*, **78,** 173-81.

Griffiths, J.R. (1991). Are cancer cells acidic? *Br J Cancer*, **64,** 425-7.

Griffiths, L., Binley, K., Iqball, S., Kan, O., Maxwell, P., Ratcliffe, P., Lewis, C., Harris, A., Kingsman, S. & Naylor, S. (2000). The macrophage - a novel system to deliver gene therapy to pathological hypoxia. *Gene Ther*, **7,** 255-62.

Grignet-Debrus, C., Cool, V., Baudson, N., Velu, T. & Calberg-Bacq, C.M. (2000). The role of cellular- and prodrug-associated factors in the bystander effect induced by the Varicella zoster and Herpes simplex viral thymidine kinases in suicide gene therapy. *Cancer Gene Ther*, **7,** 1456-68.

Haack, K., Linnebacher, M., Eisold, S., Zoller, M., von Knebel Doeberitz, M. & Gebert, J. (2000). Induction of protective immunity against syngeneic rat cancer cells by expression of the cytosine deaminase suicide gene. *Cancer Gene Ther*, **7,** 1357-64.

Haensgen, G., Krause, U., Becker, A., Stadler, P., Lautenschlaeger, C., Wohlrab, W., Rath, F.W., Molls, M. & Dunst, J. (2001). Tumor hypoxia, p53, and prognosis in cervical cancers. *Int J Radiat Oncol Biol Phys*, **50,** 865-72.

Hall, E.J. (1994). *Radiobiology for the radiologist.* Lippincott: Philadelphia.

Hall, E.J. (2000). *Radiobiology for the radiologist.* Vol. 1. Lippincott Williams and Wilkins: Philadelphia.

Hallahan, D., Geng, L., Qu, S., Scarfone, C., Giorgio, T., Donnelly, E., Gao, X. & Clanton, J. (2003). Integrin-mediated targeting of drug delivery to irradiated tumor blood vessels. *Cancer Cell*, **3,** 63-74.

Halpern, J., Jaw, J.W., Cornfield, L.J., Balfour, C. & Mash, E. (1989). Selective inactivation of rat liver cytochromes P450 by 21-chlorinated steroids. *Drug Metab Dispos*, **17,** 26-31.

Hamel, W., Magnelli, L., Chiarugi, V.P. & Israel, M.A. (1996). Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. *Cancer Res*, **56,** 2697-702.

Hamstra, D.A., Rice, D.J., Pu, A., Oyedijo, D., Ross, B.D. & Rehemtulla, A. (1999). Combined radiation and enzyme/prodrug treatment for head and neck cancer in an orthotopic animal model. *Radiat Res*, **152,** 499-507.

Hanna, N.N., Mauceri, H.J., Wayne, J.D., Hallahan, D.E., Kufe, D.W. & Weichselbaum, R.R. (1997). Virally directed cytosine deaminase/5-fluorocytosine gene therapy enhances radiation response in human cancer xenografts. *Cancer Res*, **57,** 4205-9.

Harbottle, R.P., Cooper, R.G., Hart, S.L., Ladhoff, A., McKay, T., Knight, A.M., Wagner, E., Miller, A.D. & Coutelle, C. (1998). An RGD-oligolysine peptide: a prototype construct for integrin-mediated gene delivery. *Hum Gene Ther*, **9,** 1037-47.

Harman, L.S., Carver, D.K., Schreiber, J. & Mason, R.P. (1986). One- and two-electron oxidation of reduced glutathione by peroxidases. *J Biol Chem*, **261,** 1642-8.

Hart, S.L., Arancibia-Carcamo, C.V., Wolfert, M.A., Mailhos, C., O'Reilly, N.J., Ali, R.R., Coutelle, C., George, A.J., Harbottle, R.P., Knight, A.M., Larkin, D.F., Levinsky, R.J., Seymour, L.W., Thrasher, A.J. & Kinnon, C. (1998). Lipid-mediated enhancement of transfection by a nonviral integrin- targeting vector. *Hum Gene Ther*, **9,** 575-85.

Hay, M.P., Gamage, S.A., Kovacs, M.S., Pruijn, F.B., Anderson, R.F., Patterson, A.V., Wilson, W.R., Brown, J.M. & Denny, W.A. (2003). Structure-activity relationships of 1,2,4-benzotriazine 1,4-dioxides as hypoxia-selective analogues of tirapazamine. *J Med Chem*, **46,** 169-82.

Heading, R.C., Nimmo, J., Prescott, L.F. & Tothill, P. (1973). The dependence of paracetamol absorption on the rate of gastric emptying. *Br J Pharmacol*, **47,** 415-21.

Heath-Pagliuso, S., Rogers, W.J., Tullis, K., Seidel, S.D., Cenijn, P.H., Brouwer, A. & Denison, M.S. (1998). Activation of the Ah receptor by tryptophan and tryptophan metabolites. *Biochemistry*, **37,** 11508-15.

Heim, R., Prasher, D.C. & Tsien, R.Y. (1994). Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc Natl Acad Sci U S A*, **91,** 12501-4.

Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D.D. & Kirn, D.H. (1997). ONYX-015, an E1B gene-attenuated adenovirus, causes

tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat Med*, **3,** 639-45.

Hejmadi, M.V., McKeown, S.R., Friery, O.P., McIntyre, I.A., Patterson, L.H. & Hirst, D.G. (1996). DNA damage following combination of radiation with the bioreductive drug AQ4N: possible selective toxicity to oxic and hypoxic tumour cells. *Br J Cancer*, **73,** 499-505.

Heller, L., Jaroszeski, M.J., Coppola, D., Pottinger, C., Gilbert, R. & Heller, R. (2000). Electrically mediated plasmid DNA delivery to hepatocellular carcinomas in vivo. *Gene Ther*, **7,** 826-9.

Heller, L.C. & Coppola, D. (2002). Electrically mediated delivery of vector plasmid DNA elicits an antitumor effect. *Gene Ther*, **9,** 1321-5.

Hemminki, A. & Alvarez, R.D. (2002). Adenoviruses in oncology: a viable option? *BioDrugs*, **16,** 77-87.

Hendrickson, E.A., Qin, X.Q., Bump, E.A., Schatz, D.G., Oettinger, M. & Weaver, D.T. (1991). A link between double-strand break-related repair and V(D)J recombination: the scid mutation. *Proc Natl Acad Sci U S A*, **88,** 4061-5.

Hewitt, H.B., Blake, E.R. & Walder, A.S. (1976). A critique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumours of spontaneous origin. *Br J Cancer*, **33,** 241-59.

Hicks, K.O., Fleming, Y., Siim, B.G., Koch, C.J. & Wilson, W.R. (1998). Extravascular diffusion of tirapazamine: effect of metabolic consumption assessed using the multicellular layer model. *Int J Radiat Oncol Biol Phys*, **42,** 641-9.

Hockel, M., Schlenger, K., Hockel, S. & Vaupel, P. (1999). Hypoxic cervical cancers with low apoptotic index are highly aggressive. *Cancer Res*, **59,** 4525-8.

Hockel, M., Schlenger, K., Knoop, C. & Vaupel, P. (1991). Oxygenation of carcinomas of the uterine cervix: evaluation by computerized O2 tension measurements. *Cancer Res*, **51,** 6098-102.

Hockel, M. & Vaupel, P. (2001). Biological consequences of tumor hypoxia. *Semin Oncol*, **28,** 36-41.

Holmgren, L., O'Reilly, M.S. & Folkman, J. (1995). Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med*, **1,** 149-53.

Hong, Z., Li, Z., Wang, Y., Li, L., Qiang, W., Wang, M. & Zou, J. (1994). [Pharmacokinetics and bioavailability study on acetaminophen oral drop in healthy volunteers]. *Hua Xi Yi Ke Da Xue Xue Bao*, **25,** 410-3.

Hongslo, J.K., Bjorge, C., Schwarze, P.E., Brogger, A., Mann, G., Thelander, L. & Holme, J.A. (1990). Paracetamol inhibits replicative DNA synthesis and induces sister chromatid exchange and chromosomal aberrations by inhibition of ribonucleotide reductase. *Mutagenesis*, **5,** 475-80.

Hoskin, P.J., Saunders, M.I. & Dische, S. (1999). Hypoxic radiosensitizers in radical radiotherapy for patients with bladder carcinoma: hyperbaric oxygen, misonidazole, and accelerated radiotherapy, carbogen, and nicotinamide. *Cancer*, **86,** 1322-8.

Hoskin, P.J., Sibtain, A., Daley, F.M. & Wilson, G.D. (2003). GLUT1 and CAIX as intrinsic markers of hypoxia in bladder cancer: relationship with vascularity and proliferation as predictors of outcome of ARCON. *Br J Cancer*, **89,** 1290-1297.

Hotter, G., Closa, D., Prats, N., Pi, F., Gelpi, E. & Rosello-Catafau, J. (1997). Free radical enhancement promotes leucocyte recruitment through a PAF and LTB4 dependent mechanism. *Free Radic Biol Med*, **22,** 947-54.

Howard-Flanders, P. (1958). Physical and chemical mechanisms in the injury of cells by ionizing radiations. *Adv Biol Med Phys*, **6,** 553-603.

Huang, L.E., Gu, J., Schau, M. & Bunn, H.F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2- dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A*, **95,** 7987-92.

Huber, B.E., Austin, E.A., Good, S.S., Knick, V.C., Tibbels, S. & Richards, C.A. (1993). In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. *Cancer Res*, **53,** 4619-26.

Hughes, C.S., Shen, J.W. & Subjeck, J.R. (1989). Resistance to etoposide induced by three glucose-regulated stresses in Chinese hamster ovary cells. *Cancer Res*, **49,** 4452-4.

Hui, E.P., Chan, A.T., Pezzella, F., Turley, H., To, K.F., Poon, T.C., Zee, B., Mo, F., Teo, P.M., Huang, D.P., Gatter, K.C., Johnson, P.J. & Harris, A.L. (2002). Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. *Clin Cancer Res*, **8,** 2595-604.

Humphreys, M.J., Ghaneh, P., Greenhalf, W., Campbell, F., Clayton, T.M., Everett, P., Huber, B.E., Richards, C.A., Ford, M.J. & Neoptolemos, J.P. (2001). Hepatic intra-arterial delivery of a retroviral vector expressing the cytosine deaminase gene, controlled by the CEA promoter and intraperitoneal treatment with 5-fluorocytosine suppresses growth of colorectal liver metastases. *Gene Ther*, **8,** 1241-7.

Hyacinthe, M., Jaroszeski, M.J., Dang, V.V., Coppola, D., Karl, R.C., Gilbert, R.A. & Heller, R. (1999). Electrically enhanced drug delivery for the treatment of soft tissue sarcoma. *Cancer*, **85,** 409-17.

Ido, A., Uto, H., Moriuchi, A., Nagata, K., Onaga, Y., Onaga, M., Hori, T., Hirono, S., Hayashi, K., Tamaoki, T. & Tsubouchi, H. (2001). Gene therapy targeting for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by a hypoxia-inducible enhancer linked to a human alpha-fetoprotein promoter. *Cancer Res*, **61,** 3016-21.

Irie, R.F., Irie, K. & Morton, D.L. (1974). Natural antibody in human serum to a neoantigen in human cultured cells grown in fetal bovine serum. *J Natl Cancer Inst*, **52,** 1051-8.

Jacobson, M.A., De Miranda, P., M., C.D., T., B. & E., C. (1987). Human Pharmacokinetics and tolerance of oral ganciclovir. *J American Med Assoc*, **31,** 1251-1254.

Jacobson, M.D. & Raff, M.C. (1995). Programmed cell death and Bcl-2 protection in very low oxygen. *Nature*, **374,** 814-6.

Jaffar, M., Naylor, M.A., Robertson, N., Lockyer, S.D., Phillips, R.M., Everett, S.A., Adams, G.E. & Stratford, I.J. (1998). 5-substituted analogues of 3-hydroxymethyl-5-aziridinyl-1-methyl-2-[1H-indole-4,7-dione]prop-2-en- 1-ol (EO9, NSC 382459) and their regioisomers as hypoxia-selective agents: structure-cytotoxicity in vitro. *Anticancer Drug Des*, **13,** 105-23.

Jaffar, M., Phillips, R.M., Williams, K.J., Mrema, I., Cole, C., Wind, N.S., Ward, T.H., Stratford, I.J. & Patterson, A.V. (2003). 3-substituted-5-aziridinyl-1-

methylindole-4,7-diones as NQO1-directed antitumour agents: mechanism of activation and cytotoxicity in vitro. *Biochem Pharmacol*, **66,** 1199-206.

Jamasbi, R.J. & Nettesheim, P. (1979). Increase in immunogenicity with concomitant loss of tumorigenicity of respiratory tract carcinomas during in vitro culture. *Cancer Res*, **39,** 2466-70.

John, J.A., Blogg, C.D., Murray, F.J., Schwetz, B.A. & Gehring, P.J. (1979). Teratogenic effects of the plant hormone indole-3-acetic acid in mice and rats. *Teratology*, **19,** 321-4.

Johnson, C.A., Kilpatrick, D., von Roemeling, R., Langer, C., Graham, M.A., Greenslade, D., Kennedy, G., Keenan, E. & O'Dwyer, P.J. (1997). Phase I trial of tirapazamine in combination with cisplatin in a single dose every 3 weeks in patients with solid tumors. *J Clin Oncol*, **15,** 773-80.

Jones, L.H., Abdalla, D.S. & Freitas, J.C. (1995). Effects of indole-3-acetic acid on croton oil- and arachidonic acid- induced mouse ear edema. *Inflamm Res*, **44,** 372-5.

Jounaidi, Y. & Waxman, D.J. (2000). Combination of the bioreductive drug tirapazamine with the chemotherapeutic prodrug cyclophosphamide for P450/P450-reductase-based cancer gene therapy. *Cancer Res*, **60,** 3761-9.

Kallio, P.J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H. & Poellinger, L. (1998). Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *Embo J*, **17,** 6573-86.

Kallio, P.J., Wilson, W.J., O'Brien, S., Makino, Y. & Poellinger, L. (1999). Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. *J Biol Chem*, **274,** 6519-25.

Kambara, H., Tamiya, T., Ono, Y., Ohtsuka, S., Terada, K., Adachi, Y., Ichikawa, T., Hamada, H. & Ohmoto, T. (2002). Combined radiation and gene therapy for brain tumors with adenovirus-mediated transfer of cytosine deaminase and uracil phosphoribosyltransferase genes. *Cancer Gene Ther*, **9,** 840-5.

Kanai, F., Lan, K.H., Shiratori, Y., Tanaka, T., Ohashi, M., Okudaira, T., Yoshida, Y., Wakimoto, H., Hamada, H., Nakabayashi, H., Tamaoki, T. & Omata, M. (1997). In vivo gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. *Cancer Res*, **57,** 461-5.

Keller, R.J. & Hinson, J.A. (1991). Mechanism of acetaminophen-stimulated NADPH oxidation catalyzed by the peroxidase-H2O2 system. *Drug Metab Dispos*, **19,** 184-7.

Kenten, R.H. (1955). The oxidation of indolyl-3-acetic acid by waxpod bean root sap and peroxidase systems. *Biochem J*, **59,** 110-121.

Kerbel, R.S. (1991). Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents. *Bioessays*, **13,** 31-6.

Kievit, E., Nyati, M.K., Ng, E., Stegman, L.D., Parsels, J., Ross, B.D., Rehemtulla, A. & Lawrence, T.S. (2000). Yeast cytosine deaminase improves radiosensitization and bystander effect by 5-fluorocytosine of human colorectal cancer xenografts. *Cancer Res*, **60,** 6649-55.

Kim, M., Zinn, K.R., Barnett, B.G., Sumerel, L.A., Krasnykh, V., Curiel, D.T. & Douglas, J.T. (2002). The therapeutic efficacy of adenoviral vectors for cancer gene therapy is limited by a low level of primary adenovirus receptors on tumour cells. *Eur J Cancer*, **38,** 1917-26.

Kinoshita, M., Johnson, D.L., Shatney, C.H., Lee, Y.L. & Mochizuki, H. (2001). Cancer cells surviving hypoxia obtain hypoxia resistance and maintain anti-apoptotic potential under reoxygenation. *Int J Cancer*, **91,** 322-6.

Kirn, D. (2001). Oncolytic virotherapy for cancer with the adenovirus dl1520 (Onyx-015): results of phase I and II trials. *Expert Opin Biol Ther*, **1,** 525-38.

Kishida, T., Asada, H., Satoh, E., Tanaka, S., Shinya, M., Hirai, H., Iwai, M., Tahara, H., Imanishi, J. & Mazda, O. (2001). In vivo electroporation-mediated transfer of interleukin-12 and interleukin-18 genes induces significant antitumor effects against melanoma in mice. *Gene Ther*, **8,** 1234-40.

Klatzmann, D., Valery, C.A., Bensimon, G., Marro, B., Boyer, O., Mokhtari, K., Diquet, B., Salzmann, J.L. & Philippon, J. (1998). A phase I/II study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for recurrent glioblastoma. Study Group on Gene Therapy for Glioblastoma. *Hum Gene Ther*, **9,** 2595-604.

Knight, T.R. & Jaeschke, H. (2002). Acetaminophen-induced inhibition of Fas receptor-mediated liver cell apoptosis: mitochondrial dysfunction versus glutathione depletion. *Toxicol Appl Pharmacol*, **181,** 133-41.

Knox, R. (1999). *Enzyme pro-drug strategies for cancer therapy*. Kluwer Academic/Plenum Publishers: New York.

Knox, R.J., Boland, M.P., Friedlos, F., Coles, B., Southan, C. & Roberts, J.J. (1988). The nitroreductase enzyme in Walker cells that activates 5-(aziridin-1- yl)-2,4-dinitrobenzamide (CB 1954) to 5-(aziridin-1-yl)-4-hydroxylamino- 2-nitrobenzamide is a form of NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2). *Biochem Pharmacol*, **37,** 4671-7.

Knuchel, R., Feichtinger, J., Recktenwald, A., Hollweg, H.G., Franke, P., Jakse, G., Rammal, E. & Hofstadter, F. (1988). Interactions between bladder tumor cells as tumor spheroids from the cell line J82 and human endothelial cells in vitro. *J Urol*, **139,** 640-5.

Kobayashi, S., Sugioka, K., Nakano, M., Takyu, C., Yamagishi, A. & Inaba, H. (1980). Excitation of indole acetate in myeloperoxidase-H2O2 system: possible formation of indole acetate cation radical. *Biochem Biophys Res Commun*, **93,** 967-73.

Kobori, K., Sakakibara, H., Maruyama, K., Kobayashi, T. & Yamaki, T. (1983). [A rapid method for determining urinary indoleacetic acid concentration and its clinical significance as the tumor-marker in the diagnosis of malignant diseases]. *J Uoeh*, **5,** 213-20.

Koshikawa, N., Takenaga, K., Tagawa, M. & Sakiyama, S. (2000). Therapeutic efficacy of the suicide gene driven by the promoter of vascular endothelial growth factor gene against hypoxic tumor cells. *Cancer Res*, **60,** 2936-41.

Kosower, N.S. & Kosower, E.M. (1978). The glutathione status of cells. *Int Rev Cytol*, **54,** 109-60.

Koukourakis, M.I., Giatromanolaki, A., Sivridis, E., Simopoulos, C., Turley, H., Talks, K., Gatter, K.C. & Harris, A.L. (2002). Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. *Int J Radiat Oncol Biol Phys*, **53,** 1192-202.

Kuriyama, S., Kikukawa, M., Masui, K., Okuda, H., Nakatani, T., Akahane, T., Mitoro, A., Tominaga, K., Tsujinoue, H., Yoshiji, H., Okamoto, S., Fukui, H. & Ikenaka, K. (1999a). Cancer gene therapy with HSV-tk/GCV system

depends on T-cell-mediated immune responses and causes apoptotic death of tumor cells in vivo. *Int J Cancer*, **83,** 374-80.

Kuriyama, S., Kikukawa, M., Masui, K., Okuda, H., Nakatani, T., Sakamoto, T., Yoshiji, H., Fukui, H., Ikenaka, K., Mullen, C.A. & Tsujii, T. (1999b). Cytosine deaminase/5-fluorocytosine gene therapy can induce efficient anti-tumor effects and protective immunity in immunocompetent mice but not in athymic nude mice. *Int J Cancer*, **81,** 592-7.

Kuriyama, S., Mitoro, A., Yamazaki, M., Tsujinoue, H., Nakatani, T., Akahane, T., Toyokawa, Y., Kojima, H., Okamoto, S. & Fukui, H. (1999c). Comparison of gene therapy with the herpes simplex virus thymidine kinase gene and the bacterial cytosine deaminase gene for the treatment of hepatocellular carcinoma. *Scand J Gastroenterol*, **34,** 1033-41.

Kurozumi, K., Tamiya, T., Ono, Y., Otsuka, S., Kamabara, H., Adachi, Y., Ichikawa, T., Hamada, H. & Ohmoto, T. (2004). Apoptosis induction with 5-fluorocytosine/cytosine deaminase gene therapy for human malignant glioma cells mediated by adenovirus. *J Neurooncol*, **66,** 117-127.

Kyle, A.H. & Minchinton, A.I. (1999). Measurement of delivery and metabolism of tirapazamine to tumour tissue using the multilayered cell culture model. *Cancer Chemother Pharmacol*, **43,** 213-20.

Lan, K.H., Kanai, F., Shiratori, Y., Ohashi, M., Tanaka, T., Okudaira, T., Yoshida, Y., Hamada, H. & Omata, M. (1997). In vivo selective gene expression and therapy mediated by adenoviral vectors for human carcinoembryonic antigen-producing gastric carcinoma. *Cancer Res*, **57,** 4279-84.

Liu, S.C., Minton, N.P., Giaccia, A.J. & Brown, J.M. (2002). Anticancer efficacy of systemically delivered anaerobic bacteria as gene therapy vectors targeting tumor hypoxia/necrosis. *Gene Ther*, **9,** 291-6.

Lloyd, R.V., Duling, D.R., Rumyantseva, G.V., Mason, R.P. & Bridson, P.K. (1991). Microsomal reduction of 3-amino-1,2,4-benzotriazine 1,4-dioxide to a free radical. *Mol Pharmacol*, **40,** 440-5.

Lord, E.M. (1987). Factors influencing tumour cell immunogenicity. In *Rodent tumour models in experimental cancer therapy*, Kallman, R.F. (ed) pp. 23-26. Pergamon Press: Elmsford NY.

Maccubbin, A.E., Caballes, L., Riordan, J.M., Huang, D.H. & Gurtoo, H.L. (1991). A cyclophosphamide/DNA phosphoester adduct formed in vitro and in vivo. *Cancer Res*, **51,** 886-92.

MacPhail, S.H., Banath, J.P., Yu, T.Y., Chu, E.H., Lambur, H. & Olive, P.L. (2003). Expression of phosphorylated histone H2AX in cultured cell lines following exposure to X-rays. *Int J Radiat Biol*, **79,** 351-8.

Manov, I., Hirsh, M. & Iancu, T.C. (2002). Acetaminophen hepatotoxicity and mechanisms of its protection by N-acetylcysteine: a study of Hep3B cells. *Exp Toxicol Pathol*, **53,** 489-500.

Marais, R., Spooner, R.A., Light, Y., Martin, J. & Springer, C.J. (1996). Gene-directed enzyme prodrug therapy with a mustard prodrug/carboxypeptidase G2 combination. *Cancer Res*, **56,** 4735-42.

Marais, R., Spooner, R.A., Stribbling, S.M., Light, Y., Martin, J. & Springer, C.J. (1997). A cell surface tethered enzyme improves efficiency in gene-directed enzyme prodrug therapy. *Nat Biotechnol*, **15,** 1373-7.

Marples, B., Scott, S.D., Hendry, J.H., Embleton, M.J., Lashford, L.S. & Margison, G.P. (2000). Development of synthetic promoters for radiation-mediated gene therapy. *Gene Ther*, **7,** 511-7.

Marshall, E. (1999). Gene therapy death prompts review of adenovirus vector. *Science*, **286,** 2244-5.

Mathupala, S.P., Rempel, A. & Pedersen, P.L. (2001). Glucose Catabolism in Cancer Cells. IDENTIFICATION AND CHARACTERIZATION OF A MARKED ACTIVATION RESPONSE OF THE TYPE II HEXOKINASE GENE TO HYPOXIC CONDITIONS. *J Biol Chem*, **276,** 43407-12.

Matsumura, Y. & 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,** 6387-92.

McCarthy, H.O., Yakkundi, A., McErlane, V., Hughes, C.M., Keilty, G., Murray, M., Patterson, L.H., Hirst, D.G., McKeown, S.R. & Robson, T. (2003). Bioreductive GDEPT using cytochrome P450 3A4 in combination with AQ4N. *Cancer Gene Ther*, **10,** 40-8.

McGilveray, I.J. & Mattok, G.L. (1972). Some factors affecting the absorption of paracetamol. *J Pharm Pharmacol*, **24,** 615-9.

McGuire, J.J., Canestrari, J.G. & Nagel, G.S. (1999). Characterization of the effect of AG337, a novel lipophilic thymidylate synthase inhibitor, on human head and neck and human leukemia cell lines. *Int J Oncol*, **15,** 1245-50.

McKeown, S.R., Hejmadi, M.V., McIntyre, I.A., McAleer, J.J. & Patterson, L.H. (1995). AQ4N: an alkylaminoanthraquinone N-oxide showing bioreductive potential and positive interaction with radiation in vivo. *Br J Cancer*, **72,** 76-81.

McLean, A.E. & Day, P.A. (1975). The effect of diet on the toxicity of paracetamol and the safety of paracetamol-methionine mixtures. *Biochem Pharmacol*, **24,** 37-42.

McNeish, I.A., Green, N.K., Gilligan, M.G., Ford, M.J., Mautner, V., Young, L.S., Kerr, D.J. & Searle, P.F. (1998). Virus directed enzyme prodrug therapy for ovarian and pancreatic cancer using retrovirally delivered E. coli nitroreductase and CB1954. *Gene Ther*, **5,** 1061-9.

Mesnil, M., Piccoli, C., Tiraby, G., Willecke, K. & Yamasaki, H. (1996). Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc Natl Acad Sci U S A*, **93,** 1831-5.

Mikata, K., Uemura, H., Ohuchi, H., Ohta, S., Nagashima, Y. & Kubota, Y. (2002). Inhibition of growth of human prostate cancer xenograft by transfection of p53 gene: gene transfer by electroporation. *Mol Cancer Ther*, **1,** 247-52.

Miles, B.J., Shalev, M., Aguilar-Cordova, E., Timme, T.L., Lee, H.M., Yang, G., Adler, H.L., Kernen, K., Pramudji, C.K., Satoh, T., Gdor, Y., Ren, C., Ayala, G., Wheeler, T.M., Butler, E.B., Kadmon, D. & Thompson, T.C. (2001). Prostate-specific antigen response and systemic T cell activation after in situ gene therapy in prostate cancer patients failing radiotherapy. *Hum Gene Ther*, **12,** 1955-67.

Miller, A.D. (1996). Cell-surface receptors for retroviruses and implications for gene transfer. *Proc Natl Acad Sci U S A*, **93,** 11407-13.

Mirsky, I.A. & Diengott, D. (1956). Hypoglycemic action of indole-3-acetic acid by mouth in patients with diabetes mellitus. *Proc Soc Exp Biol*, **93,** 109-110.

Mizuno, M., Ryuke, Y. & Yoshida, J. (2002). Cationic liposomes conjugation to recombinant adenoviral vectors containing herpes simplex virus thymidine kinase gene followed by ganciclovir treatment reduces viral antigenicity and maintains antitumor activity in mouse experimental glioma models. *Cancer Gene Ther*, **9,** 825-9.

Modlich, U., Pugh, C.W. & Bicknell, R. (2000). Increasing endothelial cell specific expression by the use of heterologous hypoxic and cytokine-inducible enhancers. *Gene Ther*, **7,** 896-902.

Moolten, F.L. (1986). Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res*, **46,** 5276-81.

Morse, G.D., Shelton, M.J. & O'Donnell, A.M. (1993). Comparative pharmacokinetics of antiviral nucleoside analogues. *Clin Pharmacokinet*, **24,** 101-23.

Mullen, C.A., Coale, M.M., Lowe, R. & Blaese, R.M. (1994). Tumors expressing the cytosine deaminase suicide gene can be eliminated in vivo with 5-fluorocytosine and induce protective immunity to wild type tumor. *Cancer Res*, **54,** 1503-6.

Muller, C., Readhead, C., Diederichs, S., Idos, G., Yang, R., Tidow, N., Serve, H., Berdel, W.E. & Koeffler, H.P. (2000). Methylation of the cyclin A1 promoter correlates with gene silencing in somatic cell lines, while tissue-specific expression of cyclin A1 is methylation independent. *Mol Cell Biol*, **20,** 3316-29.

Nagao, S., Kuriyama, S., Okuda, H., Tominaga, K., Nakatani, T., Tsujinoue, H., Yoshiji, H. & Fukui, H. (2001). Adenovirus-mediated gene transfer into tumors: evaluation of direct readministration of an adenoviral vector into subcutaneous tumors of immunocompetent mice. *Int J Oncol*, **18,** 57-65.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M. & Trono, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*, **272,** 263-7.

Napier, M.P., Sharma, S.K., Springer, C.J., Bagshawe, K.D., Green, A.J., Martin, J., Stribbling, S.M., Cushen, N., O'Malley, D. & Begent, R.H. (2000). Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma. *Clin Cancer Res*, **6,** 765-72.

Narayanan, R.A., Rink, A., Beattie, C.W. & Hu, W.S. (2002). Differential gene expression analysis during porcine hepatocyte spheroid formation. *Mamm Genome*, **13,** 515-23.

Nemunaitis, J., Cunningham, C., Senzer, N., Kuhn, J., Cramm, J., Litz, C., Cavagnolo, R., Cahill, A., Clairmont, C. & Sznol, M. (2003). Pilot trial of genetically modified, attenuated Salmonella expressing the E. coli cytosine deaminase gene in refractory cancer patients. *Cancer Gene Ther*, **10,** 737-44.

Neumann, E., Schaefer-Ridder, M., Wang, Y. & Hofschneider, P.H. (1982). Gene transfer into mouse lyoma cells by electroporation in high electric fields. *Embo J*, **1,** 841-5.

Nicklin, S.A., Von Seggern, D.J., Work, L.M., Pek, D.C., Dominiczak, A.F., Nemerow, G.R. & Baker, A.H. (2001). Ablating adenovirus type 5 fiber-CAR binding and HI loop insertion of the SIGYPLP peptide generate an endothelial cell-selective adenovirus. *Mol Ther*, **4,** 534-42.

Niculescu-Duvaz, D., Niculescu-Duvaz, I., Friedlos, F., Martin, J., Spooner, R., Davies, L., Marais, R. & Springer, C.J. (1998). Self-immolative nitrogen mustard prodrugs for suicide gene therapy. *J Med Chem*, **41,** 5297-309.

Nielsen, L.L. & Maneval, D.C. (1998). P53 tumor suppressor gene therapy for cancer. *Cancer Gene Ther*, **5,** 52-63.

Nishi, T., Yoshizato, K., Yamashiro, S., Takeshima, H., Sato, K., Hamada, K., Kitamura, I., Yoshimura, T., Saya, H., Kuratsu, J. & Ushio, Y. (1996). High-

efficiency in vivo gene transfer using intraarterial plasmid DNA injection following in vivo electroporation. *Cancer Res*, **56,** 1050-5.
Nishida, M., Futami, S., Morita, I., Maekawa, K. & Murota, S.I. (2000). Hypoxia-reoxygenation inhibits gap junctional communication in cultured human umbilical vein endothelial cells. *Endothelium*, **7,** 279-86.
Ogris, M. & Wagner, E. (2002). Tumor-targeted gene transfer with DNA polyplexes. *Somat Cell Mol Genet*, **27,** 85-95.
Ohwada, A., Hirschowitz, E.A. & Crystal, R.G. (1996). Regional delivery of an adenovirus vector containing the Escherichia coli cytosine deaminase gene to provide local activation of 5- fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum Gene Ther*, **7,** 1567-76.
O'Keefe, D.S., Su, S.L., Bacich, D.J., Horiguchi, Y., Luo, Y., Powell, C.T., Zandvliet, D., Russell, P.J., Molloy, P.L., Nowak, N.J., Shows, T.B., Mullins, C., Vonder Haar, R.A., Fair, W.R. & Heston, W.D. (1998). Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochim Biophys Acta*, **1443,** 113-27.
Olive, P.L. (1995). Detection of hypoxia by measurement of DNA damage in individual cells from spheroids and murine tumours exposed to bioreductive drugs. II. RSU 1069. *Br J Cancer*, **71,** 537-42.
Olive, P.L., Luo, C.M. & Banath, J.P. (2002). Local hypoxia is produced at sites of intratumour injection. *Br J Cancer*, **86,** 429-35.
O'Malley, B.W., Jr., Chen, S.H., Schwartz, M.R. & Woo, S.L. (1995). Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res*, **55,** 1080-5.
Orr, R.M. & Monia, B.P. (1998). Antisense Therapy for cancer. *Curr Res Mol Therapuetics*, **1,** 102-108.
Osaki, T., Tanio, Y., Tachibana, I., Hosoe, S., Kumagai, T., Kawase, I., Oikawa, S. & Kishimoto, T. (1994). Gene therapy for carcinoembryonic antigen-producing human lung cancer cells by cell type-specific expression of herpes simplex virus thymidine kinase gene. *Cancer Res*, **54,** 5258-61.
Pannell, D., Osborne, C.S., Yao, S., Sukonnik, T., Pasceri, P., Karaiskakis, A., Okano, M., Li, E., Lipshitz, H.D. & Ellis, J. (2000). Retrovirus vector silencing is de novo methylase independent and marked by a repressive histone code. *Embo J*, **19,** 5884-94.
Parkins, C.S., Holder, A.L., A., H.S., Chaplin, D.J. & Tozer, G.M. (2000). Determinants of anti-vascular action by combretestatin A-4 phosphate: role of nitric oxide. *Br J Cancer*, **83,** 811-816.
Patten, C.J., Thomas, P.E., Guy, R.L., Lee, M., Gonzalez, F.J., Guengerich, F.P. & Yang, C.S. (1993). Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chem Res Toxicol*, **6,** 511-8.
Patterson, A.V., Barham, H.M., Chinje, E.C., Adams, G.E., Harris, A.L. & Stratford, I.J. (1995). Importance of P450 reductase activity in determining sensitivity of breast tumour cells to the bioreductive drug, tirapazamine (SR 4233). *Br J Cancer*, **72,** 1144-50.
Patterson, A.V., Saunders, M.P., Chinje, E.C., Talbot, D.C., Harris, A.L. & Strafford, I.J. (1997). Overexpression of human NADPH:cytochrome c (P450) reductase confers enhanced sensitivity to both tirapazamine (SR 4233) and RSU 1069. *Br J Cancer*, **76,** 1338-47.

Patterson, A.V., Williams, K.J., Cowen, R.L., Jaffar, M., Telfer, B.A., Saunders, M., Airley, R., Honess, D., van der Kogel, A.J., Wolf, C.R. & Stratford, I.J. (2002). Oxygen-sensitive enzyme-prodrug gene therapy for the eradication of radiation-resistant solid tumours. *Gene Ther*, **9,** 946-54.

Patterson, L.H. (1993). Rationale for the use of aliphatic N-oxides of cytotoxic anthraquinones as prodrug DNA binding agents: a new class of bioreductive agent. *Cancer Metastasis Rev*, **12,** 119-34.

Patterson, L.H. & McKeown, S.R. (2000). AQ4N: a new approach to hypoxia-activated cancer chemotherapy. *Br J Cancer*, **83,** 1589-93.

Patterson, L.H., McKeown, S.R., Robson, T., Gallagher, R., Raleigh, S.M. & Orr, S. (1999). Antitumour prodrug development using cytochrome P450 (CYP) mediated activation. *Anticancer Drug Des*, **14,** 473-86.

Patterson, L.H., McKeown, S.R., Ruparelia, K., Double, J.A., Bibby, M.C., Cole, S. & Stratford, I.J. (2000). Enhancement of chemotherapy and radiotherapy of murine tumours by AQ4N, a bioreductively activated anti-tumour agent. *Br J Cancer*, **82,** 1984-90.

Pawelek, J.M., Low, K.B. & Bermudes, D. (1997). Tumor-targeted Salmonella as a novel anticancer vector. *Cancer Res*, **57,** 4537-44.

Pawlik, T.M., Nakamura, H., Mullen, J.T., Kasuya, H., Yoon, S.S., Chandrasekhar, S., Chiocca, E.A. & Tanabe, K.K. (2002). Prodrug bioactivation and oncolysis of diffuse liver metastases by a herpes simplex virus 1 mutant that expresses the CYP2B1 transgene. *Cancer*, **95,** 1171-81.

Pedley, R.B., Sharma, S.K., Boxer, G.M., Boden, R., Stribbling, S.M., Davies, L., Springer, C.J. & Begent, R.H. (1999). Enhancement of antibody-directed enzyme prodrug therapy in colorectal xenografts by an antivascular agent. *Cancer Res*, **59,** 3998-4003.

Peters, K.B. & Brown, J.M. (2002). Tirapazamine: a hypoxia-activated topoisomerase II poison. *Cancer Res*, **62,** 5248-53.

Phillips, M.I. (2001). Gene therapy for hypertension: the preclinical data. *Hypertension*, **38,** 543-8.

Pierrefite-Carle, V., Baque, P., Gavelli, A., Benchimol, D., Bourgeon, A., Milano, G., Saint-Paul, M.C. & Rossi, B. (2000). Regression of experimental liver tumor after distant intra-hepatic injection of cytosine deaminase-expressing tumor cells and 5-fluorocytosine treatment. *Int J Mol Med*, **5,** 275-8.

Pierrefite-Carle, V., Baque, P., Gavelli, A., Brossette, N., Benchimol, D., Bourgeon, A., Saint Paul, M.C., Staccini, P. & Rossi, B. (2002). Subcutaneous or intrahepatic injection of suicide gene modified tumour cells induces a systemic antitumour response in a metastatic model of colon carcinoma in rats. *Gut*, **50,** 387-91.

Pierrefite-Carle, V., Baque, P., Gavelli, A., Mala, M., Chazal, M., Gugenheim, J., Bourgeon, A., Milano, G., Staccini, P. & Rossi, B. (1999). Cytosine deaminase/5-fluorocytosine-based vaccination against liver tumors: evidence of distant bystander effect. *J Natl Cancer Inst*, **91,** 2014-9.

Poland, J., Sinha, P., Siegert, A., Schnolzer, M., Korf, U. & Hauptmann, S. (2002). Comparison of protein expression profiles between monolayer and spheroid cell culture of HT-29 cells revealed fragmentation of CK18 in three-dimensional cell culture. *Electrophoresis*, **23,** 1174-84.

Potter, D.W. & Hinson, J.A. (1987). Mechanisms of acetaminophen oxidation to N-acetyl-P-benzoquinone imine by horseradish peroxidase and cytochrome P-450. *J Biol Chem*, **262,** 966-73.

Potter, D.W. & Hinson, J.A. (1989). Acetaminophen peroxidation reactions. *Drug Metab Rev*, **20,** 341-58.

Potter, D.W., Miller, D.W. & Hinson, J.A. (1985). Identification of acetaminophen polymerization products catalyzed by horseradish peroxidase. *J Biol Chem*, **260,** 12174-80.

Potter, D.W., Miller, D.W. & Hinson, J.A. (1986). Horseradish peroxidase-catalyzed oxidation of acetaminophen to intermediates that form polymers or conjugate with glutathione. *Mol Pharmacol*, **29,** 155-62.

Prescott, L.F. (1980). Kinetics and metabolism of paracetamol and phenacetin. *Br J Clin Pharmacol*, **10 Suppl 2,** 291S-298S.

Prescott, L.F., Illingworth, R.N., Critchley, J.A. & Proudfoot, A.T. (1980). Intravenous N-acetylcysteine: still the treatment of choice for paracetamol poisoning. *Br Med J*, **280,** 46-7.

Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M. & Ratcliffe, P.J. (1997). Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. *J Biol Chem*, **272,** 11205-14.

Qiu, Z., Harms, J.S., Zhu, J. & Splitter, G.A. (2004). Bovine herpesvirus tegument protein VP22 enhances thymidine kinase/gancyclovir suicide gene therapy for neuroblastomas compared to herped simplex virus VP22. *J Virol*, **78,** 4224-4233.

Rainov, N.G., Kramm, C.M., Banning, U., Riemann, D., Holzhausen, H.J., Heidecke, V., Burger, K.J., Burkert, W. & Korholz, D. (2000). Immune response induced by retrovirus-mediated HSV-tk/GCV pharmacogene therapy in patients with glioblastoma multiforme. *Gene Ther*, **7,** 1853-8.

Raleigh, J.A., Miller, G.G., Franko, A.J., Koch, C.J., Fuciarelli, A.F. & Kelly, D.A. (1987). Fluorescence immunohistochemical detection of hypoxic cells in spheroids and tumours. *Br J Cancer*, **56,** 395-400.

Ram, Z., Culver, K.W., Oshiro, E.M., Viola, J.J., DeVroom, H.L., Otto, E., Long, Z., Chiang, Y., McGarrity, G.J., Muul, L.M., Katz, D., Blaese, R.M. & Oldfield, E.H. (1997). Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med*, **3,** 1354-61.

Rawlins, M.D., Henderson, D.B. & Hijab, A.R. (1977). Pharmacokinetics of paracetamol (acetaminophen) after intravenous and oral administration. *Eur J Clin Pharmacol*, **11,** 283-6.

Reid, R., Mar, E.C., Huang, E.S. & Topal, M.D. (1988). Insertion and extension of acyclic, dideoxy, and ara nucleotides by herpesviridae, human alpha and human beta polymerases. A unique inhibition mechanism for 9-(1,3-dihydroxy-2-propoxymethyl)guanine triphosphate. *J Biol Chem*, **263,** 3898-904.

Reiss, M., Brash, D.E., Munoz-Antonia, T., Simon, J.A., Ziegler, A., Vellucci, V.F. & Zhou, Z.L. (1992). Status of the p53 tumor suppressor gene in human squamous carcinoma cell lines. *Oncol Res*, **4,** 349-57.

Revesz, L. (1960). Detection of antigenic differences in isologous host-tumour systems by pretreatment with heavily irradiated tumour cells. *Cancer Research*, **20,** 443-451.

Reynolds, P.N., Nicklin, S.A., Kaliberova, L., Boatman, B.G., Grizzle, W.E., Balyasnikova, I.V., Baker, A.H., Danilov, S.M. & Curiel, D.T. (2001). Combined transductional and transcriptional targeting improves the specificity of transgene expression in vivo. *Nat Biotechnol*, **19,** 838-42.

Reynolds, T.Y., Rockwell, S. & Glazer, P.M. (1996). Genetic instability induced by the tumor microenvironment. *Cancer Res*, **56,** 5754-7.

Rice, G.C., Hoy, C. & Schimke, R.T. (1986). Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. *Proc Natl Acad Sci U S A*, **83,** 5978-82.

Richard, A.M., Hongslo, J.K., Boone, P.F. & Holme, J.A. (1991). Structure-activity study of paracetamol analogues: inhibition of replicative DNA synthesis in V79 Chinese hamster cells. *Chem Res Toxicol*, **4,** 151-6.

Rischin, D., Peters, L., Hicks, R., Hughes, P., Fisher, R., Hart, R., Sexton, M., D'Costa, I. & von Roemeling, R. (2001). Phase I trial of concurrent tirapazamine, cisplatin, and radiotherapy in patients with advanced head and neck cancer. *J Clin Oncol*, **19,** 535-42.

Rizzuto, G., Cappelletti, M., Maione, D., Savino, R., Lazzaro, D., Costa, P., Mathiesen, I., Cortese, R., Ciliberto, G., Laufer, R., La Monica, N. & Fattori, E. (1999). Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation. *Proc Natl Acad Sci U S A*, **96,** 6417-22.

Rofstad, E.K. & Sutherland, R.M. (1989). Growth and radiation sensitivity of the MLS human ovarian carcinoma cell line grown as multicellular spheroids and xenografted tumours. *Br J Cancer*, **59,** 28-35.

Rosenberg, S.A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M.T., Yang, J.C., Topalian, S.L. & et al. (1990). Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med*, **323,** 570-8.

Rosenfeld, M.A., Siegfried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stier, L.E., Paakko, P.K., Gilardi, P., Stratford-Perricaudet, L.D., Perricaudet, M. & et al. (1991). Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium in vivo. *Science*, **252,** 431-4.

Ross, D., Larssson, R., Norbeck, K. & P, M. (1985). Reactive species formed during peroxidase-catalyzed oxidations. *Life Chemistry Reports*, **3,** 112-119.

Rysanek, K. & Vitek, V. (1959). Increased excretion of 5-hydroxy-indole-acetic acid after the administration of 3-indole-acetic acid (heteroauxine). *Experimentia*, **15,** 217-218.

Sakai, T., Takadate, A. & Otagiri, M. (1995). Characterization of binding site of uremic toxins on human serum albumin. *Biol Pharm Bull*, **18,** 1755-61.

Samulski, R. (1993). Adeno-associated virus: integration at a specific chromosome locus. *Curr OPin Genet Dev*, **3,** 74-80.

Sandhu, J.K., Haqqani, A.S. & Birnboim, H.C. (2000). Effect of dietary vitamin E on spontaneous or nitric oxide donor- induced mutations in a mouse tumor model. *J Natl Cancer Inst*, **92,** 1429-33.

Sandmair, A.M., Loimas, S., Puranen, P., Immonen, A., Kossila, M., Puranen, M., Hurskainen, H., Tyynela, K., Turunen, M., Vanninen, R., Lehtolainen, P., Paljarvi, L., Johansson, R., Vapalahti, M. & Yla-Herttuala, S. (2000). Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. *Hum Gene Ther*, **11,** 2197-205.

Sanna, K. & Rofstad, E.K. (1994). Hypoxia-induced resistance to doxorubicin and methotrexate in human melanoma cell lines in vitro. *Int J Cancer*, **58,** 258-62.

Saunders, M.P., Jaffar, M., Patterson, A.V., Nolan, J., Naylor, M.A., Phillips, R.M., Harris, A.L. & Stratford, I.J. (2000a). The relative importance of NADPH:

cytochrome c (P450) reductase for determining the sensitivity of human tumour cells to the indolequinone EO9 and related analogues lacking functionality at the C-2 and C-3 positions. *Biochem Pharmacol*, **59,** 993-6.

Saunders, M.P., Patterson, A.V., Chinje, E.C., Harris, A.L. & Stratford, I.J. (2000b). NADPH:cytochrome c (P450) reductase activates tirapazamine (SR4233) to restore hypoxic and oxic cytotoxicity in an aerobic resistant derivative of the A549 lung cancer cell line. *Br J Cancer*, **82,** 651-6.

Saveliev, A., Everett, C., Sharpe, T., Webster, Z. & Festenstein, R. (2003). DNA triplet repeats mediate heterochromatin-protein-1-sensitive variegated gene silencing. *Nature*, **422,** 909-13.

Schindl, M., Schoppmann, S.F., Samonigg, H., Hausmaninger, H., Kwasny, W., Gnant, M., Jakesz, R., Kubista, E., Birner, P. & Oberhuber, G. (2002). Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clin Cancer Res*, **8,** 1831-7.

Schuler, G. & Steinman, R.M. (1997). Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J Exp Med*, **186,** 1183-7.

Semenza, G.L., Roth, P.H., Fang, H.M. & Wang, G.L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem*, **269,** 23757-63.

Sen, S. & Stevenson, E.L. (1977). A scanning electron microscopic study of IAA-induced tumors in bean (Phaseolus vulgaris L.) embryos. *Cytobios*, **18,** 27-36.

Sena-Esteves, M.S.Y., Fraefe C, Breakefield XO. (2000). HSV-1 amplicon vector - simplicity and versatility. *Mol Ther*, **2,** 9-15.

Senan, S., Rampling, R., Graham, M.A., Wilson, P., Robin, H., Jr., Eckardt, N., Lawson, N., McDonald, A., von Roemeling, R., Workman, P. & Kaye, S.B. (1997). Phase I and pharmacokinetic study of tirapazamine (SR 4233) administered every three weeks. *Clin Cancer Res*, **3,** 31-8.

Sersa, G., Cemazar, M., Parkins, C.S. & Chaplin, D.J. (1999). Tumour blood flow changes induced by application of electric pulses. *Eur J Cancer*, **35,** 672-7.

Sesardic, D., Boobis, A.R., Murray, B.P., Murray, S., Segura, J., de la Torre, R. & Davies, D.S. (1990). Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man. *Br J Clin Pharmacol*, **29,** 651-63.

Seung, L.P., Mauceri, H.J., Beckett, M.A., Hallahan, D.E., Hellman, S. & Weichselbaum, R.R. (1995). Genetic radiotherapy overcomes tumor resistance to cytotoxic agents. *Cancer Res*, **55,** 5561-5.

Seymour, L.W., Duncan, R., Strohalm, J. & 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 Mater Res*, **21,** 1341-58.

Sheldon, P.W. & Hill, S.A. (1977). Hypoxic cell radiosensitizers and local control by X-ray of a transplanted tumour in mice. *Br J Cancer*, **35,** 795-808.

Shen, J., Hughes, C., Chao, C., Cai, J., Bartels, C., Gessner, T. & Subjeck, J. (1987). Coinduction of glucose-regulated proteins and doxorubicin resistance in Chinese hamster cells. *Proc Natl Acad Sci U S A*, **84,** 3278-82.

Sherman, M.L., Datta, R., Hallahan, D.E., Weichselbaum, R.R. & Kufe, D.W. (1990). Ionizing radiation regulates expression of the c-jun protooncogene. *Proc Natl Acad Sci U S A*, **87,** 5663-6.

Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H. & Tsujimoto, Y. (1995). Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. *Nature*, **374,** 811-3.

Sivridis, E., Giatromanolaki, A., Gatter, K.C., Harris, A.L. & Koukourakis, M.I. (2002). Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. *Cancer*, **95,** 1055-63.

Smith, I.E., Courtenay, V.D., Mills, J. & Peckham, M.J. (1978). In vitro radiation response of cells from four human tumors propagated in immune-suppressed mice. *Cancer Res*, **38,** 390-2.

Sommadossi, J.P., Bevan, R., Ling, T., Lee, F. & Mastre, B. (1988). Clinical pharmacokinetics of ganciclovir in patients with normal and impaired renal function. *Rev Infect Dis*, **10,** S507-S514.

Souhami, R.L., Craft, A.W., Van der Eijken, J.W., Nooij, M., Spooner, D., Bramwell, V.H., Wierzbicki, R., Malcolm, A.J., Kirkpatrick, A., Uscinska, B.M., Van Glabbeke, M. & Machin, D. (1997). Randomised trial of two regimens of chemotherapy in operable osteosarcoma: a study of the European Osteosarcoma Intergroup. *Lancet*, **350,** 911-7.

Springer, C.J., Antoniw, P., Bagshawe, K.D., Searle, F., Bisset, G.M. & Jarman, M. (1990). Novel prodrugs which are activated to cytotoxic alkylating agents by carboxypeptidase G2. *J Med Chem*, **33,** 677-81.

Springer, C.J., Poon, G.K., Sharma, S.K. & Bagshawe, K.D. (1994). Analysis of antibody-enzyme conjugate clearance by investigation of prodrug and active drug in an ADEPT clinical study. *Cell Biophys*, **25,** 193-207.

Stackhouse, M.A., Pederson, L.C., Grizzle, W.E., Curiel, D.T., Gebert, J., Haack, K., Vickers, S.M., Mayo, M.S. & Buchsbaum, D.J. (2000). Fractionated radiation therapy in combination with adenoviral delivery of the cytosine deaminase gene and 5-fluorocytosine enhances cytotoxic and antitumor effects in human colorectal and cholangiocarcinoma models. *Gene Ther*, **7,** 1019-26.

Stribbling, S.M., Friedlos, F., Martin, J., Davies, L., Spooner, R.A., Marais, R. & Springer, C.J. (2000). Regressions of established breast carcinoma xenografts by carboxypeptidase G2 suicide gene therapy and the prodrug CMDA are due to a bystander effect. *Hum Gene Ther*, **11,** 285-92.

Stripecke, R., Carmen Villacres, M., Skelton, D., Satake, N., Halene, S. & Kohn, D. (1999). Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther*, **6,** 1305-12.

Sutherland, R.M. (1988). Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science*, **240,** 177-84.

Suzuki, S., Tadakuma, T., Kunitomi, M., Takayama, E., Sato, M., Asano, T., Nakamura, H. & Hayakawa, M. (2001). Liposome-Mediated Gene Therapy Using HSV-TK/Ganciclovir under the Control of Human PSA Promoter in Prostate Cancer Cells. *Urol Int*, **67,** 216-23.

Tannock, I.F. (1968). The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Br J Cancer*, **22,** 258-73.

Tarrus, E., Cami, J., Roberts, D.J., Spickett, R.G., Celdran, E. & Segura, J. (1987). Accumulation of caffeine in healthy volunteers treated with furafylline. *Br J Clin Pharmacol*, **23,** 9-18.

Teicher, B.A., Lazo, J.S. & Sartorelli, A.C. (1981). Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. *Cancer Res*, **41,** 73-81.