Development of a novel enzyme/prodrug system for hypoxia- and radiation-mediated gene therapy of cancer

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Abstract

Solid tumours often present a disorganised and inadequate vascular supply that results in multiple foci of low oxygen tension (hypoxia). Despite being an adverse prognostic factor, hypoxia represents a physiological difference that can be exploited for selective cancer treatment. In particular, high tumour specificity may be achieved by hypoxia-mediated gene-directed enzyme/prodrug therapy (GDEPT), whereby the target cells are genetically modified to synthesise an enzyme able to convert a prodrug into a cytotoxin.

In the present work a novel system consisting of the horseradish peroxidase (HRP) and the non-toxic plant hormone indole-3-acetic acid (IAA) is proposed as an enzyme/prodrug combination for cancer gene therapy. The cytotoxic potential of HRP/IAA GDEPT and the induction of a bystander effect were demonstrated \textit{in vitro} under normoxic as well as hypoxic conditions. Further improvements were achieved by adopting novel IAA derivatives. The chemical agents and the cellular targets involved in HRP/IAA-induced toxicity are yet to be identified, but the results presented indicate that an apoptotic pathway may be activated. With a view to combining hypoxia-targeted GDEPT with a standard radiotherapy protocol, the interaction of HRP/IAA with therapeutically significant doses of ionising radiation (IR) was evaluated, and oxic and anoxic enhancement of IR toxicity was observed. Finally, to limit prodrug activation to the tumour site, selective transgene expression in hypoxic and irradiated cells was demonstrated by the use of synthetic promoters containing hypoxia- and IR-responsive regulatory elements.

Taken collectively, the results indicate that HRP and IAA represent an effective system for use in hypoxia- and radiation-mediated cancer gene therapy. A combinational approach, exploiting hypoxic and radiation-response elements to control HRP gene expression, may overcome some of the limitations of tumour biology associated with conventional radiotherapy regimes.
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Gene delivery under hypoxia

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Hypoxia-targeted gene expression

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The bystander effect

Herpes simplex virus type-1 thymidine kinase/ganciclovir

Cytosine deaminase/5-fluorocytosine

Nitroreductase/CB1954

Cytochrome P450 reductase/bioreductive drugs

Cytochrome P450/cyclophosphamide
Carboxypeptidase G2/CMDA

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<th>Description</th>
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<tbody>
<tr>
<td>ADEPT</td>
<td>antibody-directed enzyme/prodrug therapy</td>
</tr>
<tr>
<td>Ad</td>
<td>adenovirus</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine sulfoximine</td>
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<tr>
<td>CDDP</td>
<td>cisplatin</td>
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<td>CD</td>
<td>cytosine deaminase</td>
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<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CP</td>
<td>cyclophosphamide</td>
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<tr>
<td>Cpd I/II</td>
<td>compound I/II</td>
</tr>
<tr>
<td>CPG2</td>
<td>carboxypeptidase G2</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotide</td>
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<tr>
<td>5-FC</td>
<td>5-fluorocytosine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>Egr</td>
<td>early growth response</td>
</tr>
<tr>
<td>Epo</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>GCV</td>
<td>ganciclovir</td>
</tr>
<tr>
<td>GDEPT</td>
<td>gene-directed enzyme/prodrug therapy</td>
</tr>
<tr>
<td>Glut</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia regulatory element</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus type-1</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>----------</td>
<td>------------------------------------------------</td>
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<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
</tr>
<tr>
<td>i.p.</td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td>IR</td>
<td>ionising radiation</td>
</tr>
<tr>
<td>i.v.</td>
<td>intra-venous</td>
</tr>
<tr>
<td>MOI</td>
<td>3-methylene-2-oxindole</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NTR</td>
<td>nitroreductase</td>
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<tr>
<td>ODN</td>
<td>oligo-deoxyribonucleotide</td>
</tr>
<tr>
<td>OER</td>
<td>oxygen enhancement ratio</td>
</tr>
<tr>
<td>o/n</td>
<td>overnight</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDEPT</td>
<td>polymer-directed enzyme/prodrug therapy</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SER</td>
<td>sensitivity enhancement ratio</td>
</tr>
<tr>
<td>SHMT</td>
<td>single-hit multi-target</td>
</tr>
<tr>
<td>SI</td>
<td>selectivity index</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
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</table>
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Chapter 1
Introduction

1.1 Hypoxia in solid tumours

Multiple factors contribute to the resistance of solid malignancies to therapy, including intrinsic genetic and extrinsic physiological determinants. Properties such as blood flow, tissue oxygenation, nutrient supply, pH distribution and bioenergetic status can markedly influence therapeutic response to treatment.

An important role in the micromilieu of tumours is played by the pathophysiology of vasculature and blood flow. Blood vessels within the tumour mass are highly irregular, tortuous and elongated, with blind ends, incomplete endothelial linings, increased vascular permeability and irregular blood flow (Figure 1.1; Jain, 1988; Brown and Giaccia, 1998; Vaupel and Höckel, 1998). This abnormal vascular system results in reduced or even abolished O$_2$ delivery to the neoplastic and stromal cells. In addition, anaemia and the formation of methemoglobin or carboxyhemoglobin can reduce O$_2$ transport capacity (Fyles et al., 2000).

The inadequate vascular geometry relative to the volume of oxygen consuming tumour cells creates diffusion-limited O$_2$ delivery (chronic hypoxia; Figure 1.1.A; Thomlinson and Gray, 1955). Moreover, the dynamic changes in microregional blood flow in tumours have been related to the formation of areas of perfusion-limited O$_2$ delivery (acute or transient hypoxia; Figure 1.1.B; Brown, 1979; Chaplin et al., 1987; Kimura et al., 1996). Both chronic and transient hypoxia have been detected in animal tumour models and clinical data confirm that they occur in human cancers as well (Hill et al., 1996; Brizel et al., 1997; Raleigh et al., 1998; Vaupel and Höckel, 1998). Low oxygen tension in tumours is frequently associated with low glucose concentration, high lactate levels and low extracellular pH (Vaupel et al., 1989). Biochemists usually define hypoxia as O$_2$-limited electron transport (Boyer et al., 1977); clinicians as a state of reduced O$_2$ availability or O$_2$ partial pressure below critical values (Zander and Vaupel, 1985); radiobiologists refer to the proportion of clonogenic cells in tumours which
exhibit maximal radioresistance, defined in animal models by the paired survival curve assay (Hall, 1994).

![Diagram](image)

**Figure 1.1.** Diagrammatical representation of hypoxic regions in solid tumours (adapted from Brown and Giaccia, 1998).

A. **Diffusion-limited O\(_2\) delivery.** In the proximity of a capillary three tumour cell populations can be identified: well-oxygenated cells (white), chronic hypoxic viable cells (light grey) and necrotic cells (dark grey). Proliferation decreases as a function of the distance from the vasculature.

B. **Perfusion-limited O\(_2\) delivery.** Blood vessels in tumour are tortuous, with incomplete walls, increased vascular permeability and irregular blood flow. They present areas of acute hypoxia, cyclic with reoxygenation.

A variety of invasive and non-invasive methods have been developed to measure the oxygenation status of solid malignancies (Stone et al., 1993). Hypoxia in human tumours has been estimated indirectly by measuring tumour vascularization (e.g. intercapillary distance, vascular density; West et al., 2001); the degree of DNA damage after radiation (Partridge et al., 2001); the expression of hypoxia-regulated genes (section 1.2.3; Airley et al., 2001) and by hypoxia marker techniques (misonidazole,
Chapman et al., 1983; pimonidazole, Arteel et al., 1995; the 2-nitroimidazole drug EF5, Evans et al., 2001). Where the facilities are available in a clinical setting, nuclear magnetic resonance spectroscopy, imaging techniques (Stubbs, 1999) and non-invasive tomographic detection of bioreducible hypoxic markers have been performed to estimate tumour oxygenation (Chapman et al., 1998). Direct measurements of tissue oxygen partial pressure (pO\textsubscript{2}) have been performed by utilising oxygen sensitive polarographic electrodes (Eppendorf, Hamburg, Germany; e.g. Vaupel et al., 1991) and luminescence-based optical sensors (Collingridge et al., 1997). Eppendorf probes are currently considered a “gold standard”, even though their use is limited to accessible and fairly large tumours.

Using oxygen electrodes, it has been observed that the majority of solid tumours present median pO\textsubscript{2} levels lower than their normal tissue of origin. For example, measurements carried out in normal breast revealed a median pO\textsubscript{2} of 65 mmHg (8.6% O\textsubscript{2}), whereas in breast carcinomas of stages pT1-4 the median pO\textsubscript{2} was 28 mmHg (3.9% O\textsubscript{2}; Vaupel and Höckel, 1998). More than 30% of the breast cancers investigated exhibited pO\textsubscript{2} values between 0 and 2.5 mmHg (0.3% O\textsubscript{2}). In contrast, values <12.5 mmHg (1.6% O\textsubscript{2}) could not be detected in the normal breast.

In several tumour types, high heterogeneity in the hypoxic distribution has been observed both within the tumour mass and between tumours of same clinical stage and grade (e.g. Nordsmark et al., 2001 b). In metastatic lesions, as in primary tumours, red cell flux was found to be anisotropic and compromised compared to surrounding normal tissue (Hill et al., 1996; Pigott et al., 1996). In metastases of carcinomas of the head and neck, breast and rectal cancers, lower median pO\textsubscript{2} values and higher hypoxic fractions were recorded, compared not only to the normal surrounding tissue, but also to the primary tumours (Becker et al., 1998; Vaupel and Höckel, 1998).

1.1.1 Hypoxia and response to treatment

The relevance of tumour oxygenation to radiocurability was first proposed by Gray and co-workers in 1953, and its role in the response to radiation has since been recognised both in experimental and clinical systems. Anoxic cells are ~3-fold more radioresistant than normoxic cells, with a steep increase in radiosensitivity between 2 and 10 mmHg.
This phenomenon, known as the “oxygen effect”, is observed when oxygen is present during or within milliseconds after radiation exposure, and thought to result from $O_2$ reaction with the ionised target molecules, generating poorly repairable peroxide lesions (oxygen-fixation hypothesis; Hall, 1994).

![Graph](image)

**Figure 1.2.** Radiosensitivity of cultured mammalian cells under different oxygenation conditions (from Vaupel et al., 1989).

The $O_2$ partial pressure ($pO_2$) values found in blood, normal and malignant tissues, and at which the sensitising effect is half-maximal (3-4 mmHg) are indicated. Clonogenic survival is the endpoint considered.

A correlation between the presence of hypoxia and response to therapy has been shown in a range of human tumour sites. Studies performed in patients with soft tissue sarcomas (Brizel et al., 1996; Nordsmark et al., 2001a), carcinomas of the uterine cervix (Höckel et al., 1996; Fyles et al., 1998; Knocke et al., 1999) and of the head and neck (Nordsmark et al., 1996; Brizel et al., 1997, 1999; Nordsmark and Overgaard, 2000) indicated that the presence of hypoxic regions adversely affects locoregional...
control and/or disease-free survival after primary radiotherapy. Radiation resistance induced by the oxygen effect is unlikely to be the only explanation, since tumour oxygen status has been observed to be the most important prognostic factor for treatment outcome and metastatic free survival in cervical carcinoma after radiotherapy as well as surgery (Höckel et al., 1996). Hypoxia-driven cellular modifications may contribute to this poor prognostic outlook, giving rise to more aggressive locoregional disease, invasive capacity and angiogenesis (Brizel et al., 1996; Sundfør et al., 1998; Graham et al., 1999; Höckel et al., 1999; Walenta et al., 2000).

Oxygen dependency has been shown for some chemotherapeutic agents both in vitro and in vivo (Teicher et al., 1981; Grau and Overgaard, 1988; Chaplin et al., 1989). A number of factors may be involved. Firstly, hypoxic cells slow down or arrest their rate of progression through the cell division cycle (Figure 1.1. A; Durand and Raleigh, 1998). Experiments conducted in mammalian cells of different origin showed that prolonged hypoxia causes accumulation in G1 and inhibition of DNA replication, effects reversible upon reoxygenation (Åmellem and Pettersen, 1991; Giaccia, 1996; Krtolica and Ludlow, 1996; Schmaltz et al., 1998; Koritzinsky et al., 2001). In experimental tumours, the fraction of proliferating cells and/or the rate of cell proliferation decreased as the distance from the vasculature increased (Figure 1.1.A; Tannock, 1968; Minchinton et al., 1990; Rodriguez et al., 1994). This can result in a loss of efficacy of most anticancer agents, which are primarily effective against rapidly diving cells. Secondly, because of drug metabolism by the cells in the intermediate layers, the limited penetration of plasma-borne agents determines a reduction in the dose of drug reaching the hypoxic population (Figure 1.1.A). Also, the extracellular pH is lower in hypoxic than in well-oxygenated areas, while the intracellular pH is kept constant (Griffiths, 1991). Thus, the uptake and activity of weak acids such as chlorambucil is increased, while that of weak bases like vinblastine is decreased (Chaplin et al., 1998). Finally, hypoxia can induce the production of stress proteins responsible for resistance to doxorubicin, etoposide and methotrexate (Shen et al., 1987; Hughes et al., 1989; Sanna and Rofstad, 1994). To date, studies on human tumours demonstrating a direct correlation between the presence of hypoxic areas and response to chemotherapy are lacking.
The hypoxic microenvironment in solid tumours, which affects neoplastic cells and non-neoplastic stromal cells such as macrophages and fibroblasts, is likely to have profound effects on tumour propagation if one considers the molecular changes demonstrated \textit{in vitro} and \textit{in vivo} under hypoxic conditions. These changes may result from the stimulation or inhibition of gene expression and from post-transcriptional and post-translational modifications (section 1.2.3).

The pathophysiological microenvironment of tumours is generally considered to be mutagenic. Point mutations, deletions and gene amplification after hypoxia/reoxygenation may occur as a result of errors in DNA repair and/or replication, oxidative damage leading to DNA strand breakage and impaired activity of enzymes such as topoisomerases, helicases and ligases (Janssen et al., 1993; Russo et al., 1995; Reynolds et al., 1996).

Experimental evidence suggests that low oxygen levels cause apoptosis in normal and neoplastic cells (Yao et al., 1995; Graeber et al., 1996). p53 accumulated under hypoxia and was required for induction of apoptosis. However, although reduced, hypoxia-induced apoptotic death was also detected in p53 null cells, indicating the presence of p53-independent pathways (Graeber et al., 1996; Kim et al., 1997; Schmaltz et al., 1998). Involvement of \textit{Apaf-1}, caspase-9 and genes of the \textit{bcl-2} family has also been reported (Shimizu et al., 1995; Soengas et al., 1999). Moreover, hypoxia can select for tumour cells that have acquired \textit{p53} mutations and have lost their apoptotic potential, providing them with a growth advantage (Alarcon et al., 1996; Graeber et al., 1996; Kim et al., 1997). However, recent clinical data are conflicting. Squamous cell carcinomas of the uterine cervix, characterised by pronounced hypoxia (Sundfor et al., 1998) and low apoptotic index (Höckel et al., 1999), showed a high probability for lymphatic spread and recurrence, despite adjuvant treatment with radiation or chemotherapy in addition to radical surgery (Höckel et al., 1999). On the other hand, in patients with primary soft tissue sarcoma, no association was found between median pO\textsubscript{2} and \textit{p53} status (Nordsmark et al., 2001 a). In squamous cell carcinoma of the cervix, the pre-radiotherapy apoptotic index did not correlate with pre-treatment oxygenation (both hypoxic fraction and median pO\textsubscript{2}), but correlated significantly with the change in
oxygenation post-treatment, ascribed by the authors to reduced interstitial fluid pressure as a result of cell loss via apoptosis (Sheridan et al., 2000).

Hypoxia-induced tumour progression and metastatic spread has been related to the up-regulation of gene products that may promote tumour progression, by enabling tumour cells to adapt to nutritional deprivation or to escape a hostile environment. Proangiogenic factors attract new vasculature, glucose transporters and glycolytic enzymes allow the switch to energy saving glycolysis and oncoproteins give hypoxic tumour cells a growth advantage (details on hypoxia-mediated gene expression will be discussed in section 1.2.3). At the same time, hypoxia can reduce adhesion to the extracellular matrix and down-regulation of cell adhesion molecules such as integrins, in order to facilitate tumour cell detachment (Hasan et al., 1998).

An association between primary tumour oxygenation and the likelihood of distant metastasis has been reported in clinical studies involving patients with high-grade soft tissue sarcoma (Brizel et al., 1996) and advanced squamous cell carcinoma of the uterine cervix (Sundfør et al., 1998). Also, a positive correlation has been observed between tumour lactate concentration and the presence of metastatic deposits in cervix and head and neck squamous cell carcinomas, while ATP and glucose concentration did not show significant correlation (Rofstad, 2000).

Thus, hypoxia not only provides an environment directly facilitating chemo- and radio-resistance, but also encourages the evolution of phenotypic changes inducing permanent resistance to treatment.

1.1.2 How to overcome tumour hypoxia

The main strategies adopted to overcome the resistance of hypoxic tumours to therapy include to increase the delivery of oxygen (or oxygen-mimetic drugs), or to exploit the unique environmental conditions of solid tumours by using hypoxia-selective cytotoxic agents.

One of the first attempts in the clinic to improve tumour oxygenation was the use of hyperbaric oxygen in combination with radiotherapy (Churchill-Davidson, 1968). Multicentre clinical trials showed a significant increase in locoregional control and
survival in patients with cervix or advanced head and neck carcinoma treated with radiation and hyperbaric oxygen, compared with radiotherapy given in air (reviewed by Overgaard and Horsman, 1997). However these studies were discontinued mainly because of problems with patient compliance. Subsequently, chemical radiosensitisers, in particular electron-affinic radiosensitisers, were introduced by Adams and co-workers (1969, 1972). The rational for their use is that they can diffuse out of the tumour blood supply and, not being metabolised in cellular respiration, can reach hypoxic areas and radiosensitise them in an oxygen-mimicking fashion. Of these drugs, misonidazole, a 2-nitroimidazole, showed remarkable results in laboratory studies (Sheldon et al., 1974; Brown, 1975), but, mainly because of dose limitations due to high neurotoxicity, the clinical experience was disappointing (Dische, 1985). New nitroimidazole compounds were then developed, without major clinical benefit (Riese et al., 1997).

In order to overcome both chronic and transient hypoxia, the combination of carbogen (95% O\(_2\) and 5% CO\(_2\)), effective against the first, and the vitamin B derivative nicotinamide, which is believed to prevent transient blood flow fluctuation (Chaplin et al., 1990), has been successfully tested in animal models (Kjellen et al., 1991). In clinical studies, the treatment was given in combination with standard or accelerated radiotherapy (ARCON). Phase I, II and III trials indicated feasibility and some therapeutic efficacy, in particular in the treatment of bladder carcinoma, albeit limited by the gastro-intestinal toxicity of nicotinamide (Hoskin et al., 1997, 1999; Miralbell et al., 1999; Bernier et al., 2000).

The physiology of solid tumours, even though involved in resistance to treatment, represents an attractive target for selective cancer therapy. Bioreductive drugs, such as the indolequinones mitomycin C and EO9, SR 4233 (later known as tirapazamine, TPZ), RSU 1069 and CB1954, have been extensively utilised as hypoxia-selective cytotoxins (HSCs; Stratford and Workman, 1998). In order for bioreductive drugs to be activated, the cells need to be hypoxic and to produce the appropriate set of reductase enzymes. In normal tissue, bioactivation is restricted by the presence of oxygen. The concentration of bioreductive drug required to induce the same level of killing in hypoxic vs. normoxic cells (hypoxic cytotoxicity ratio, HCR) may vary
depending on the cell type and the level of hypoxia. HCR values for the prototype bioreductive drug mitomycin C did not exceed 10 (Rauth et al., 1983), to be compared with values in the 7-300 range for TPZ (Brown and Wang, 1998), 10-200 for nitroimidazoles (Mohindra and Rauth, 1976; Hill et al., 1986; Stratford et al., 1986) and up to 5000 for E08 and E09 (Jaffar et al., 1998). Nevertheless, clinical evaluation of mitomycin C in combination with radiotherapy for the treatment of squamous cell carcinoma of the head and neck indicated significant improvement in locoregional control and survival, compared with radiation treatment alone (Haffty et al., 1997). In the case of TPZ, an improvement in survival was observed in conjunction with radiotherapy, but it did not reach statistical significance (Del Rowe et al., 2000). However, the combination of TPZ, cisplatin and radiotherapy resulted in remarkably good and durable clinical responses in patients with advanced head and neck cancers (Rischin et al., 2001).

Accurate targeting of oxygen-deprived cells within a tumour mass may also be achieved by hypoxia-targeted gene therapy. This recent approach will be discussed in detail in the following sections.

1.2 Gene therapy of cancer

One of the major goals of antitumour therapies is to target tumour cells selectively and specifically, whilst sparing normal tissue from damage. This may be achieved by gene therapy that can combine highly specific gene delivery with highly specific gene expression. Gene therapy can be described as the transfer of DNA or RNA to modify transiently or permanently the genetic repertoire of target cells for therapeutic purposes. The first clinical gene therapy protocol was approved in 1989 (Rosenberg et al., 1990), and since then more than 400 clinical trials have been undertaken world-wide, of which over half relate to cancer (Hum Gene Ther, 2000).

For gene therapy, three separate issues need to be considered: 1) delivery of a gene to the target cells, 2) regulation of gene expression and 3) therapeutic efficacy. Each of these aspects is outlined below, with a view to gene therapy of the hypoxic tumour environment.
1.2.1 Gene delivery

The administration of gene therapy vectors requires that they be not only targeted, but also protected from degradation, sequestration or immune attack, in order to reach the appropriate sites for transfection. The ideal DNA vehicle should be capable of hosting an expression cassette carrying one or more genes of the size needed for the clinical application, and should be easy to produce and purify in large quantities and high concentrations. It should not induce inflammation and be safe for the recipient and the environment. Finally, it should express the gene for as long as required, in an appropriately regulated fashion.

The efficient delivery of DNA to tumour sites remains a formidable task, but progress has been made in recent years using both viral and non-viral methods. Vehicles such as retro- and adenoviruses, liposomes and naked DNA injection are adopted in clinical studies (Table 1.1; reviewed by Gómez-Navarro et al., 1999). New therapeutic modalities are currently being explored in order to overcome the limitation of poor gene transfer and patient toxicity, including immunoporation (Bildirici et al., 2000), macrophages and bacteria (next section), adeno-associated and herpes simplex viruses, lentiviruses, cationic polymer-DNA complexes and electroporation (Table 1.1).

<table>
<thead>
<tr>
<th>Viral methods</th>
<th>Non-viral methods</th>
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<tbody>
<tr>
<td>Retroviruses</td>
<td>Calcium phosphate precipitation</td>
</tr>
<tr>
<td>Adenoviruses</td>
<td>Nanoparticles</td>
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<tr>
<td>Lentiviruses</td>
<td>Liposome-DNA complexes (lipoplex)</td>
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<tr>
<td>Herpes Simplex viruses</td>
<td>Gene gun</td>
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<tr>
<td>Adeno-associated viruses</td>
<td>DNA injection</td>
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<tr>
<td>Chimeric (e.g. retro/adeno) viruses</td>
<td>DNA electroporation</td>
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<tr>
<td>Poxviruses</td>
<td>Immunoporation</td>
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<tr>
<td>Vaccinia viruses</td>
<td>Ultrasound</td>
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<tr>
<td>Parvoviruses</td>
<td>Cationic agents-DNA complexes (polyplex)</td>
</tr>
</tbody>
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Table 1.1. Summary of common viral and non-viral DNA delivery systems.
Viral vectors capitalise on the ability of the viruses to efficiently enter the cells through specific receptors and transfer their genome. Viral genes need to be removed to permit the insertion of the expression cassette and make the virus non-toxic. In order to achieve higher levels of infection and antitumour activity, it is currently becoming more and more common to adopt replication-competent rather than non-replicative viral vectors (reviewed by Kirn et al., 2001). In this case, tumour-specific replication is mandatory.

Retroviruses are small RNA viruses that replicate through a DNA intermediate. Viruses deleted of one or more of their structural genes can infect packaging cells that express the missing genes (reviewed by Robbins and Ghivizzani, 1998). Retroviral vectors host 9-10 kilobase pairs (kbp) of foreign genetic material and integrate the therapeutic gene into the genome of the infected cells that will maintain it during subsequent mitotic divisions. This can be an advantage when treating hereditary and chronic disorders, but can also present a number of risks, including insertional mutagenesis and toxicity associated with overexpression of the therapeutic gene. However, the major limitation of retroviral vehicles, as for example those derived from the Moloney murine leukaemia virus (MoMLV), is that the target cells must be proliferating at the time of infection (Miller et al., 1990). Specifically, they are likely to be highly inefficient in transfecting slowly dividing hypoxic cells. In the clinical trials, safety has been demonstrated, but transfection efficiencies were low (Ram et al., 1997; Klatzmann et al., 1998 a, b).

Of the viral delivery systems, adenoviruses (Ads) are probably the best characterised and most extensively used. Unlike retroviruses, they do not need packaging cells. They are stable and easy to manipulate, can be grown to high titres (~$10^{13}$ viral particles/ml) and E1/E3 deleted viruses can accommodate up to 8.5 kbp of foreign DNA (Zhang, 1999). Also, Ads can infect different cell types independently from their proliferation status. Because of their non-integrative nature, vector sequences are not inherited in progeny cells, resulting in a transient expression of foreign genes. Mutant Ads have been designed to replicate selectively in tumour cells, e.g. in cells lacking functional p53 (ONYX-015; Bischoff et al., 1996; Khuri et al., 2000). Targeted delivery could be achieved by modifying the viral capsid proteins, fibre and penton base
to permit the recognition of cell-specific receptors (Haism et al., 1999; Harari et al., 1999). The main drawbacks of Ads are a strong T cell-mediated inflammatory response that reduces the effectiveness of repeated administration (Harvey et al., 1999), and, in clinical settings, concerns about patient safety (Marshall, 1999). In targeting hypoxic cells, it is necessary to keep in mind that hypoxia causes a marked downregulation of integrin $\alpha_v\beta_3$ (Hasan et al., 1998), necessary for the initial binding, internalisation and uncoating of Ad vectors (Zhang, 1999).

Adeno-associated viruses (AAVs) are paroviruses, with potential for long-term gene expression. They are non-pathogenic, can also infect non-proliferating populations and, compared to adenoviruses, are less immunogenic and present a broad tissue tropism (Robbins and Ghivizzani, 1998). However, AAVs have limited packaging capacity (only 4-5 kbp of DNA insert), are difficult to prepare in high titres, and depend on “helper viruses”.

Vectors based on the Herpes simplex virus type-1 (HSV), able to infect non-dividing cells, can be “helper” virus dependent (amplicons) or independent (Robbins and Ghivizzani, 1998). Amplicons can accommodate as much as 150 kbp and efficiently express integrated transgenes. Drawbacks are some instability (due to selective advantage for small amplicons), contamination with helper viral DNA and low ratio of amplicon to helper virus. Helper virus-independent vectors have deletions in essential viral genes, and therefore need to be grown in appropriate cells, but can host up to 40 kbp of foreign DNA. A clinical trial involving the delivery of a conditionally-replicating HSV vector to brain tumours has shown safety of this approach (Markert et al., 2000).

Lentiviruses, such as the human immunodeficiency virus type-1 (HIV), are unique among retroviruses because of their ability to infect both replicating and non-replicating cells, mediating efficient delivery and long-term expression. High transduction has been observed in the lympho-hemopoietic and the central nervous systems, without significant inflammatory response, while liver and muscle were characterised by poor transgene expression (reviewed by Trono, 2000). The development of efficient vector packaging systems and addressing the issue of biosafety will determine the potential of these vectors.
Compared to viral vectors, non-viral systems are particularly suitable for gene therapy with respect to simplicity of use, lack of immune response, ease of large scale production and DNA packaging. Moreover, non-viral systems can be controlled and characterised as well as any other pharmaceutical products, in terms of delivery components, complex size, DNA and vector concentration. However, there are some drawbacks, mainly low efficiency in gene transfer. Non-viral systems can be broadly divided into physical (electroporation, sonoporation or gene gun) and chemical (lipids, polymers, proteins).

In electroporation, brief high or low voltage electric pulses are applied to cells in culture and tissues in vivo to induce transient pores in the cell membranes. This method is applied in clinical settings for delivery of chemotherapeutic drugs, with high antitumour efficiency and negligible side effects (Mir et al., 1998). Recent studies have shown delivery of plasmid DNA to different tissues in vivo (Somiari et al., 2000; Cemazar et al., 2002). Electric pulse parameters are important for optimal gene expression, but they appear to differ greatly depending on the target tissue.

Cationic liposomes (Figure 1.3) are clinically well tolerated, easy to produce, non-infectious, non-immunogenic and can package large DNA molecules. Even though liposome-DNA complexes can also transfect slowly dividing cells, the overall transfection efficiency in vivo remains low.

The introduction of foreign gene products into human cells via DNA complexes consists of a sequence of discrete steps (Figure 1.3). First the vector must bind to the cell surface, then enter the cell by an endocytic or phagocytic process. Efficient escape from the vesicle is necessary to evade enzymatic degradation in the acidified environment of the maturing endosome. Finally the DNA must enter the nucleus either by penetrating the envelope through the nuclear pores or during mitosis when the envelope breaks down. Receptor-mediated gene delivery may further improve both safety and efficiency, by preventing undesirable delivery to normal tissues and sequestration of the vector before it reaches the target. Ligands to surface receptors have included asialoglycoprotein (Wu and Wu, 1987), basic fibroblast growth factor (Hoganson et al., 1998), transferrin (Wagner et al., 1990) and adhesion molecules (reviewed by Parkes and Hart, 2000).
Figure 1.3. Liposome-mediated DNA delivery (from Crystal, 1995).

The liposomes used for gene therapy are usually cationic, to electrostatically bind to DNA. The complexes enter the target cells by fusing with the plasma membrane and, once escaped from the endosome, can enter the cell nucleus. It is likely that most of the DNA that enters the nucleus remains episomal.

Peptides containing an arginine-glycine-aspartic acid (RGD) motif with high affinity for integrins (Hart et al., 1994) and a short polylysine segment for electrostatic binding of DNA could efficiently transfer genetic material to different cell types (Hart et al., 1995, 1997; Harbottle et al., 1998). The transfection efficiency was enhanced by 100-fold by incorporating the cationic liposome Lipofectin (L) into the peptides (P)/DNA (D) complex (LPD; Figure 1.4). LPD vectors have been shown to promote receptor-mediated endocytosis and reduce endosomal degradation via lipid-mediated destabilisation of the endosomal membrane (Hart et al., 1998). Efficient transfection was demonstrated in a number of cell types in vitro (Hart et al., 1998; Compton et al., 2000; Dachs et al., 2000; Estruch et al., 2001; Wright et al., 2001) and in bronchial and alveolar cells in vivo, with transgene expression sustained for at least three to seven days (Jenkins et al., 2000). However, direct intramyocardial (Wright et al., 2001) or intratumour (Cemazar et al., 2002) injection of LPD complexes resulted in negligible marker gene expression.
Figure 1.4. Complexes of Lipofectin, integrin-targeted peptides and DNA (LPD).

After 2 h-incubation at room temperature, vesicles of 44 nm in diameter containing plasmid DNA are formed. The peptide component bears a 16-lysine DNA-binding domain and a cyclised integrin-binding domain. The cationic charge properties of the complex contribute to non-specific cell binding and transfection properties. The lipid component acts as an adjuvant to transfection by mediating endosomal escape of the plasmid component.

**Gene delivery under hypoxia**

In targeting hypoxic cells, a further obstacle to gene delivery is represented by their reduced metabolism, proliferation rate, gene transcription and translation (Heacock and Sutherland, 1986), which could affect DNA uptake and gene expression. However, by using LPD complexes it has been demonstrated that *in vitro* transfection and transgene expression could be obtained even under the extreme tumour conditions of anoxia (Dachs et al., 2000). Although *in vivo* data on specific transfer of genetic material to hypoxic cells are still lacking, promising approaches are under investigation. Bacteria and macrophages, although not delivering DNA itself, represent interesting cellular vehicles to transfer therapeutic modalities.

Examples of prokaryotic vectors include anaerobic bacteria of the genera *Clostridium*, *Bifidobacterium* and tumour-invasive *Salmonella* auxotroph. The ability of *Clostridia* to selectively germinate and replicate in necrotic and hypoxic regions of solid tumours (Malmgren and Flanigan, 1955; Carey et al., 1967) makes them a promising
tumour-selective vehicle for gene therapeutics. Spores of *C. beijerinckii* genetically engineered to produce the *Escherichia coli* (*E. coli*) enzyme nitroreductase (NTR) have been intravenously (i.v.) injected in tumour-bearing mice, and NTR protein was detected in all tumours tested but not in normal tissues (Lemmon et al., 1997). *In vitro* conversion of the prodrug CB1954 (see section 1.2.5) into a cytotoxic agent by *Clostridia*-produced NTR demonstrated the therapeutic potential of this approach (Fox et al., 1996; Lemmon et al., 1997). *In vivo*, i.v. injection of spores of cytosine deaminase (CD)-transfected *C. sporogenes* followed by systemic administration of the prodrug 5-fluorocytosine (5-FC; section 1.2.5) induced significant antitumour activity (Liu et al., 2000). Tumour selective spore germination was also observed in rhabdomyosarcoma-bearing rats injected with five different bacterial strains, the most efficient species being *C. acetobutylicum* and *C. oncolyticum* (Lambin et al., 1998).

Attenuated hyperinvasive auxotrophic mutants of *Salmonella typhimurium* can selectively target tumour tissues and amplify in necrotic spaces to levels in excess of $10^9$ bacteria per gram of tissue (Pawelek et al., 1997). While the ability to replicate in tumour tissue provides inherent anti-tumour activity, it is their ability to deliver therapeutic proteins to cancer cells *in vivo* that may confer utility for gene therapy strategies.

*Bifidobacteria* are Gram-positive non-pathogenic anaerobes, found in the gastrointestinal tract. Immediately after i.v. inoculation of *B. longum* to tumour-bearing mice viable bacilli could be detected throughout the animal body, but following 4-7 days the bacteria proliferation was restricted to the tumour mass (Yazawa et al., 2000). Compared to *Clostridia* and *Salmonella*, the advantage of *Bifidobacterium* strains, used in the preparation of fermented milk products, could be their lack of pathogenesis in humans.

Macrophages may be effective vehicles for hypoxia-selective gene therapy (Griffiths et al., 2000). It is known that macrophages infiltrate solid malignancies to form a significant proportion of the tumour solid mass, dominating in areas of hypoxia and necrosis (Leek et al., 1997). Differentiated macrophages transduced with an adenoviral vector containing the human cytochrome P450 2B6 (CYP 2B6) gene were found to infiltrate human tumour spheroids. Tumour cell death was induced when the
spheroids were incubated with the prodrug cyclophosphamide (CP, section 1.2.5), converted by CYP 2B6 into a cytotoxin (Griffiths et al., 2000). A hypoxia-responsive promoter (see section 1.2.3) conferred an additional level of selectivity to the system. The macrophages themselves did not appear to be affected by the CYP 2B6/CP treatment. However, CYP 2B6/CP may not be the ideal combination for hypoxia-regulated gene therapy, since the rate limiting activation of CP is an oxygen-dependent reaction (section 1.2.5).

1.2.2 Gene regulation

The control of the initiation of gene transcription is the predominant form of regulation for most genes, although other mechanisms can be activated later in the pathway from mRNA to protein to modulate the synthesis of the final product. In order to engineer cancer-targeted gene expression, transcription regulation specific to certain tissues, diseases, conditions or stimuli (such as hypoxia or radiation) can be exploited. For instance, DNA-regulatory sequences from several cancer-selective genes, including α-fetoprotein (hepatoma), tyrosinase (melanoma), c-erbB2 and DF3/MUC1 (breast cancer), carcinoembryonic antigen (epithelial cancers) and prostate-specific antigen (PSA) have been shown to direct expression of therapeutic genes in the indicated tumour cell types (reviewed by Nettelbeck et al., 2000).

1.2.3 Hypoxia-mediated gene induction

The adaptive response to cellular hypoxia involves the modulation of the synthesis of multiple proteins controlling processes such as glucose homeostasis, angiogenesis, vascular permeability and inflammation. Hypoxia is an independent stimulus of gene expression, unrelated to ionising radiation (IR) or heat shock. It can to some extent be mimicked by carbon monoxide, by the iron chelator desferrioxamine, cobalt, nickel and manganese, but not by zinc, iron or cadmium. The exact molecular mechanism by which cells detect O₂ concentration remains elusive. Potassium channels have been implicated in O₂ sensing by carotid body type I and a number of different vascular cells (reviewed by Wang and Semenza, 1996). On the other hand, studies on hypoxia-regulated erythropoietin (Epo) gene expression suggest that the O₂ sensor is a haem-containing
protein that exists in different conformations (oxy, deoxy) dependent on O₂ binding (Wang and Semenza, 1996). Carbon monoxide would simulate hypoxic induction by irreversibly substituting for oxygen, while hypoxia-mimicking metals are thought to displace iron in the porphyrin ring and their low affinity for oxygen would lock the haem protein in the deoxy conformation, triggering the generation of reactive oxygen species (ROS). ROS were also found to increase in mitochondria after hypoxia, and the inhibition of the mitochondrial electron transport chain blocked the induction of hypoxia-dependent transcription factors (Chandel et al., 1998). Finally, it has recently been suggested that a prolyl hydroxylase may act as a cellular oxygen sensor (Ivan et al., 2001; Jaakkola et al., 2001).

The signal is then transduced through an interactive network rather than a linear pathway, which is not surprising given the importance of O₂ homeostasis. Both tyrosine phosphorylation and redox regulation seem to be implicated, but the exact mechanism is still unclear.

Many genes controlling tumour biology are oxygen-regulated and the purpose of their products is to counteract the detrimental effects of hypoxia (reviewed by Dachs and Chaplin, 1998; Dachs and Tozer, 2000). These include:
- cytokines, growth factors and other proteins that regulate angiogenesis, vascular permeability and inflammation, such as Epo, endothelin-1, inducible nitric oxide synthase (iNOS), platelet-derived growth factor-B, tyrosine kinase receptor Tie2 and vascular endothelial growth factor (VEGF);
- metabolic enzymes, like aldolase (ALD)-A and -C, carbonic anhydrase (CA), enolase (Eno)-1, glucose transporter (Glut)-1 and -3, lactate dehydrogenase (LDH)-A, phosphofructokinase (PFK)-L and -C, phosphoglycerate kinase (PGK)-1 and pyruvate kinase-M;
- transcription factors, including Activator Protein (AP)-1, hypoxia-inducible factor (HIF)-1, nuclear factor (NF)-κB and p53, which regulate transcription of genes encoding proteins governing growth control, apoptosis, differentiation and metastasis;
stress proteins, such as the heat shock proteins (HSPs) and cellular redox regulators, to prepare the cells to deal with reactive oxygen metabolites after the oxidative stress of reoxygenation and reperfusion.

The DNA regulatory elements controlling the expression of oxygen-responsive genes have been defined in most cases, and involve the specific binding and trans-activation by various inducible, phosphorylation-dependent and/or redox sensitive transcription factors. Published evidence indicates that only HIF-1 is specifically oxygen-responsive, while the other transcriptional systems appear to contribute to the response to hypoxia via related redox and metabolic changes.

The control of Epo, the main regulator of red blood cell production, was studied as a model to gain an understanding of how cells sense and respond to changes in oxygen tension. Madan and Curtin (1993) defined the minimal DNA region necessary for hypoxia response of the Epo gene as a 24 bp sequence, able to regulate transcription regardless of orientation of and distance from the coding region. Beck et al. (1993) identified a 120 kDa protein that is upregulated by hypoxia and binds to the Epo hypoxia enhancer sequence. This nuclear transcription factor was defined as HIF-1 and found to be common to a variety of Epo-producing and non-producing mammalian cells.

Affinity purification and molecular cloning of HIF-1 showed it to function as a heterodimer, consisting of two basic-helix-loop-helix proteins from the Per-aryl hydrocarbon receptor nuclear translocator (ARNT)-Sim (PAS) family of transcription factors: HIF-1α (120 kDa) and HIF-1β (91-94 kDa; previously identified as ARNT, part of the xenobiotic response; Wang et al., 1995). Recently the endothelial PAS protein (EPAS)-1 has been reported to show close sequence homology and similar properties to HIF-1α, and therefore renamed HIF-2α (Wenger and Gassmann, 1997).

HIF-1 is common to all mammalian cells and human tissues and organs tested to date (Maxwell et al., 1993; Wiener et al., 1996) and has an important pathophysiological role within solid tumours. Compared with the wild type tumours, mouse Hepa-1 hepatomas from HIF-1β-deficient cells presented reduced hypoxia-mediated gene expression, vascularity and growth rate (Maxwell et al., 1997). Analogously, HIF-1β−/− knock-out embryonic stem (ES) cells failed to activate hypoxia-responsive genes and to respond to a decrease in glucose concentration (Maltepe et al., 1997).
Embryos were not viable past embryonic day 10.5 and showed defective angiogenesis of the yolk sac and branchial arches, stunted development and embryo wasting. Similar effects were seen in HIF-1α-deficient embryos (Ryan et al., 1998). In subcutaneously implanted HIF-1α-ES cells, vessel perfusion, oxygenation and VEGF expression were significantly reduced (Carmeliet et al., 1998; Ryan et al., 1998). Tumour growth rate and apoptosis were increased in one instance (Carmeliet et al., 1998), decreased in the other (Ryan et al., 1998). Disruption of the interaction between HIF-1α and p300/CBP led to attenuation of hypoxia-inducible gene expression and tumour growth (Kung et al., 2000). In breast cancer cells in vitro, the cumulative induction of HIF-1α and HIF-2α protein levels was inversely related to clonogenic survival under hypoxia (Blancher et al., 2000). HIF-1 was stabilised after inactivation of the von Hippel-Lindau (VHL) tumour suppressor gene, and amplified by other oncogenic mutations (Jiang et al., 1997; Maxwell et al., 1999).

Increased levels of HIF-1α protein in human prostate cancer cell lines correlated with increased metastatic potential (Zhong et al., 1998). More importantly, the HIF-1α subunit was overexpressed in 13/19 clinical tumour types and not in non-malignant tumours such as breast fibroadenoma and uterine leiomyoma (Zhong et al., 1999). HIF-1α production was a strong independent prognostic marker in early stage cervical carcinoma (Birner et al., 2000), cancer of the oropharynx (Aebersold et al., 2001) and the oesophagus (Koukourakis et al., 2001), but not in patients with ovarian cancer (Birner et al., 2001). However, a significant reduction in overall survival after platinum-based chemotherapy was observed in ovarian cancer patients with overexpression of both p53 and HIF-1α (Birner et al., 2001).

Hypoxia primarily upregulates the DNA binding activity of HIF-1 (Wang and Semenza, 1993). It is generally accepted that HIF-1β is not oxygen sensitive and that HIF-1α is hypoxia-regulated at the post-translational level (Figure 1.5; Pugh et al., 1997). The levels of HIF-1α increase during hypoxia and the protein subunit is degraded within 3-5 minutes during reoxygenation (Huang et al., 1996). HIF-1α is poly-ubiquinated in air, by targeting it to the ubiquitin-proteasome degradation pathway, a process mediated by the VHL protein (Maxwell et al., 1999), whereas ubiquination is
largely reduced under hypoxia (Figure 1.5; Huang et al., 1998; Kallio et al., 1999). Hydroxylation of a HIF-1α proline residue (HIF-1α P564) by a prolyl-4-hydroxylase, strictly dependent on the presence of oxygen as a co-substrate and of iron as a cofactor, was found to modulate the interaction of HIF-1α with the VHL protein (Figure 1.5; Jaakkola et al., 2001).

![Figure 1.5. Hypoxia-mediated transcriptional regulation.](image)

In oxygenated cells the HIF-1α subunit is oxidatively modified by a prolyl hydroxylase (HIF-1α PH). This iron-dependent process is necessary for binding of HIF-1α to the VHL protein, followed by the activation of the ubiquitin-E3 ligase (UL) and HIF-1α proteasome degradation. Under hypoxia, HIF-1α complexes with HIF-1β to form the functional transcription factor. Recruitment of co-activators such as p300 and binding to the hypoxia regulatory elements (HREs) initiate hypoxia-regulated gene transcription.
In addition, the hypoxic increase in HIF-1α protein depends on a redox-dependent increase in protein stabilisation, which in turn originates from a change in conformation upon dimerisation with HIF-1β. Phosphorylation of HIF-1α via p42/p44 mitogen activated protein kinase (MAPK) appears to also play a role in its activation pathway (Richard et al., 1999). Finally, the two subunits form the functional transcription factor HIF-1 and dimerisation is required for stable association with the nuclear compartment (Chilov et al., 1999).

To modulate gene expression, the HIF-1 heterodimer interacts with the p300/CBP and SRC-1 family of co-activators (Arany et al., 1996; Carrero et al., 2000; Kung et al., 2000), and binds to a cognate hypoxia regulatory element (HRE; Figure 1.5). HREs are enhancers containing the core sequence 5’-(A/G)CGT(G/C)(G/C)-3’, localised at varying distances from and orientations to the coding sequence region of HIF-1-regulated genes (Table 1.2).

<table>
<thead>
<tr>
<th>HRE core sequence</th>
<th>gene</th>
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<tr>
<td>GGGCCCTACGTGCTGCCCTCGCATGGC</td>
<td>Epo, murine</td>
</tr>
<tr>
<td>GGGCCCTACGTGCTGCTTCACACACGC</td>
<td>Epo, human</td>
</tr>
<tr>
<td>TTGTCACGTCTGCACAGA</td>
<td>PGK-1, murine</td>
</tr>
<tr>
<td>GTGAGACGTGCGGCTTCC</td>
<td>PGK-1, human</td>
</tr>
<tr>
<td>CCAGCGGACGTGCGGGAAACCACGTG</td>
<td>LDH-A, murine</td>
</tr>
<tr>
<td>TCCACAGGCGGTGCCTGACACGCA</td>
<td>GLUT-1, murine</td>
</tr>
<tr>
<td>AGTGCAATACGTGGTTTCACAGGTC</td>
<td>VEGF, murine</td>
</tr>
<tr>
<td>AGTGCAATACGTGGGCTTCACAGGTC</td>
<td>VEGF, rodent</td>
</tr>
<tr>
<td>AGTGCAATACGTGGGCTCCACACAGGTC</td>
<td>VEGF, human</td>
</tr>
<tr>
<td>GGGCCGGACGTGGGGGCCACAGGGAAGAGCGA</td>
<td>Eno-1, human</td>
</tr>
<tr>
<td>CTGGCGTGACTACGTGCTGCAG</td>
<td>PFK-L, murine</td>
</tr>
<tr>
<td>TGACTACGTGCTGCCTAG</td>
<td>iNOS, human</td>
</tr>
</tbody>
</table>

Table 1.2. Hypoxia regulatory elements (HREs) from hypoxia-inducible genes.
The proposed HIF-1 binding site is in bold. The second underlined sequence is also involved in the response to hypoxia.
Hypoxia-targeted gene expression

The high frequency of HIF-1 expression across many human tumours of diverse origin represents a possible therapeutic target for HRE-directed gene therapy of the hypoxic environment. Transgene expression regulated by the murine PGK-1 HRE could be induced in hypoxic tumour cells (Dachs et al., 1997). Production of the marker protein CD2 in stably transfected human fibrosarcoma cells HT1080 increased with increasing length and severity of hypoxia. Compared to oxygen levels typical of normal tissues, radiobiologically relevant hypoxia (O2 concentration <0.3%) induced a 3-fold increase in gene expression (Table 1.3). Following anoxia and subsequent reoxygenation a 7-8-fold induction was observed. When the transfected tumour cells were grown as xenografts in nude mice, expression of CD2 was limited to peri-necrotic areas, indicative of hypoxia. This system was analysed on a single cell basis, by exposing tumour-bearing mice to the bioreductive drug RSU 1069 (which induces DNA cross-links in hypoxic cells) and X-rays (preferentially generating DNA strand breaks in oxic cells). By analysing individual tumour cells with single-cell electrophoresis combined with CD2 immunostaining, it could be demonstrated that increased CD2 expression was only seen in hypoxic tumour cells. Similarly, murine C2C12 myoblasts engineered to express the human Epo gene regulated by the murine PGK-1 promoter showed a 2.7-fold increase in gene expression in anoxia and a 3.2-fold induction at 1.3% O2 (Rinsch et al., 1997). Serum Epo levels in mice with C2C12-Epo cells implanted in the dorsal flank exposed to 7% O2 were 2-fold higher than in controls kept at 21% O2. In a more recent study, Koshikawa et al. (2000) observed a ~2-fold anoxic induction when the murine VEGF promoter controlled the expression of either luciferase or the enhanced green fluorescent protein (EGFP) in Lewis lung carcinoma A11 cells. The markers were replaced with the gene for the HSV thymidine kinase (TK) and retrovirally transduced cells were incubated in air with the prodrug ganciclovir (GCV), a substrate for HSV TK (section 1.2.5). Significant toxicity was only observed when HSV TK expression was triggered by a 16 h hypoxic pre-exposure.

To achieve significant gene expression in a therapeutic context, specific and robust transcriptional activation is required. In order to increase the hypoxic/oxic inducibility ratio of hypoxia-responsive promoters, a series of DNA constructs
containing fragments of the murine PGK-1, murine LDH-A, human Epo and human Eno-1 genes were inserted into the context of the basal simian virus (SV) 40 promoter (Table 1.3; Boast et al., 1999). In transiently transfected human mammary tumour T47D cells, the Epo HREs exhibited the most stringent regulation in hypoxia (255-fold induction at 0.1% O₂), while the PGK-1 HREs showed the highest absolute levels of expression (more than the strong cytomegalovirus (CMV) promoter), at the expense of selective regulation (146-fold at 0.1% O₂; see Table 1.3). The hypoxia response was augmented by 2-fold by inserting at the C-terminus of the reporter gene luciferase a 150 bp oligonucleotide spanning the 3’ untranslated region (UTR) of VEGF, which is involved in hypoxia-induced mRNA stability. The PGK-1 HREs were inserted in an adenoviral vector and in a panel of transduced cell lines a low basal level of β-galactosidase (β-gal) transgene expression was observed in normoxia, with levels of hypoxic induction comparable to the full-length CMV (Binley et al., 1999).

The hypoxic regulation system appears to be specifically affected by the cellular background, since cell lines of diverse origin were characterised by different responses (Table 1.3). In fibrosarcoma HT1080 cells transiently transfected with constructs containing fragments of the human Epo and VEGF genes, the best differential response to hypoxia was obtained by combining five copies of the 35 bp VEGF HREs with the adenoviral E1b minimal promoter (Shibata et al., 1998, 2000). In this cell line, a 6 h-hypoxic (0.02% O₂) incubation induced a 40-50-fold increase in luciferase activity (Shibata et al., 1998). An even higher hypoxic/oxic ratio (~500) was obtained when the five VEGF HREs were linked to the minimal CMV, with a marker protein production similar to the full-length CMV (Shibata et al., 2000). However, the same promoter construct induced 4-34-fold increase in gene expression in hypoxic human neurons (Cao et al., 2001). Interestingly, in the study by Shibata and co-workers (2000) the inclusion of the 3’ VEGF UTR decreased hypoxic gene expression. This may be due to the fact that the VEGF mRNA contains destabilising elements not only in its 3’ UTR, but also in its 5’ UTR and coding region, and selective mRNA stabilisation in response to hypoxia depends on the co-operation of elements in different regions (Dibbens et al., 1999).
Table 1.3. In vitro studies of hypoxia-regulated transgene expression, utilising different hypoxia-responsive promoters.

The basic components of the DNA constructs and the cell lines used are indicated. Gene expression has been evaluated after various hypoxic (* = 0.02% \( \text{O}_2 \); ** = 0.1% \( \text{O}_2 \), *** = 0.3% \( \text{O}_2 \)) or anoxic incubation intervals (Dachs, 1997; Boast, 1999; Binley, 1999; Ruan, 2001: 16 h. Rinsch, 1999: 24 h. Shibata, 1998; Shibata, 2000; Cao, 2001: 6 h. Shibata, 2000; Koshikawa, 2000: 18 h). m: murine, h: human.
It is not clear whether the large fold-inductions by HRE-controlled genes in response to hypoxia in later reports (Shibata et al., 1998, 2000; Binley et al., 1999; Boast et al., 1999) represent a clear improvement over the constructs used in earlier studies (Dachs et al., 1997; Rinsch et al., 1997), since transient rather than stable transfection methods were employed. Also, the end point in early studies was the immunological detection of a protein, whereas luciferase and β-gal assays measure the conversion by the enzyme of multiple substrate molecules into detectable units. The enzyme assays therefore further amplify any increase in transcription, making a direct comparison between assays difficult.

### 1.2.4 Radiation-mediated gene induction

In clinical settings, hypoxia-regulated gene therapy is likely to be used in combination with conventional treatments that can efficiently target well-oxygenated tumour areas. With the majority of human tumours treated with radiotherapy, ionising radiation (IR) is the most obvious candidate. Moreover, IR itself can be exploited for selective transgene expression in the tumour mass. In the majority of patients receiving radiotherapy, radiation is directed to the tumour volume, providing some degree of localisation for controlling the expression of therapeutic genes. Combined gene therapy and radiotherapy protocols could increase the effectiveness of a standard treatment, sparing normal tissue from damage.

IR has been shown to activate a number of genes, many of which are "immediate early response genes" involved in cell cycle progression, DNA damage sensing and repair (reviewed by Fornace, 1992). These include members of the Jun/fos family, tumour necrosis factor (TNF)-α, early growth response (Egr)-1, p21WAF1/CIP1 and GADD45. Some of these genes, such as p21 (el-Deiry et al., 1993) and GADD45 (Kastan et al., 1992) operate within p53-dependent pathways, and thus may not function efficiently in tumours exhibiting mutant p53 phenotypes. However, since activation of Egr-1 is p53-independent, its promoter has been adopted for the regulation of therapeutic genes in the majority of the experimental models of radiation-mediated gene therapy.
Egr-1 encodes a nuclear phosphoprotein involved in the transition from G₀ to G₁, DNA synthesis and growth suppression in response to a variety of stress stimuli (Christy and Nathans, 1989 a; Cao et al., 1990; Hallahan et al., 1995 a). X-ray-induced activation of Egr-1 was shown to take place at the transcriptional level, and the essential sequences for IR-induction were identified as 10 nucleotide motifs of the consensus sequence CC(A/T)₆GG, also known as CARG elements (Datta et al., 1992). In the Egr-1 promoter there are six CARG elements; however, only four grouped in the 5' distal enhancer region appear to contribute to radiation responsiveness. Reactive oxygen species (ROS) were recognised to be involved in CARG-mediated induction, since a similar activation pathway has been observed after cell exposure to H₂O₂ or IR (Datta et al., 1993). It is still unclear if the response is mediated by the binding of a transcription factor, if it is due to a variation of DNA conformation after cellular redox change, or if DNA damage per se is the initiating signal. In addition, CARG elements are known to be involved in the regulation of a number of immediate-early genes (e.g. Egr-1, c-fos, β-actin) following mitogenic stimulation (Gius et al., 1990), and are thus often referred to as serum-response elements (SREs). This growth factor-induced response is governed by the binding of the serum response factor (SRF), but whether this transcription factor is also involved in the activation of the Egr-1 gene by IR has not as yet been determined. The human and murine Egr-1 promoters also contain putative binding sites for the Sp-1 transcription factor, the Fos-Jun heterodimer AP-1 as well as for cyclic AMP and Egr-1 itself (Tsai-Morris et al., 1988; Christy and Nathans 1989 a, b; Sakamoto et al., 1991), all of which may influence radiation-mediated promoter induction.

The therapeutic potential of Egr-1 radiation-inducibility was demonstrated by positioning a 425 nucleotide region (E425), located at the 5' end of the coding sequence of the murine Egr-1 gene, upstream of the cDNA of the tumoricidal cytokine TNF-α (Weichselbaum et al., 1994). This DNA construct was delivered to human epidermoid carcinoma xenografts (SQ-20B) by using cell carriers (Weichselbaum et al., 1994), liposomes (Seung et al., 1995) or adenoviral vectors (Ad5, Hallahan et al., 1995 b; Mauceri et al., 1996). The combination of a fractionated radiation dose schedule of 40 Gy (5 Gy fractions twice a day for 4 days) and intratumourally delivered Ad5-E425-
TNF-α resulted in increased tumour regression by up to 90% of control values compared with maxima of 50% for vector or radiation alone (Hallahan et al., 1995 b; Staba et al., 1998). Similarly, Joki et al. (1995) and Takahashi et al. (1997) used a plasmid construct containing the E425 promoter to regulate the expression of HSV TK. Glioma cells containing this vector were sensitised to GCV after exposure to a single radiation dose of 20 Gy. In vivo, human hepatoma Huh-7 cells xenografted into nude mice regressed significantly after liposome delivery of a E425-HSV TK construct followed by 20 Gy γ-irradiation and GCV administration, but not after IR alone (Kawashita et al., 1999).

In order to produce a radiation-responsive promoter without potentially antagonistic binding sites, four isolated CArG elements of the same sequence were introduced in the basal CMV promoter (Marples et al., 2000; Scott et al., 2000). Using EGFP as reporter, the synthetic promoter was shown to be induced by radiation doses as low as 1 Gy, and to be more radiation-responsive than the wild-type Egr1 counterpart (3.1- vs. 1.9-fold, values at 3 Gy; Marples et al., 2000; Scott et al., 2000). When tumour cell cultures were transfected with CArG-HSV TK-based vectors and grown in the presence of GCV after a single 2 Gy trigger dose, cell survival was ~70%, compared to 90% for mock-transfected cultures treated similarly (Marples et al., 2000). The potential of using the synthetic radiation-responsive promoters was also evaluated in an in vivo preliminary study in MCF-7 breast adenocarcinoma xenografts (B. Marples, Gray Cancer Institute/Karmanos Cancer Institute, Detroit, MI, personal communication). CArG-HSV TK-transfected tumours treated with 50 μM GCV intra-peritoneal (i.p.) for two days prior, during and for three days after three daily fractions of 3 Gy required 33 days to reach a 500 mm³ volume (growth delay), to be compared with 15 days for tumours treated with GCV only and 25 days for radiation alone. The small size of the study did not allow to draw any conclusions on the significance of the results.

1.2.5 Gene-directed enzyme/prodrug therapy (GDEPT)
Genetic immunopotentiation, mutation compensation and molecular chemotherapy are the three major approaches in the design of therapeutic genes for cancer gene therapy. In the first case, tumour immunogenicity is enhanced by the delivery of genes that encode immunomodulators, such as cytokines (Dranoff et al., 1993, 1997; Soiffer et al., 1998)
or co-stimulatory molecules (Townsend and Allison, 1993). Mutation compensation aims to inactivate oncogenes or induce tumour suppressor gene expression (Takahashi et al., 1992; Bischoff et al., 1996; Roth et al., 1996); while in molecular chemotherapy, or gene-directed enzyme/prodrug therapy (GDEPT; Figure 1.6) a "suicide" gene is delivered to the tumour cells. In this latter approach, the enzyme encoded by the therapeutic gene is non-toxic per se, but is able to convert a prodrug into a potent cytotoxin. Only molecular chemotherapy will be discussed in detail below.

The concept of enzyme/prodrug therapy has been analysed prior to the advent of gene therapy. Prodrugs are chemicals that are inert even at relatively high doses, but can be converted to a toxic species at the target. Ideally, specific activation of a prodrug is the result of the metabolism by an enzyme that is either unique to the tissue or at significantly higher concentrations at the tumour site. Even though prodrug treatment of animal tumours with high levels of endogenous activating enzymes has been successful (Connors and Whisson, 1966; Khan and Ross, 1967; Cobb et al., 1969), clinical results were disappointing, since human cancers that contained satisfactory levels of the enzymes were rare and not associated with any particular type of tumour (Connors, 1995). To overcome this problem and achieve high levels of enzyme at the target, two different approaches are being investigated, antibody-directed enzyme/prodrug therapy (ADEPT; Melton and Sherwood, 1996) and GDEPT.

In the choice of the appropriate enzyme/prodrug combination, priority should be given to the enzyme. From past experience, it is likely that suitable prodrugs can be designed for almost any enzyme substrate specificity (Connors, 1995). For ease of handling and possible protein modification, the enzyme should be monomeric, of low molecular weight and with no requirement for glycosylation. It should have high catalytic activity under physiological conditions, fast and efficient prodrug activation even at low concentrations of the substrate (high $k_{cat}$ and low $K_m$), without dependence on further catalysis by other cellular enzymes. Expression of the enzyme itself, even at a high rate, should not lead to cytotoxic effects; the bystander effect required (see next section) would not be achieved if the cells were killed by the action of the enzyme alone. The reaction pathway should also be different from any endogenous enzyme, in order to avoid cytotoxic activation of the prodrug in normal tissues. This is the main
drawback of proteins of human origin, which, on the other hand, have the potential advantage of avoiding complications of acquired immunity, in particular after prolonged administration or protein synthesis.

**Figure 1.6.** Gene-directed enzyme/prodrug therapy (GDEPT).

A stimulus-regulated enzyme-encoding gene is delivered to the tumour. Only a fraction of the target cells will express the foreign gene and synthesise the enzyme. After systemic injection of the prodrug, cytotoxic activation takes place at the target only, and the bystander effect allows the eradication of neighbouring untransfected cells.

- Transfected cell
- Untransfected cell
- : enzyme
- : activated drug
- : dead cells
- : immune response

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The selected prodrug should be freely diffusible throughout the tumour (possibly a neutral species), chemically stable under physiological conditions and have suitable pharmacological and pharmacokinetic properties. For significant therapeutic gain, the released drug should be at least 100-fold more toxic than the prodrug.

**The bystander effect**

The bystander phenomenon, initially described by Moolten (1986), can be defined as an extension of the killing effects of the active drug to untransfected neighbouring cells (Figure 1.6). This implies that even if only a fraction of the target cells are genetically modified and express the therapeutic gene, tumour eradication may still be achieved. The bystander effect is crucial for a successful GDEPT strategy, since with the protocols currently adopted in the clinical trials the transfection efficiency is unlikely to be greater than 10%.

Two major categories of bystander effect have been identified: local and immune-mediated. In the first case, the killing of adjacent cells is due to the transfer of toxic metabolic products through gap junctions, via apoptotic vesicles, or through the diffusion of soluble toxic metabolites. Relying only on gap junctions could be restrictive, since cell-to-cell contact is required, and a number of tumour tissues and conditions, in particular hypoxia, have been shown to down-regulate intercellular gap junctional communication (Holder et al., 1993; Mesnil et al., 1996; Touraine et al., 1998; Nishida et al., 2000). In the case of freely diffusible species, a key role in obtaining a considerable but localised bystander effect is played by the activated drug half-life, which, assuming diffusion ranges in tumours of 100 to 200 μm, should be of about one minute (Patterson and Harris, 1999).

*In vivo* evidence in animal models suggests that a systemic immune response may play an important role in inducing bystander killing (Freeman et al., 1997). The presence of an intense inflammatory infiltrate has been described in the regressing tumours of immunocompetent animals treated with GDEPT systems, whereas the bystander effect was significantly reduced in immunodeficient athymic mice (Vile et al., 1994; Gagandeep et al., 1996; Pavlovic et al., 1996; Bi et al., 1997; Kuriyama et al., 1999 a) and after sublethal irradiation (Ramesh et al., 1996).
The impact of the bystander effect on the success of gene therapy strategies is so important that a number of studies are currently focused on its enhancement. Gap junctional communication, for instance, can be improved by regulating the production of connexins (Cxs), membrane proteins considered the building blocks of gap junctions (Mesnil and Yamasaki, 2000). The transfer of Cx-encoding genes (Cx43, Cx32, Cx40) or chemically induced Cx-overexpression has been shown in vitro (Elshami et al., 1996; Ghoumari et al., 1998; Kunishige et al., 1998) and in vivo (Dilber et al., 1997; Park et al., 1997; Touraine et al., 1998) to increase intercellular communication and the transfer of toxic agents. It has been noticed that in some human tumour cells not only expression but also correct surface localisation of Cx43 are necessary components of the bystander effect (McMasters et al., 1998), and that Cx-co-transfection appears not applicable to all tumour systems (Cirenei et al., 1998).

Another attractive tool to enhance the bystander effect is the HSV virion protein VP22. Once synthesised in infected cells, VP22 can spread very efficiently via a Golgi-independent pathway to surrounding uninfected cells, where it specifically accumulates in the nucleus (Elliott and O'Hare, 1997). Due to these peculiar trafficking properties, delivery of DNA constructs containing the VP22 coding sequence fused to the gene for the marker EGFP resulted in a significant spread of the VP22-EGFP fusion to the nucleus of untransfected cell monolayers (Elliott and O'Hare, 1999). The VP22-EGFP spread appeared to be a general phenomenon, common to all cell types tested to date (Wybranietz et al., 1999). Therapeutic advantage of this “biologically active” bystander effect for GDEPT was demonstrated by coupling the VP22 to the HSV TK coding sequence, which produced significant bystander killing in vitro and tumour regression in vivo, regardless of cellular gap junction activity (Dilber et al., 1999).

To enhance the immunological response to the tumour, novel strategies aim to combine tumour immunisation with GDEPT systems. Co-transfection of cytokine- and suicide gene-based vectors followed by prodrug treatment has met with varying results. In an experimental model for the treatment of glioblastoma, no enhancement of tumour eradication could be observed when HSVTK/GCV was combined with interleukin (IL)-2 gene delivery (Ram et al., 1994). On the other hand, Chen et al. (1995) reported improved antitumour activity of a HSV TK-IL-2 fusion protein for the treatment of
colorectal liver metastases. Also, safety and some clinical benefit was demonstrated in four patients with glioblastoma multiforme (GBM), who underwent standard surgery and radiotherapy followed by HSV TK/GCV and IL-2 gene therapy (Palù et al., 1999).

Several combinations have been proposed for GDEPT. Most of them do not fulfil all the above requirements, including those currently adopted in the clinical trials. In some cases active DNA replication (S phase) is an essential requirement for cytotoxicity (e.g., HSV TK/GCV); in other systems, the prodrug requires further metabolism by endogenous enzymes (CD/5-FC; NTR/CB1954; HSV TK/GCV). Some activated drugs are characterised by a long half-life (CD/5-FC), others are not membrane-permeable and depend upon cell-to-cell contact to diffuse into neighbouring cells (HSV TK/GCV). Nevertheless, some promising preclinical and clinical data have been reported, and they will be discussed in the following sections.

*Herpes simplex virus type-1 thymidine kinase/ganciclovir*

To date, the best studied enzyme/prodrug strategy in cancer GDEPT is undoubtedly HSV TK with the nucleoside analogue GCV. GCV and related agents, widely used in the treatment of HSV infection in humans, are poor substrates for the mammalian nucleoside monophosphate kinase, but can be converted (1000-fold more efficiently) to the monophosphate by TK from HSV (Figure 1.7). Subsequent reactions catalysed by cellular kinases lead to a number of toxic metabolites, the most active ones being the triphosphates (Figure 1.7). GCV-triphosphate competes with deoxyguanosine triphosphate for incorporation into elongating DNA during cell division, causing inhibition of the DNA polymerase and single strand breaks (Elion, 1983; Mar et al., 1985). These characteristics make the HSV TK/GCV combination particularly suitable for the eradication of rapidly dividing tumour cells invading non-proliferating tissue. On the other hand, since activated GCV is an S-phase specific cytotoxin, it is necessary that the target cells are actively dividing at the time of the exposure and that the prodrug is continuously administered to allow them to start replicating the DNA.
Figure 1.7. Metabolism of the prodrug ganciclovir (GCV).

GCV is specifically phosphorylated by HSV TK to its monophosphate, which is subsequently converted to the di- and triphosphate forms by guanylate kinase and other cellular kinases. GCV-triphosphate can be incorporated into elongating DNA, causing inhibition of the DNA replication and single strand breaks.

In the last 15 years more than 400 papers have discussed the potential of HSV TK/GCV for cancer GDEPT. Preclinical studies using adeno- and retroviral vectors were performed in many different animal models and positive results were reported for established rodent liver metastases (Caruso et al., 1993) and glioblastomas (Short et al., 1990; Culver et al., 1992), murine hepatocellular carcinomas (Kuriyama et al., 1999 b), human head and neck carcinomas (O’Malley et al., 1995) and mesotheliomas (Smythe et al., 1995), and other tumour types. Nevertheless, the HSV TK/GCV-induced mechanisms of cell killing have not been completely elucidated, and both apoptotic (Freeman et al., 1993; Wei et al., 1999; Beltinger et al., 2000; Thust et al., 2000) and non-apoptotic (Kaneko and Tsukamoto, 1995; Vile et al., 1997; Melcher et al., 1998) pathways have been reported.
The relationship between \textit{p53} gene status, \textit{p53}-mediated apoptosis and the sensitivity of human tumours to HSV TK/GCV is controversial. \textit{p53} played a significant role in the \textit{in vivo} response of oesophageal cancer, as demonstrated in two human lines, T.Tn and TE2 cells, with truncated and wild type \textit{p53}, respectively, implanted in nude mice (Matsubara et al., 1999). Exposure to HSV TK/GCV induced \textit{p53} accumulation and translocation of the receptor \textit{CD95} to the cell surface, with subsequent recruitment of Fas-associated death domain and trigger of the apoptotic caspase cascade, in human SH-EP neuroblastoma cells (Beltinger et al., 1999) and in murine tumours (Wei et al., 1999). Mitochondria appeared to have a major involvement in HSV TK/GCV-induced apoptosis, by regulating both the initiation (through \textit{p53} accumulation) and the effector phase of apoptosis (through the release of cytochrome \textit{c} into the cytosol and caspase activation; Beltinger et al., 2000). On the other hand, endogenous \textit{p53} status did not correlate with sensitivity to HSV TK/GCV in human SKOV-3 ovarian and Hep3B hepatocellular carcinoma cells (Xie et al., 1999) and in some breast cancer cell lines (Li et al., 1999).

One of the main drawbacks of the HSV TK/GCV system is that the highly charged triphosphate is insoluble in lipid membranes. This impairs the diffusion of the drug and makes cell-to-cell contact necessary for bystander killing. Nevertheless, preclinical studies showed that tumour regression could be achieved when only 10\% of the tumour cells expressed \textit{HSV TK} (Caruso et al., 1993; Freeman et al., 1993). This phenomenon has been proposed to result from transfer of activated GCV through gap junctions (Elshami et al., 1996; Dilber et al., 1997; Touraine et al., 1998; Mesnil and Yamasaki, 2000) or exchange of apoptotic vesicles (Freeman et al., 1993; Colombo et al., 1995). Transfer of toxicity in some murine cell lines occurred before there was evidence of apoptotic degeneration, indicating that apoptosis could be the result, not the cause, of the bystander effect (Denning and Pitts, 1997). It is likely that a major role in the \textit{in vivo} bystander killing is played by the host immune system. HSV TK/GCV treatment resulted in infiltration of CD4\(^+\) and CD8\(^+\) T cells and macrophages, as well as increased production of IL-2, IL-12, IFN-\(\gamma\), TNF-\(\alpha\) and granulocyte/macrophage colony-stimulating factor, suggesting that the induced cell killing creates a cytokine-rich
immunostimulatory environment (Gagandeep et al., 1996; Vile et al., 1997). The generation of immunostimulatory signals in vivo appeared to be predominant in non-apoptotic tumours, and associated with the induction of genes of the inducible HSP family (Melcher et al., 1998). An immune-related antitumour response could also account for the “distant bystander effect” (Dilber and Smith et al., 1997). GCV treatment of head and neck squamous cell carcinoma xenografts in nude mice resulted not only in the eradication of TK⁺ tumours, but also in the regression of untransduced tumours in the contra-lateral flank (Wilson et al., 1996). This effect was abrogated in mice with severe combined immunodeficiency (SCID; Bi et al., 1997). Interestingly, in a plasmacytoma model, local and some distant bystander effects could be observed also in SCID mice (Dilber et al., 1996), suggesting that T and B lymphocytes, absent in SCID mice, may not be involved in HSV TK/GCV-mediated distant bystander killing.

On the basis of animal studies, the first gene therapy trial using HSV TK/GCV to treat ovarian cancer was approved in 1991 (Freeman et al., 1995), and since then several other clinical studies have been undertaken. These include gene therapy of brain tumours, particularly GBM (Oldfield et al., 1993; Eck et al., 1996; Izquierdo et al., 1996; Ram et al., 1997; Stockhammer et al., 1997; Klatzmann et al., 1998 a; Shand et al., 1999), of metastatic melanoma (Klatzmann et al., 1998 b) and prostate carcinoma (Herman et al., 1999; Shalev et al., 2000). Gene delivery has been performed by injecting HSV TK-containing replication-deficient Ads (Eck et al., 1996; Herman et al., 1999; Shalev et al., 2000) or retroviral vector-producing cells (VPCs; Oldfield et al., 1993; Izquierdo et al., 1996; Ram et al., 1997; Klatzmann et al., 1998 a, b; Shand et al., 1999). In the phase I clinical trials only moderate toxic events were reported, which were mostly resolved at the termination of the therapy course. Some complications (seizures, intratumoural haemorrhage) occurred in patients with brain tumours as a result of the biopsies to implant the VPCs (Ram et al., 1997; Shand et al., 1999), but no safety hazards due to the use of xenogeneic cells or in situ release of retroviral vectors within the tumour were identified. Moderate therapeutic response was observed in some of the patients, especially those with small tumours (<3 mm³; Ram et al., 1997). In a phase I/II study for recurrent glioblastoma, injection of retroviral producing cells in the surgical
cavity margins after tumour debulking followed by i.v. GCV resulted in the absence of recurrence in four of 12 patients at four months, and in one patient at 2.8 years after treatment (Klatzmann et al., 1998 a). In another clinical trial for recurrent GBM, four of 48 patients had some tumour regression, but the therapeutic benefit was transitory and not associated with any regrowth delay (Shand et al., 1999). Finally, a highly powered multicentre, randomised, controlled phase III trial involving 248 patients with untreated GBM failed to show any advantage of retroviral HSV TK followed by GCV as regards to tumour progression or overall survival (Rainov, 2000).

These relatively poor responses could be due to insufficient gene transfer and limited distribution within the tumour mass, which only allowed the treatment of small tumours with a high concentration of foreign DNA (Ram et al., 1997). The growth rate of the tumour cells might also play an important role in the response to HSV TK/GCV and explain the higher sensitivity of fast growing experimental tumours compared to slower growing spontaneous human malignancies. Furthermore, because of GCV toxicity particularly to the bone marrow (Caruso, 1996), the maximum dose used in humans (10 mg/kg/day) was much lower than the doses used in most animal experiments (up to 300 mg/kg/day).

There are numerous possibilities for ameliorating treatment efficacy, notably through the improvement of gene delivery as well as a better understanding of the molecular mechanisms of the bystander effect. Significant benefits could also arise from the introduction of new nucleoside analogues with a higher affinity for HSV TK, fewer side effects than GCV and marked cytotoxic activity towards HSV TK-transfected cells (Balzarini et al., 1994, 1998; Degrève et al., 1999; Thust et al., 2000). Moreover, HSV TK mutants have been engineered to increase specificity and activity towards the prodrug (Black et al., 1996, 2001).

**Cytosine deaminase/5-fluorocytosine**

The enzyme CD, found in certain bacteria and fungi but not in mammalian cells, catalyses the hydrolytic deamination of cytosine to uracil. It is also able to convert the non-toxic prodrug 5-FC to 5-fluorouracil (5-FU), which is then transformed by cellular enzymes to potent pyrimidine antimetabolites (5-FdUMP, 5-FdUTP, 5-FUTP; Figure...
1.8). Three pathways are involved in the induced cell death: thymidylate synthase inhibition, formation of (5-FU) RNA and of (5-FU) DNA complexes (Springer and Niculescu-Duvaz, 1996). 5-FU is widely used in cancer chemotherapy and is the drug of choice in the treatment of colorectal carcinoma. It is characterised by an array of side effects and relatively high dose levels are required for tumour response. Although not cell cycle phase-specific, 5-FU has both proliferation-dependent and independent actions.

The CD gene used for GDEPT has been cloned from E. coli (Austin and Huber, 1993) and shown in a number of in vitro studies to enhance mammalian cell sensitivity to 5-FC by up to 2000-fold (Ge et al., 1997). However, in vitro studies showed loss of cytotoxicity of 5-FU and CD/5-FC in hypoxic tumour cells (G.U. Dachs, Gray Cancer Institute, unpublished data), which might result in limited therapeutic efficacy in solid tumours. In vivo antitumour activity of the CD/5-FC combination has been demonstrated in several animal models, including fibrosarcomas (Mullen et al., 1994), carcinomas (Huber et al., 1993, 1994; Ohwada et al., 1996; Kanai et al., 1997; Bentires-Alj et al., 2000), gliomas (Ge et al., 1997; Ichikawa et al., 2000) and metastatic formations of different origin (Consalvo et al., 1995; Topf et al., 1998).

One of the main advantages of the CD/5-FC system is a strong bystander effect that does not require cell-to-cell contact, since 5-FU can diffuse into and out of cells by non-facilitated diffusion (Domin et al., 1993). Experiments conducted in vitro by exposing mixed transfected and untransfected populations to 5-FC showed that 1-30% of cells expressing CD could generate sufficient 5-FU to inhibit the growth of the neighbouring cells not producing the enzyme, even when the cells were sparsely seeded (Huber et al., 1994; Ichikawa et al., 2000). Significant amounts of 5-FU were found in the culture medium of treated CD+ cells (Huber et al., 1994), and the transfer of the supernatant from transfectants exposed to 5-FC to untransfected cells (medium-switch) resulted in their death (Lawrence et al., 1998). Bystander killing was also observed in cells negative for gap junctions (Lawrence et al., 1998). However, studies with [5-3H]5-FC showed that moderate and unsaturable amounts of the drug accumulated intracellularly (Haberkorn et al., 1996), indicating that 5-FC uptake might be a
bottleneck in this treatment strategy. Also, the \textit{in vitro} bystander effect was not detected in the early reports of CD/5-FC (Mullen et al., 1992) and appears to be reduced in some cell lines (Bentires-Alj et al., 2000).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure18.png}
\caption{Conversion of 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) by the \textit{E. coli} cytosine deaminase (CD). The formation of the antimetabolites 5-fluorodeoxyuridine-5'-monophosphate (5-FdUMP), 5-fluorodeoxyuridine-5'-triphosphate (5-FdUTP) and 5-fluorouridine-5'-triphosphate (5-FUTP) is shown.}
\end{figure}

Figure 1.8. Conversion of 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) by the \textit{E. coli} cytosine deaminase (CD). The formation of the antimetabolites 5-fluorodeoxyuridine-5'-monophosphate (5-FdUMP), 5-fluorodeoxyuridine-5'-triphosphate (5-FdUTP) and 5-fluorouridine-5'-triphosphate (5-FUTP) is shown.
Nevertheless, significant bystander killing has been observed in preclinical studies. 5-FC treatment of xenografts grown from mixtures of CD and CD\(^+\) human WiDr colorectal carcinoma cells in nude mice caused tumour regression even when only 4% of the inoculated cells expressed the enzyme (Huber et al., 1994; Trinh et al., 1995). A comparable antitumour effect (60% cure rate) was induced by GCV in tumours composed of 50% HSV TK\(^+\) cells (Trinh et al., 1995). Similar to HSV TK/GCV, the CD/5-FC combination induced an immune-mediated distant bystander effect, associated with an infiltration of natural killer cells within the tumours (Pierrefite-Carle et al., 1999). Immunocompetent mice pre-treated with CD/5-FC GDEPT exhibited significant resistance when re-challenged with wild type tumours (Mullen et al., 1994; Consalvo et al., 1995; Kuriyama et al., 1999 a). This “vaccination effect” appeared to be dependent on the novel CD\(^+\) malignancies, as, for example, the eradication of CD\(^+\) adenocarcinomas conferred no protection against fibrosarcomas (Mullen et al., 1994). It is important to note that the CD protein itself is immunogenic (Mullen et al., 1994).

Despite encouraging preclinical results, it becomes clear from the clinical reports that the treatment with a single GDEPT strategy may at best lead to a partial response. Therefore, combinations of several genes or treatment modalities have been investigated. Based on the extensive clinical experience with 5-FU as both a chemotherapeutic agent and a radiosensitiser (reviewed by McGinn and Kinsella, 1993), the combination of CD/5-FC GDEPT with IR was tested. CD-expressing SK-ChA-1 cholangiocarcinoma cells (Pederson et al., 1997, 1998), B16F melanoma (Szary et al., 1997) and WiDr cells (Khiel et al., 1996; Stakhouse et al., 2000) exposed to 5-FC were selectively sensitised to radiation in a drug concentration-dependent fashion, with maximum killing enhancement after 72 h-incubation pre-irradiation (Khiel et al., 1996) or, in contrast, at 3 h post-irradiation (Szary et al., 1997). Analysis of the linear quadratic or single-hit multi-target parameters of the survival curves indicated a significant reduction in cell survival at both low and high doses of radiation (Pederson et al., 1997; Stackhouse et al., 2000). In xenografts of squamous cell carcinoma of the head and neck daily administration of 5-FC (800 mg/kg) concomitant with radiation therapy (50 Gy, 5 Gy/day) induced significant volumetric regression and cure in three of seven
CD-transfected tumours, while radiation or GDEPT alone produced no significant tumour control (Hanna et al., 1997). A robust bystander effect was induced, as a significant increase in animal survival was observed when 25% of the tumour population expressed CD (Hamstra et al., 1999b). The combined GDEPT and radiotherapy strategy produced a measurable antitumour effect when clinically relevant dose regimens of radiation (2 or 5 Gy per fraction over one week) were analysed (Stackhouse et al., 2000).

Specific gene expression and prodrug activation offer the possibility of combining different GDEPT systems to enhance the antitumour activity of the single treatments, without increasing systemic toxicity. Delivery of CD-HSV TK fusion genes followed by GCV and 5-FC treatment conferred upon gliosarcoma, mammary carcinoma and prostate tumour cells prodrug sensitivity (Rogulski et al., 1997a; Uckert et al., 1998; Blackburn et al., 1998, 1999) and radiosensitisation (Rogulski et al., 1997a; Blackburn et al., 1999), equivalent to or better than those observed for each system independently. The mechanism underlying the synergistic cytotoxicity of concomitant CD/5-FC and HSV TK/GCV therapies is unclear, and it appears to be related to the enhancement of GCV phosphorylation by HSV TK after 5-FU treatment (Aghi et al., 1998). In vivo analysis showed high efficacy of the combined systems, further enhanced by radiotherapy (Rogulski et al., 1997b; Kim et al., 1998; Uckert et al., 1998).

Improved antitumour activity and radiosensitisation of CD/5-FC has been recently achieved by using the catalytically superior Saccharomyces cerevisiae CD (Hamstra et al., 1999a; Kievit et al., 1999, 2000) or by co-transfecting cells with the genes for CD and uracil phosphoribosyltransferase, able to directly convert 5-FU to 5-FUMP at the first step of its activating pathway (Adachi et al., 2000; Erbs et al., 2000; Koyama et al., 2000).

5-FC has been extensively used in the past as an antifungal agent, therefore its toxicological properties in humans are well established. A phase I clinical trial involving local injection of a plasmid containing the CD gene regulated by the tumour-selective erbB-2 promoter, followed by systemic 5-FC administration (200 mg/kg/day), demonstrated safety of this GDEPT approach for the treatment of breast cancer (Pandha...
et al., 1999). CD-immunohistochemistry and mRNA in situ hybridisation showed tumour-selective gene expression in 11 of 12 patients. In four patients there was evidence of tumour regression, even though two of them did not receive the prodrug, which may be due to the immunogenicity of CD. In an ongoing phase I clinical study, a CD-containing adenovirus was intratumourally administered to 18 patients diagnosed with metastatic liver disease associated with colorectal carcinoma, followed by oral administration of 5-FC (Crystal et al., 1997).

**Nitroreductase/CB1954**

The mustard prodrug CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] is a weak monofunctional alkylator, but it can be efficiently activated by the rodent enzyme DT diaphorase into a potent DNA cross-linking agent (Knox et al., 1988 b). CB1954 resulted in the single agent cure of Walker carcinosarcoma in rats, but its use against human cancers was limited by low reactivity with the human DT diaphorase (Khan and Ross, 1967). This problem was overcome when an *E. coli* NTR was found to reduce this prodrug 90 times faster than the Walker rat DT diaphorase (Anlezark et al., 1992) and to convert it into a cytotoxin when added to mammalian cells (Knox et al., 1992). The metabolism of CB1954 is shown in Figure 1.9. The prodrug is converted by NTR, by an oxygen-independent mechanism, to the difunctional alkylator 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, which after further reactions with cellular thioesters, such as acetyl coenzyme-A, is able to form poorly repairable DNA interstrand cross-links (Knox et al., 1988 b). Since nitroreductases require NADH or NADPH as cofactors (Knox et al., 1988 a), activation of the prodrug can only take place intracellularly, limiting the use of the NTR/CB1954 combination for ADEPT. Its application for GDEPT was demonstrated after cloning (Michael et al., 1994) and insertion of the *E. coli* NTR gene into retroviral, adenoviral and plasmid DNA vectors. NTR expression in several human and murine cells resulted in up to 2600-fold increase in sensitivity to CB1954, compared to the parental lines (Bridgewater et al., 1995; Bailey et al., 1996; Green et al., 1997; Friedlos et al., 1998; McNeish et al., 1998; Weedon et al., 2000). In transgenic mice, NTR expression under the control of a T cell-specific promoter induced selective CB1954 toxicity in the thymus and the spleen of systemically treated animals,
whereas non-lymphoid tissues were unaffected (Drabek et al., 1997). Selective tissue ablation was also demonstrated in transgenic mice engineered to specifically express the NTR gene in the luminal epithelial cells of the mammary gland (Clark et al., 1997). Even if transgenics provide a useful model for studying the in vivo efficacy of gene therapy strategies, they may not be predictive for clinical scenarios, where levels of gene transfer are unlikely to be as high. However, retro- or adenoviral-mediated NTR transfer followed by CB1954 treatment has been shown to cause regression and significantly prolonged median survival of nude mice bearing human tumour xenografts of pancreatic (McNeish et al., 1998; Weedon et al., 2000), hepatocellular and squamous cell carcinomas (Djeha et al., 2000).

**Figure 1.9.** Bioactivation of the prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) by the *E. coli* nitroreductase (NTR). Thioesters, such as acetyl coenzyme-A, convert 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide into a potent cross-linking agent.
Apoptosis was induced in NTR-transfected thymocytes and mammary epithelial cells of CB1954-treated animals (Clark et al., 1997; Drabek et al., 1997; Cui et al., 1999), which appeared to be a consequence of DNA cross-linking and strand breakage (Clark et al., 1997; Friedlos et al., 1998). Although NTR/CB1954 treatment increased the expression of wild-type p53, functional p53 was not required to induce apoptosis (Cui et al., 1999).

The NTR/CB1954 system appears to act more rapidly than most combinations, as significant cytotoxic effects have been noted after relatively short exposure times, such as 4 h in vitro (Bridgewater et al., 1995) and 24 h in vivo (Cui et al., 1999). This may be due to the fact that the activated drug is a DNA cross-linking agent able to kill both proliferating and non-proliferating cells, which do not need to enter the S-phase for cytotoxicity to take place (Bridgewater et al., 1995; Clark et al., 1997; Green et al., 1997; Weedon et al., 2000).

An efficient bystander effect was demonstrated in a number of cell lines and in animal models, regardless of cell-to-cell contact and gap junction status (Bridgewater et al., 1997). The primary NTR-metabolites of CB1954, 4-hydroxylamines, are membrane-permeable and, after further metabolism, they are able to induce single strand breaks and interstrand cross-links, respectively, in neighbouring parental cells (Friedlos et al., 1998). Mixed populations with 30% NTR+ pancreatic cancer cells were 1000-fold more sensitive to CB1954 than untransfected cells (Green et al., 1997), and bystander killing was measurable when only 5% of the exposed population expressed NTR (McNeish et al., 1998). On the other hand, in cell culture experiments with 5-10% NTR+ murine L cells (Drabek et al., 1997) and in mammary glands of transgenic RED20 and RED40 mice (Clark et al., 1997) no bystander effect could be observed. Also, SCID mice implanted with tumours containing either 30% or 100% of NTR-expressing Burkitt lymphoma cells treated with CB1954 i.p. (20 mg/kg/day for 10 days) were growth inhibited but not cured (Westphal et al., 2000).

The versatility that NTR offers by metabolising a range of nitro-prodrug substrates may have clinical relevance in view of the cross-resistance observed among alkylating agents (Frei et al., 1998). Structural variations of CB1954 included N-dihydroxypropyl and (N-dimethylamino)ethyl carboxamide side chains, the use of chloro-, bromo-,
mesyl- and iodo- leaving groups on the mustards, and regioisomeric changes (Friedlos et al., 1997). In Chinese hamster V79 fibroblasts, at least four analogues showed increased toxicity and bystander killing efficacy compared to the model compound CB1954 (Friedlos et al., 1997).

**Cytochrome P450 reductase/bioreductive drugs**

The redox-sensitive flavoprotein NADPH:cytochrome P450 reductase (P450R) is an important endogenous bioactivator of many nitroaromatic, aromatic N-oxide and quinone “triggered” hypoxia-selective cytotoxins (HSCs; section 1.1.2). P450R overexpression in human fibrosarcoma (HT1080) or breast cancer (MDA231) cells conferred increased sensitivity to tirapazamine, RSU 1069, EO9, mitomycin C and porfiromycin (Patterson et al., 1997; Saunders et al., 2000). Selective hypoxic targeting could be further refined by incorporating an optimised PGK-1 HRE-SV40 chimeric promoter to regulate the expression of P450R (Patterson and Stratford, 1999). In transfected HT1080 cells anoxic incubation produced a 3.4-fold increase in enzyme activity (Patterson and Stratford, 1999) and a 1000-fold enhancement of in vitro hypoxic cytotoxicity of RSU 1069 (Patterson et al., 2000). HT1080 tumour xenografts were established and treated with a combination of 10 Gy X-rays and RB 6145, a precursor of RSU 1069 (Patterson et al., submitted manuscript). Compared to radiation alone, a significant increase in specific growth delay was observed in the transfected tumours, but not in the untransfected xenografts (50% cure rate in HRE-P450R xenografts, 100% mortality in empty vector control xenografts).

**Cytochrome P450/cyclophosphamide**

The oxazaphosphorine prodrug cyclophosphamide (CP) is activated by metabolism of liver cytochrome P450 (CYP) via a 4-hydroxylation reaction (Figure 1.10). The 4-hydroxy intermediate breaks down to form the difunctional alkylating toxin phosphoramide mustard, which leads to DNA cross-links, G2-M arrest and apoptosis in a cycle-independent fashion (Chen et al., 1996). It has also been proposed that acrolein, formed in equimolar amounts with the phosphoramide mustard upon activation of CP, sensitises the cells to the mustard (Chen and Waxman, 1995). The isomeric analogue
ifosfamide (IP) is activated in a similar way, and both CP and IP reactions require NADH and O₂.

**Figure 1.10.** Pathways of cytochrome P450 (CYP)-catalysed cyclophosphamide (CP) and ifosfamide (IP) activation.

The phosphoramide mustard is able to induce DNA alkylation. Acrolein, produced in equimolar amounts, maybe responsible for protein alkylation.

CYP is a very complex enzymatic system, involving a number of isoenzymes with different substrate specificity and activity against CP or IP. For example, in rats three liver CYP enzymes, 2B1 (Phenobarbital-inducible), 2C6 and 2C11 (both constitutively expressed) are involved in activating CP (Clarke and Waxman, 1989), while the CYP
3A isoenzyme additionally contributes to the activation of IP (Weber and Waxman, 1993). CYP 2B6 and CYP 3A4 are responsible for these processes in human liver (Chang et al., 1993) and CYP 2B1 is the most active one (Clarke and Waxman, 1989).

CYPs are primarily synthesised in the liver and in some human cancers, including colon, breast, lung, liver, kidney and prostate, which are known to express isoforms of the 3A and 1A subfamilies (Patterson et al., 1999). To reduce CP and IP side effects towards critical host tissues such as bone marrow, kidney and heart, it was hypothesised that overexpression of CYP enzymes in genetically engineered tumour cells could lead to selective sensitisation to oxazaphosphorines. Transfected human B-lymphoblastoid cells producing catalytically competent CYP 2A6, 2B6, 2C8, 2C9 and 3A4 were significantly growth-inhibited by CP and IP (Chang et al., 1993). CYP 2B1 gene overexpression sensitised rat glioma and human breast carcinoma cells to CP both in vitro and in vivo (Wei et al., 1994, 1995; Chen and Waxman, 1995; Chen et al., 1996; Manome et al., 1996). In co-culture experiments, a 75% decrease in proliferation was induced when only 10% of the CP-exposed population were transfected with CYP 2B1, independently of cell-to-cell contact (Chen and Waxman, 1995; Wei et al., 1995; Chen et al., 1996). Because the CP metabolite phosphoramidate mustard does not diffuse efficiently across cell membranes, it is likely that this bystander effect is due to diffusible precursors, such as 4-hydroxy-CP and its tautomer aldophosphamide (Figure 1.10; Wei et al., 1995).

In a recent phase I/II clinical study, human embryonic 293 cells were stably transfected with the CYP 2B1 gene, microencapsulated using cellulose sulphate and polydiallyldimethyl ammonium (CapCell) and angiographically placed into a tumour-feeding artery of patients with inoperable pancreatic adenocarcinoma (Löhr et al., 1999, 2001). On day two and for three consecutive days, 1 g/m² body surface IP was administered. The seventeen patients in the study showed no evidence of pancreatitis, vessel occlusion or allergic response. The prodrug was well tolerated and no toxicity beyond grade II was detected. By the end of the study, two patients had a partial response (more than 50% reduction in tumour volume for one month), two had minor
response (tumour reduction by 25-50%), eight had stable disease (75-125% of the initial tumour size). All treated patients had longer survival times than matched controls.

Carboxypeptidase G2/CMDA

In the enzyme/prodrug systems described so far the prodrug is converted to an intermediate metabolite, which requires further catalysis by cellular enzymes to form the active drug. Lack or reduced expression of these enzymes in the target cells would lead to tumour resistance. The bacterial enzyme carboxypeptidase G2 (CPG2), which has no human analogue, is able to cleave the glutamic acid moiety from the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid (CMDA), releasing the DNA-crosslinking mustard drug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoic acid without further catalytic requirements (Figure 1.11; Springer et al., 1990). The application of the CPG2/CMDA combination in ADEPT strategies has been successfully demonstrated in a number of tumour models, and phase I clinical trials showed no conversion to the active species by human enzymes and no CMDA-related toxicity (Springer et al., 1994; Bagshawe et al., 1995; Martin et al., 1997). The feasibility of CPG2-mediated GDEPT was investigated in vitro in COS (monkey kidney) cells and four human tumour epithelial lines (Marais et al., 1996). Transfection with the CPG2 gene led to the production of an active enzyme, able to convert CMDA to a cytotoxic agent within a 19 h-incubation. Compared to mock-transfected controls, CPG2-transfectants were characterised by an increase in sensitivity to the prodrug spanning from 11- to 95-fold (Marais et al., 1996). However the breast tumour cell line MDA MB361 showed only modest sensitivity to CMDA activated by intracellular expression of CPG2, mainly because the prodrug could not enter the cells (Marais et al., 1997). To overcome this problem, CPG2 was expressed tethered to the outer surface of mammalian cells, resulting in effective cell kill by CMDA in vitro and in vivo (Marais et al., 1997; Stribbling et al., 2000).
Figure 1.11. Conversion of the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid (CMDA) into a cytotoxin (adapted from Marais et al., 1996). The bacterial enzyme carboxypeptidase G2 (CPG2) is able to cleave the glutamic acid moiety from the prodrug releasing the DNA-crosslinking mustard drug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoic acid without further catalytic requirements.

The CPG2/CMDA system has been shown to induce a robust bystander effect. Exposure to the prodrug induced 50% and 100% WiDr cell kill in monolayers containing 2% and 12% CPG2+ cells, respectively (Marais et al., 1996). In nude mice, regression and cure of breast carcinoma xenografts could be obtained when only 10% of the cells expressed the tethered CPG2 (Stribbling et al., 2000). Evidence of the in vivo bystander effect was obtained by measuring the proportion of apoptotic cells in the treated tumours, which was higher than the percentage of cells expressing CPG2. The effect of the surface tethered CPG2 on host immunicity has not yet been reported.

To improve the efficacy of the system, a number of self-immolative prodrugs have been developed in combination with CPG2, some of them resulting in a significant increase in cytotoxicity (Niculescu-Duvaz et al., 1998, 1999).
1.3 Horseradish peroxidase/indole-3-acetic acid

Peroxidases are a group of haem-containing hydrogen peroxide-utilising enzymes, which act on a wide variety of substrates and have diverse biological roles. The peroxidase from horseradish (*Armoracia rusticana*; HRP) is probably the best characterised of these enzymes. The dominant isoform of HRP, isoenzyme C, is a monomeric glycoprotein of 308 residues, with four disulphide bridges, two Ca$^{2+}$ ions per molecule of protein and eight carbohydrate side chains, which account for about 20% of its molecular weight (~43 kDa; Welinder, 1979). Glycosylation is not required for its peroxidase action, since the recombinant enzyme produced in *E. coli*, which is not glycosylated, is a competent catalyst (Smith et al., 1990). HRP is extensively used in enzyme immunoassays and histochemistry as a reporter.

The plant hormone indole-3-acetic acid (IAA; 1, Figure 1.12) is involved in the regulation of cellular growth, division and differentiation, as well as senescence and abscission of leaves (Engvild, 1989). The activity of this auxin is regulated through irreversible conversion via oxidation by peroxidases, leading to decarboxylation, or non-decarboxylation reactions. IAA can be produced in humans through deamination of tryptamine by monoamine oxidase (Weissbach et al., 1959), at concentrations of 0.61-3.32 μM in plasma (Martínez et al., 1983), and 23-30 pM in the cerebrospinal fluid (Anderson et al., 1984). Plasma concentration can rise up to 11 μM in case of renal dysfunction (Qureshi and Baig, 1993). An increase in IAA was also observed in human oesophageal cancer tissues (Shimojo et al., 1997). Cancer tissue biopsies from five patients contained 4700-7400 ng IAA/g, while in the surrounding normal tissues IAA content was 90-390 ng/g. In each patient IAA levels in cancer specimens was always higher than in normal tissue (Shimojo et al., 1997). Other routes of IAA production in animals are ingestion of fibres and metabolism by intestinal bacteria (Weissbach et al., 1959).

The reaction between HRP and IAA has been analysed in depth but not yet completely elucidated. In contrast to most peroxidase reactions, it does not require H$_2$O$_2$ (Dunford, 1999). The native HRP enzyme is a Fe$^{III}$ species, and the key oxidants in the catalytic cycle are a [Fe$^{IV}$=O]$^{+}$ radical cation (compound (Cpd) I; Figure 1.12) and a
Fe^{IV}=O species (Cpd II). The pathway involving Cpd III (also termed "oxidase" pathway) is relevant at low enzyme/substrate ratios (Smith et al., 1982) and explains the formation of superoxide radicals (O$_2^-$). However, at pH 7.4 neither the ferrous enzyme nor Cpd III were reported to be formed, and this pathway appeared less relevant under physiological conditions (Dunford, 1999). At neutral pH, the reaction is initiated by autoxidation of IAA (1), by a highly efficient branched-chain mechanism (Dunford, 1999). This is followed by a one-electron oxidation of IAA to an indolyl radical cation (2), which very rapidly undergoes scission of the exocyclic carbon-carbon bond to yield the carbon-centred skatole radical (3). In the presence of oxygen, the skatole radical rapidly forms a peroxyl radical (4), which then decays in two ways. Combination and elimination by the Russell mechanism, in which two peroxyl radicals combine, form indole-3-aldehyde (5), indole-3-carbinol (6) and a singlet oxygen. This pathway appears not to occur at physiological pH (Candeias et al., 1994), and it is more likely that reduction and protonation of the peroxyl radical form skatole hydroperoxide (7), which reacts further with Cpd I to form indole-3-carbinol (6). Each hydroperoxide molecule can initiate another enzyme cycle, or decompose non-enzymatically to 3-methylene-2-oxindole (MOI, 8). MOI can react with cellular nucleophiles, e.g. protein thiols and DNA, to form adducts (9). In anoxic solution, decarboxylation of the radical cation can still take place and the carbon-centred radical preferentially reacts with hydrogen donors (Augusto, 1993; Candeias et al., 1994).
Figure 1.12. Possible mechanisms involved in the cytotoxic activation of indole-3-acetic acid (IAA) by horseradish peroxidase (HRP; adapted from Folkes and Wardman, 2001).

HRP can be activated to compound (Cpd) I by trace peroxides or IAA itself. IAA (1) is oxidised by HRP Cpd I and II to the radical cation (2), which fragments rapidly to yield the skatole radical (3). In anoxia this probably forms adducts, e.g. with DNA. In air, 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).
When activated by purified HRP, IAA was shown to inhibit colony formation in mammalian cells, while neither enzyme nor prodrug alone were cytotoxic at the concentration or times analysed (Folkes et al., 1998, 1999). To date, the activated drug and the cellular targets have not yet been identified. The initial hypothesis was that the peroxyl radical (4) would initiate membrane lipid peroxidation (Folkes et al., 1998). This was indeed detected in phosphatidylcholine-cholesterol liposomes incubated with HRP and IAA (Candeias et al., 1995), and found to be prevented in the presence of antioxidants such as ascorbate, Trolox, α-tocopherol and β-carotene (Candeias et al., 1996). Moreover, lipid peroxidation in the presence of activated IAA analogues correlated well with their reaction rate with HRP (Candeias et al., 1995). However, no lipid peroxidation was detected in mammalian cells exposed to cytotoxic doses of HRP/IAA and, although Trolox was protective against cytotoxicity probably intercepting indole radicals, pre-loading the cells with α-tocopherol was ineffective (Folkes et al., 1999). On the other hand, incubation of the activated drug with plasmid DNA in a cell-free system resulted in the formation of adducts and strand breaks, indicating that DNA damage could be involved in the observed cell kill (Folkes et al., 1999).

The HRP/IAA system shows potential as an enzyme/prodrug combination, and besides GDEPT, specific HRP-targeting has been proposed using HRP-conjugated antibodies (ADEPT; Folkes et al., 1998) or polymers (PDEPT, polymer-directed enzyme/prodrug therapy; Connors et al., 1995; Folkes and Wardman, 2001). Non-specific activation of IAA in normal tissues is unlikely to take place, since in the absence of hydrogen peroxide IAA is a poor substrate for mammalian peroxidases (Kobayashi et al., 1980). Endogenous myeloperoxidase (MPO) in human promyelocytic leukaemia lymphocytes (Folkes et al., 1998) and rat white blood cells (Pires de Melo et al., 1997, 1998) was notably less efficient than HRP in converting IAA into a cytotoxic in therapeutically significant prodrug doses. MPO could oxidise IAA to the skatole radical, but the reaction was inhibited after only a few minutes (Folkes et al., 1998), probably because of the formation of non-cycling intermediates (Kettle and Winterbourn, 1996). Although a detailed clinical study has not been performed, the effect of IAA on blood glucose levels was evaluated in twelve patients with diabetes.
mellitus and six healthy volunteers (Mirsky and Diengott, 1956). After oral administration of 100 mg/kg IAA (0.57 mmol/kg), a small reduction in blood sugar concentration was observed, but no other effects were reported. In another study, eleven volunteers received between 3 and 10.5 g IAA orally, with no toxicity observed (Rysánek and Vítek, 1959).

1.4 Aim of the project

The aim of this PhD project was the development of a novel enzyme/prodrug combination for hypoxia- and radiation-mediated gene therapy.

Regions of reduced oxygenation are known to occur in solid tumours and have been observed to affect the outcome of different treatment modalities. However, since severe hypoxia is characteristic of the neoplastic microenvironment, such physiological condition may be exploited to provide a novel, selective gene therapy approach (Dachs et al., 1997). In this project the use of the HRP/IAA system in hypoxia-regulated gene therapy was investigated. Materials and methods are described in Chapter 2. The cytotoxic potential and bystander killing of HRP/IAA GDEPT was analysed \textit{in vitro} in a panel of cell lines of human origin and under different oxygenation conditions (Chapter 3). The mechanisms of HRP/IAA-induced cell death was studied in detail in one of the cell lines adopted (Chapter 4). To further improve the efficacy of this GDEPT approach, the action of ten IAA analogues in combination with HRP was evaluated under normoxic as well as extreme anoxic tumour conditions (Chapter 5). It is likely that, to be effective, gene therapy will need to be combined with conventional treatments such as radiotherapy. The effect of clinically relevant doses of radiation on HRP/IAA GDEPT was therefore studied (Chapter 6). Moreover, specific gene regulation was achieved by utilising synthetic promoters that respond to radiation as well as to hypoxia (Chapter 7). Chimeric promoters containing hypoxia regulatory elements (HREs), radiation responsive CArG elements or their combination were utilised for specific hypoxia- and/or radiation-mediated gene expression and prodrug activation.
Chapter 2
General materials and methods

2.1 Cell culture

Four established human cell lines were used in this study (Table 2.1). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma Aldrich, Gillingham, UK), 2 mM L-glutamine (Life Technologies), in a humidified incubator at 37°C and 5% CO2/air (normoxia).

Cell growth and viability were monitored by cell counting and trypan blue (Sigma Aldrich) exclusion assay. Cells were suspended in medium with trypan blue at the final concentration of 0.2%, and living cells, which exclude the dye, were counted under the microscope.

All cells utilised were negative for mycoplasma infection, as tested by using the MycoTect kit (Life Technologies).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Source/Ref</th>
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<tr>
<td>T24</td>
<td>Bladder carcinoma</td>
<td>European Collection of Cell Cultures, Salisbury, UK</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Mammary carcinoma</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>FaDu</td>
<td>Nasopharyngeal squamous</td>
<td>American Type Culture Collection, Manassas, VA</td>
</tr>
<tr>
<td></td>
<td>carcinoma</td>
<td>Ades at al., 1992</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>Dermal microvascular</td>
<td></td>
</tr>
<tr>
<td></td>
<td>endothelium</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.1. Cell lines utilised in this work.*
2.2 Hypoxic conditions

For experiments under anoxic and hypoxic conditions, cells were plated in 6 cm oxygen-impermeable dishes (Permanox, Nalge Nunc International, Loughborough, UK). Permanox contains less dissolved oxygen than ordinary plastic, such as polystyrene, perspex, polypropylene and polycarbonate. Plastic dishes retain oxygen for many hours, which can diffuse to the attached cells (Chapman et al., 1970). Glass dishes, on the other hand, are not always a good alternative, since some cell lines do not attach as well as to plastic.

Experiments under anoxic conditions were carried out at 37°C in an anaerobic glove cabinet (DON Whitley Scientific Limited, Shipley, UK) with 5% CO₂, 5% H₂, 90% N₂ and palladium catalyst. Alternatively, the cells were flushed continuously in airtight boxes with a humidified gas mixture containing 5% CO₂ and 95% N₂ (BOG Gases, London, UK), certified to contain <0.0005% O₂. The anoxia achieved in anaerobic glove cabinets was found to be more severe than that induced by gassing with CO₂ and N₂, and differences could be detected for gene regulation, drug sensitivity and other assays (Dachs et al., 1997). Glove cabinets were designed for culturing obligate anaerobic bacteria and can maintain stringent anoxic conditions. They allow manipulations such as changing media, trypsinising and plating cells. The palladium catalyst combines any incoming oxygen with H₂ to form water vapour, which is removed by using silica gel. The presence of oxygen in the cabinet was continuously monitored with the Anaerobic indicator BR55 (Oxoid Ltd., Basingstoke, UK).

Hypoxic conditions were obtained by flushing airtight perspex boxes with a humidified gas mixture of 0.1% O₂, 5% CO₂ and balance N₂ (BOC).

For all anoxic/hypoxic experiments, cultures were manipulated in the anaerobic cabinet, and plastics and fluids were pre-incubated in the cabinet for 24-48 h prior to use to remove residual oxygen.

2.3 DNA manipulations

DNA manipulations were carried out by standard procedures (Sambrook et al., 1989).
2.3.1 Enzyme reactions

DNA was digested with restriction endonucleases (Life Technologies; Promega, Southampton, UK), using the manufacturers’ recommended reaction buffers and temperatures. Multiple digests were carried out in buffers compatible with the different endonucleases used. Digestion volumes varied depending on the amount of DNA digested. In general, total volumes of 20-40 µl were composed of 10% DNA (0.1-5 µg), 1x buffer, 2-5 units of enzyme/µg DNA and distilled water (dH2O). A unit is defined as the amount of restriction enzyme necessary to digest 1 µg of DNA in 1 h.

In order to ligate incompatible restriction sites, double-stranded DNA ends were blunted by filling in with 1-5 units of DNA Pol I Large Klenow fragment (Promega)/µg DNA, the recommended reaction buffer and 0.05 mM deoxynucleotides (dNTPs), in a total volume of 20-60 µl, for 30 min at 37°C. The reaction was stopped at 75°C for 10 min. Alternatively, the DNA was blunted with 0.5-1 unit of Mung Bean endonuclease (Life Technologies)/µg DNA in 1x reaction buffer for 20 min at 30°C. The reaction was stopped by addition of gel tracking dye (next section).

To prevent self-ligation of vectors linearised with a single restriction enzyme, 5’ phosphates were removed from the DNA fragments by using calf intestinal alkaline phosphatase (CIP; Life Technologies). The linearised plasmids were incubated with 1 unit CIP/µg DNA, in a total volume of 20-50 µl, for 30 min at 37°C. The reaction was stopped at 75°C for 10 min.

Enzymes were removed with the QIAquick PCR Purification kit (QIAGen, Crawley, UK), according to manufacturer’s instructions. The protocol ensures removal of primers <10 bases, enzymes, salts and unincorporated nucleotides. 10 volumes of buffer PN were added and mixed with the reaction sample. Briefly, the mix was applied to a QIAquick column, previously placed in a 2 ml collection tube, and centrifuged for 1 min at 6000 rpm. The column was washed with 750 µl 80% EtOH/buffer (PE) and centrifuged for 1 min at 6000 rpm. An additional centrifugation for 1 min at 13000 rpm allowed complete removal of residual EtOH. The DNA was eluted by adding 60 µl elution buffer EB to the column and centrifuging.
2.3.2 Gel electrophoresis

For sizing and separation of DNA samples, a horizontal submerged agarose gel electrophoresis system was used. The concentration of agarose (Ultra Pure agarose for electrophoretic grade, Life Technologies) varied according to the size of the fragments of interest. For example, 1% gels were used for 500-10^4 bp fragments, 2% for 50-500 bp fragments, 3% for <50 bp. Gels were prepared by dissolving the agarose in 1x TAE buffer (see below), containing ethidium bromide (final concentration of 100 µg/ml; Sigma Aldrich). The molten agarose was cast in a horizontal tray fitted with well-forming combs, and, once set, was submerged in 1x TAE buffer in the flat-bed apparatus.

Prior to loading into individual wells, 1/6 total volume of gel tracking dye was added to the DNA solutions. The samples were electrophoresed at a constant voltage of 60-100 V, in parallel with an appropriate double-stranded DNA marker (bacteriophage λ digested with Pst I, Life Technologies, or 100 bp DNA ladder, Promega). DNA bands were visualised using a 254 nm transilluminator. Gels were photographed using a Polaroid MP4 Land Camera and Polaroid 667 film type (Sigma Aldrich).

Buffers and solutions:

**Tris-acetate EDTA (TAE) (50x):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>242 g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1 ml</td>
</tr>
<tr>
<td>EDTA (0.5 M, pH 8.0)</td>
<td>100 ml</td>
</tr>
<tr>
<td>Distilled water (dH₂O)</td>
<td>to 1000 ml</td>
</tr>
</tbody>
</table>

This buffer can be stored at room temperature (rt).

**Gel tracking dye (6x):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol blue</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40.0 g</td>
</tr>
<tr>
<td>EDTA (0.5 M, pH 8.0)</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 100 ml</td>
</tr>
</tbody>
</table>

It can be stored at rt.
2.3.3 Extraction of DNA from gels

DNA bands were visualised with UV light and cut from the gel using a sterile blade. They were weighed in a 1.5 ml plastic tube, dissolved in three volumes of NaI solution (BIO-101, Vista, CA) and melted at 55°C. 10-15 µl EZ-glassmilk solution (BIO-101) was added to the molten slice and incubated for 5 min at rt. After centrifugation at 13000 rpm in a microcentrifuge, the pellet was eluted with 25 µl sterile dH2O for 5-15 min at 55°C. The solution was then vortexed, centrifuged and the supernatant was transferred to a new tube and stored at -20°C.

2.3.4 Ligation of DNA fragments into plasmid vectors

A number of different plasmid vectors were used in this study. They are described with the specific manipulations in the results sections (Chapters 3-7). A general procedure for ligation is outlined here.

Both the insert and the vector were digested with their respective restriction enzymes. The DNA was electrophoresed, specific bands excised and extracted from the gel as described above. To estimate the amount of DNA extracted, the samples were run on a gel in parallel with markers of known molecular weight and concentration, and the intensities of the isolated bands compared under UV light. Dividing the estimated DNA weight by the size of the fragment, an approximation of the relative molarities of both vector and insert was derived. The following reactions were set up: vector and insert at molecular ratios of 1:2, 1:4, 1:8; vector alone and water, as negative controls.

Ligations were carried out overnight (o/n) at rt in 10 µl total volume, with 1 µl T4 DNA ligase (Life Technologies), and 2 µl 5x ligation buffer (Life Technologies).

2.4 Transformation of competent bacteria

The commercial competent cells used were E. coli Top 10 cells (Invitrogen). Their transformation efficiency is given by the manufacturer as >1.0 x10⁸ colonies/µg supercoiled plasmid DNA. Briefly, frozen cells were thawed on ice. 2-5 µl DNA (10-50 ng) was added to 35 µl cells, and incubated for 30 min on ice to allow the DNA to be taken up. The samples were incubated for 30 s at 42°C in a waterbath and then for 2 min on ice. This treatment induces the enzymes involved in the repair of DNA and other
cellular components, allowing the cells to recover from the unusual conditions of the transformation process. It also substantially increases the transformation efficiency. 60-200 µl pre-warmed SOC medium (Invitrogen) was added to each transformation mixture (without antibiotic for selection) and the cells were shaken at 200-300 rpm for 1 h at 37°C to allow expression of the plasmid. Half the content of each vial (~100 µl) was spread on LB agar (Sigma Aldrich) plates with the appropriate antibiotic for selection (30 µg/ml kanamycin, or 100 µg/ml ampicillin, both from Life Technologies) and incubated o/n at 37°C. A number of bacterial colonies were picked at random and grown in small-scale cultures (section 2.5.1) for subsequent DNA analysis.

2.5 Preparation of plasmid DNA

2.5.1 Small scale plasmid preparation

Single bacterial colonies were inoculated in 1 ml of culture broth with the appropriate antibiotic for selection and grown o/n shaking at 200-300 rpm at 37°C. Cells were harvested by centrifugation at 13000 rpm for 1 min. The pellet was resuspended in 200 µl Solution 1 (see below) and incubated for 5 min at rt. 400 µl Solution 2 was added, the sample was mixed and incubated for 5 min on ice. 300 µl ice cold Solution 3 was added, the sample was mixed and incubated for 5 min on ice. Cellular debris and denatured chromosomal DNA were pelleted by centrifugation at 13000 rpm for 10 min. 750 µl supernatant was removed to a fresh tube and an equal volume of isopropanol was added. The plasmid DNA was precipitated by centrifugation at 13000 rpm for 10 min. The supernatant was discarded and the pellet washed with 100 µl 70% EtOH. The plasmid DNA was pelleted by centrifugation at 13000 rpm for 1 min, the supernatant was discarded and the pellet was air-dried and resuspended in 10-15 µl TE with 20 µg/ml RNase A (Sigma Aldrich).
Buffers and solutions:

**Solution 1 (10x stock):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl pH 8 (1 M)</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>Glucose (20%, w/v)</td>
<td>45.5 ml</td>
</tr>
<tr>
<td>EDTA (0.5 M, pH 8.0)</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 100 ml</td>
</tr>
</tbody>
</table>

This solution can be stored at rt.

**Solution 2 (1x):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH (10 N)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>SDS (25%, w/v)</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 100 ml</td>
</tr>
</tbody>
</table>

To be prepared fresh.

**Solution 3 (1x):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-acetate</td>
<td>147.0 g</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>pH to 4.8</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 500 ml</td>
</tr>
</tbody>
</table>

It can be stored at rt.

**Tris-EDTA (TE):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>1.21 g</td>
</tr>
<tr>
<td>EDTA 0.5 M pH 7.6</td>
<td>2 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 500 ml</td>
</tr>
</tbody>
</table>

It can be stored at rt.

### 2.5.2 Large scale isolation of plasmid DNA

All the solutions were provided by the QIAfilter Plasmid Maxi kit (QIAGen).

Single bacterial colonies were inoculated into 100 ml of culture broth with the appropriate antibiotic for selection and grown o/n shaking at 200-300 rpm at 37°C. 1 ml
was removed and glycerol was added to the final concentration of 10%. The glycerol stabs were stored at -70°C for future use.

Following manufacturer’s instructions, cells were harvested by centrifugation at 5000 rpm for 5 min in polycarbonate tubes. The pellet was resuspended in 10 ml Buffer P1. 10 ml Buffer P2 was added, the sample was mixed and incubated for 5 min at rt. 10 ml ice cold Buffer P3 was added to the lysate and the sample was mixed, poured into the QIAfilter Cartridge and incubated for 10 min at rt. A QIAGEN-tip 500 was equilibrated by adding 10 ml Buffer QBT and allowing the column to empty by gravity flow. The plunger was inserted into the cartridge and the lysate filtered in the QIAGEN-tip. The lysate was allowed to enter the resin by gravity flow. The QIAGEN-tip containing DNA bound to a resin was washed twice with 30 ml Buffer QC. The plasmid DNA was eluted with 15 ml Buffer QF pre-heated at 65°C. 10.5 ml isopropanol was added and the plasmid DNA was precipitated by centrifugation at 10000 rpm for 30 min. The supernatant was discarded and the pellet washed with 5 ml 70% EtOH. The plasmid DNA was pelleted by centrifugation at 10000 rpm for 10 min, the supernatant was discarded, the pellet was air-dried and resuspended in 0.5 ml TE.

DNA concentration and purity were measured with a UV/visible spectrophotometer (Ultraspec II, LKB Biochrom, Cambridge). The concentration of double stranded DNA in ng/μl is obtained by multiplying the absorption reading at 260 nm by the scaling factor 50 and the dilution factor. The purity is assessed from the ratio of the readings at 260 and 280 nm. An optimum value is in the range 1.8-2.0. A ratio greater than 2.0 indicates RNA contamination, one less than 1.8 suggests proteins in the sample. Samples were stored at -20°C.

2.6 DNA sequencing

The sequence integrity of the plasmids was confirmed using a Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, Amersham, UK) and a Gene Readir DNA Analyzer (LI-COR, Lincoln, USA). The kit consists of four reagents each containing dNTP and ddNTP terminators of the same type and a DNA polymerase. The mix was added to a primer labelled with an infrared tag emitting at 800 nm, to allow fluorescence-based sequencing.
Primers
To sequence the promoter region of the plasmids constructed (Chapter 7), the following custom primers (purchased from MWG-Biotech) were designed, based on the regions flanking the cloning sites in the commercial vector pCI-neo (Clontech, Basingstoke, UK):
To read in the forward direction (5'→3'):
5'-AAA CGC CAG CAA CGC GGC C-3'
and in the reverse direction (3'→5'):
5'-GGT TCA CTA AAC GAG CTC TGC-3'

To determine the sequence of the fragments inserted in the vector pBluescript-KS (Stratagene, Amsterdam, The Netherlands; Chapter 7), the following primers were designed according to manufacturer's recommendations:
5'-AAT TAA CCC TCA CTA AAG GG-3' (forward)
5'-GTA ATA CGA CTC ACT ATA GGG C-3' (reverse)

Sequencing
For a 0.2 mm gel (41 cm gel system):
21 g urea
23 ml ddH₂O
4 ml formamide
8 ml Rapid gel (Amersham Pharmacia Biotech)
5 ml 10x TBE
were mixed in the above order, stirred at rt until solubilised and sonicated for 2-4 min to degas.
Before pouring the gel between the assembled glass plates, 75 ml TEMED (Sigma Aldrich) and 350 μl 10% ammonium persulphate (Sigma Aldrich) were added to the gel mixture. The gel was left to polymerise horizontally for at least 90 min.

For each dNTP, 1.5 μl terminator reagent (Amersham Pharmacia Biotech) was added to 4.5 μl master reagent mix in a Costar 96-well PCR plate. The reaction was
overlaid with 20 μl Chill-out liquid wax (MJ Research Inc, Waltham, MA). The plate was placed in a thermocycler (Mastercycler gradient, Eppendorf, Cambridge, UK) and the programme, the profile of which is specified below, was started.

At the end of the programme, 4 μl loading dye (Amersham Pharmacia Biotech) was added to each reaction. The mixtures were heated to 65°C for 2 min, and 1 μl was loaded to each lane of the sequencing gel. The reactions can be stored at -20°C for a maximum of 24 h after addition of the loading dye.

The samples were electrophoresed o/n at a constant voltage of 1800 V. The sequence was analysed using the BASEIMAGEIR v4.0 software (LI-COR).

Programme:
95°C for 1 min
15 cycles of:
  95°C for 10 s
  Tm (melting temperature of the primer)-3°C for 30 s
  65°C for 30 s
15 cycles of:
  95°C for 10 s
  70°C for 30 s
hold at 15°C.

Buffers and solutions:
Tris-borate-EDTA (TBE, 10x)
Tris-base 107.8 g
Boric Acid 27.5 g
EDTA 9.3 g
dH2O to 1 l
The pH should be between 8.3 and 8.7.
Sequencing master reagent mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1.4 µl</td>
</tr>
<tr>
<td>Primer</td>
<td>1.5 pmole</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>130 ng/kbp</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>to 21 µl</td>
</tr>
</tbody>
</table>

2.7 Eukaryotic cell transfection

A variable fraction of cells incorporate the exogenous DNA after transfection (transfection efficiency), which depends on the cell type and the method adopted. Transient transfectants can express the foreign gene for 1-7 days, but during replication the exogenous DNA is lost or integrated into their chromosomal DNA at one or more random locations. By adding a selectable marker (e.g., puromycin or neomycin) to the DNA vector, it is possible to identify stably transfected clones. These can be isolated and assayed for gene expression over many generations of cell divisions.

A number of non-viral methods have been developed for eukaryotic transfection (see section 1.2.1), including calcium phosphate precipitation, electroporation and cationic vesicles. In this study two types of cationic vesicles were used, Lipofectin coupled with integrin-targeted peptides (Hart et al., 1998), or Lipofectamine.

2.7.1 Lipofectin and integrin-targeted peptides

Prior to use, the integrin-targeted peptides (sequence K₁₆GACRRETAWACG, Institute of Child Health, London, UK; Hart et al., 1998) were cyclised o/n at 4°C at 0.1 mg/ml in OptiMEM (Life Technologies) and subsequently filter-sterilised and stored at -20°C.

24 h prior to transfection, exponentially growing mammalian cells were harvested, counted and plated in normoxia at 1.5 x 10⁵ per 6 cm dish (4-18 x 10³ cells/cm²).

The transfection mix was freshly prepared, containing a ratio of 0.75:1.8:1 by weight of Lipofectin (Life Technologies), peptides and plasmid DNA:

1.5 µl Lipofectin diluted in 200 µl OptiMEM
3.6 µg peptide, at 0.1 µg/ml in OptiMEM
2 µg DNA in 200 µl OptiMEM.
The components were mixed in the above order and left to form complexes for 2 h at rt. Prior to adding the mixture to the cells, OptiMEM was added to a total volume of 1 ml.

The culture medium was removed and the cells were washed with excess PBS, followed by a wash in OptiMEM. 1 ml of transfection mix was added per dish and the cells were incubated for 5 h or o/n at 37°C in a humidified incubator. The transfection mix was removed and replaced with complete DMEM.

Transient transfectants were assayed for gene expression 24-48 h after transfection.

To obtain stable transfectant lines, clones were selected over two weeks in complete medium containing the neomycin analogue G418 (Life Technologies) at a concentration of 0.5 mg/ml active drug. Separate clones were picked using a fine pipette into 24-well culture dishes, and cultures expanded in the presence of the selecting agent. Transgene expression was tested by using the appropriate assays.

### 2.7.2 Lipofectamine

Transfections were carried out as described above. The transfection mix in this case was:

- 10 µl Lipofectamine (Life Technologies) diluted in 45 µl OptiMEM
- 5 µg DNA in 45 µl OptiMEM.

The components were left to form complexes for 30-45 min at rt, and, prior to adding the mixture to the cells, 1 ml OptiMEM was added. The cells were exposed to the transfection mix for 5-7 h at 37°C in the humidified incubator.

### 2.8 Detection of enhanced green fluorescent protein by flow cytometry

The enhanced green fluorescent protein (EGFP) is widely used as a marker for transfection efficiency and gene expression, although care should be taken when using it as a reporter under certain tumour conditions (Coralli et al., 2001). For gene regulation studies, conclusions about the rate of transcription are drawn by analysing the strength of the fluorescence signal. Work conducted in our group has shown that EGFP fluorescence was absent under anoxia and significantly lower under severe hypoxic
conditions (0.02% O₂), when compared to normoxic conditions (21% O₂). After 5-10 h of reoxygenation a full recovery of the fluorescence signal was observed. These findings suggest that it may be problematic to analyse EGFP production under complete anoxia and that, after severe hypoxia, it is necessary to incubate the cells in normoxia for 5-10 h prior to fluorescence analysis.

Transfectants containing EGFP were harvested using trypsin/EDTA, washed in PBS and resuspended in phenol red-free Hanks’ balanced salt solution (HBSS, Life Technologies) at 5-10 x10⁵ cells/ml. Relative fluorescence was detected by fluorescence activated cell sorting (FACS) analysis on a Becton Dickinson FACScan. As single cells pass through a laser beam, they scatter light and emit fluorescence, which varies depending on the physical characteristics of the cells. EGFP has the following spectral characteristics: excitation maximum 488 nm, emission maximum 507 nm, and could be detected by the FL-1 detector at the FACScan utilised. Settings were left unchanged during all of the readings in individual experiments.

The data were analysed using dedicated software (CELLQuest for Apple Macintosh, Becton Dickinson, Franklin Lakes, NJ). Fluorescence was normalised to that of cells transfected with a control plasmid while gating against cell debris using forward vs. side scatter (FCS vs. SSC). Cells were scored as positive if they showed an increase in fluorescence with respect to the control. The fraction of cells expressing EGFP was calculated by subtracting the control from the sample test profile.

2.9 Immunofluorescence staining for HRP
1-2 x10⁵ cells were plated on a coverslip in a 6 cm dish. The cells were fixed in 3% paraformaldehyde/PBS for 20 min at rt, rinsed with PBS and incubated in 15 mM glycine/PBS for 20 min at rt. Non-specific binding was blocked by two washes in wash buffer performed over a period of 20 min. The primary antibody (rabbit polyclonal anti-HRP; Dako, Ely, UK) was diluted 1:200 in 10% FCS/wash buffer, centrifuged for 10 min at 13000 rpm and incubated with the cell monolayers in a humidified chamber for 1 h at rt. Following extensive rinsing in wash buffer (at least six washes of 10 min each), the cells were exposed to the secondary antibody (TRITC-conjugated swine anti-rabbit immunoglobulins; Dako), diluted 1:200, as above. The six washes were repeated,
followed by two rinses in PBS over 10 min. The coverslips were mounted on a microscope slide using Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA) and viewed using an Olympus BH-2 fluorescence microscope.

Alternatively, antibody-labelling was performed on cell suspensions, and was followed by FACS-analysis.

Buffers and solutions:

Wash buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Tween 20 (Sigma Aldrich)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>PBS</td>
<td>to 100 ml</td>
</tr>
</tbody>
</table>

To be prepared fresh.

2.10 HRP activity assay

Cells in exponential growth were harvested by trypsinisation and resuspended in 0.5% hexadecyltrimethylammonium bromide (Sigma Aldrich). The cells were lysed by freeze/thawing three times in liquid nitrogen/37°C and by centrifugation in a microcentrifuge at 13000 rpm for 15 min. Supernatants were stored at -20°C.

Enzyme reactions were carried out at rt by diluting 40 μl supernatant in 80 mM phosphate buffer (pH 5.4), with 20 μl of 88 mM 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB, Sigma Aldrich) in 10% DMSO (Sigma Aldrich) and 3.0 mM H$_2$O$_2$, in a total volume of 2 ml. The absorbance was read at rt on a Hewlett Packard model 8452A diode array spectrophotometer. At 652 nm the absorbance spectrum (Figure 2.1.A) presented a peak that depends on HRP concentration (Bos et al., 1981). The absorbance at 652 nm (A652) was measured over 30 min at 1 min intervals. In the first 10 min, the absorbance increased linearly with time (Figure 2.1.B), and the rate was proportional to the amount of HRP in the samples, as assessed in calibration curves using purified HRP enzyme (Figure 2.1.C). Samples were kept on ice during the assay.

The total cellular protein content in the samples was determined by using a commercial protein assay kit (Bio-rad, Hemel Hempstead, UK) against a standard protein curve. The HRP activity was expressed as units of enzyme per μg of total
cellular protein in the sample. A unit is defined as the amount of enzyme that produces an increase of one A652 unit per minute.

**Figure 2.1. Detection of HRP activity.**

In the presence of HRP and H$_2$O$_2$, the absorption spectrum of the oxidation product of the substrate TMB showed two peaks, at 370 and 652 nm (A). The absorbance at 652 nm (A652) was followed for 30 min using different concentrations of purified HRP (B). In the first 10 min a linear increase of A652 was observed. The rate of increase depended linearly on enzyme concentration (C).
Buffers and solutions:

**Phosphate buffers:**

50 mM PO₄ buffer in sterile H₂O, from 100 mM stock (Sigma Aldrich). Adjust pH to 6.0.

80 mM PO₄ buffer in sterile H₂O, from 100 mM stock. Adjust pH to 5.4.

Filter sterilise. These buffers can be stored at 4°C.

**0.5% Hexadecyltrimethylammonium Bromide:**

Hexadecyltrimethylammonium Bromide (Sigma Aldrich) 100 mg

50 mM PO₄ buffer, pH 6 20 ml.

This solution can be stored at -20°C.

### 2.11 Western blot

For Western blot analysis, cell monolayers were washed in ice-cold PBS and lysed in 100 µl ice-cold lysis buffer. Equal quantities of proteins (about 20 µg) from such whole cell extracts were suspended in sample buffer, denatured at 95°C for 5 min, loaded into the wells of pre-cast polyacrylamide gels (Invitrogen-Novex, Groningen, Netherlands) in 1x running buffer (National Diagnostics, Hessle, UK) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) at a constant voltage of 125 V.

Proteins in the gel were transferred to nitrocellulose membranes (Genetic Research Instrumentation Ltd, Rayne, UK) by using an LKB-Pharmacia-Biotech semi-dry blotter, for 1 h at 0.8 mA/cm² membrane surface area.

The membrane was rinsed in 0.1% Tween 20/PBS and incubated for 5-10 h in blocking solution at 4°C. After washing in Tween 20/PBS, immunoblotting was performed with primary antibodies for 1 h at rt or o/n at 4°C, on a shaker. The membrane was rinsed four times in Tween 20/PBS over 20 min and incubated with secondary HRP-conjugated immunoglobulins shaking for 1 h at rt. After four washes over 30 min, immunoreactive bands were detected by using the enhanced chemiluminescence technique (ECL kit, Amersham Pharmacia Biotech), according to manufacturer’s instructions. The membrane was exposed to a diographic film for 3-60
The total protein content on the gel was visualised by staining at rt with a Coomassie blue stain solution for 2 h, and by removing excess staining solution o/n.

Buffers and solutions:

**Lysis buffer:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
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<td>Tris-HCl, pH 7.5</td>
<td>50 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>100 mM</td>
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<tr>
<td>Triton-X</td>
<td>1%</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
</tr>
<tr>
<td>Protease inhibitor cocktail (Sigma Aldrich)</td>
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**Sample buffer:**

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<tr>
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<td>0.05% bromophenol blue</td>
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**Membrane Blocking solution:**

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<tr>
<td>Dry-powdered milk</td>
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<tr>
<td>PBS</td>
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Antibody solution:

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</thead>
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</tr>
<tr>
<td>BSA</td>
<td>1 g</td>
</tr>
<tr>
<td>PBS</td>
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Coomassie blue stain:

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<tr>
<td>Acetic acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>Coomassie blue</td>
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</tr>
<tr>
<td>dH₂O</td>
<td>to 100 ml</td>
</tr>
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</table>

Filter the solution.

Destain solution:

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</tr>
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<tbody>
<tr>
<td>Methanol</td>
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<tr>
<td>Acetic acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 100 ml</td>
</tr>
</tbody>
</table>

2.12 Irradiation procedure

Cells were irradiated using a Pantak IV X-ray generator, operated at 240 kV (HVL 1.3 mm Cu), at a dose rate of 1.6 Gy/min.

2.13 Clonogenic assay

Exponentially growing cells were harvested, plated at low density and allowed to adhere for 4-6 h. The cells were exposed to the drugs in HBSS in the 37°C incubator.

For experiments under anoxia/hypoxia, cells were preplated in the anaerobic cabinet and, after incubation for 5-6 h to ensure anoxic/hypoxic conditions, were exposed to the prodrugs in the cabinet or in perspex boxes flushed continuously with 0.1% O₂.

Following drug exposure, the cells were rinsed with PBS and grown for 8-15 days in complete DMEM supplemented with feeder cells (V79 cells exposed to 250 Gy ⁶⁰Co
irradiation). After fixation and staining with 2.5% crystal violet (Sigma Aldrich) w/v in isomethylated spirit (IMS), colonies of >50 cells were scored.

2.14 MTS proliferation assay

This assay consists of a colorimetric method for determining the number of viable cells after drug exposure. The CellTiter 96™ AQueous Assay (Promega) kit consists of 3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent (phenazine methosulfate; PMS). MTS is bioreduced by dehydrogenase enzymes in metabolically active cells into a formazan that is soluble in the tissue culture medium. The amount of formazan formed, which is proportional to the number of living cells in culture, can be measured directly using a multiwell spectrophotometer at 492 nm, without additional processing.

The MTS assay was performed according to manufacturer's instructions. Briefly, exponentially growing cells were plated in 96-well plates (2000 cells/well; 8 wells/drug concentration) and exposed to the prodrugs. After drug exposure, the cells were grown until the control wells (exposed to HBSS only) reached confluence. The culture medium was supplemented with MTS and PMS, at the final concentrations of 166 µg/ml and 12.5 µM, respectively. The reaction was left to take place for 2 h at 37°C in the dark, the plates were agitated to ensure complete mixing and scanned on a multiplate reader (Labsystems Multiskan MCC/340) at 492 nm. Cell growth was evaluated relative to HBSS-treated controls.

Buffers and solutions:

**MTS stock:**

<table>
<thead>
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<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTS</td>
<td>42 mg</td>
</tr>
<tr>
<td>PBS</td>
<td>21 ml</td>
</tr>
</tbody>
</table>

Stir for 15 min or until MTS is completely dissolved. Adjust pH to 6.5 and filter sterilise. The stock can be stored at -20°C protected from light.
PMS stock:
- PMS 9.2 mg
- PBS 10 ml
Adjust pH to 6.5 and filter sterilise. The stock can be stored at -20°C protected from light.

2.15 Statistical analysis
Significance tests were carried out on the data groups by using analysis of variance (ANOVA) followed by pair-wise comparison between specific groups (Student t-test). Linear least square regressions and correlation analysis were performed by using a fitting package (JMP, SAS Institute Inc., Marlow, UK). Values of p<0.05 were considered as significant.
Chapter 3

Horseradish peroxidase/indole-3-acetic acid: a novel enzyme/prodrug combination for gene therapy of cancer

3.1 Introduction

GDEPT is a two-phase suicide gene therapy. Firstly, a gene encoding a foreign enzyme is delivered; secondly, a prodrug is administered and selectively converted into a cytotoxin by the enzyme synthesised at the target.

In the choice of the appropriate enzyme/prodrug combination for cancer treatment a number of issues have to be taken into account (section 1.2.5). Since 1986, when Moolten introduced the concept of suicide genes, several different GDEPT systems have been proposed, but none seem to fulfil all the required criteria (Connors, 1995; Knox, 1999). Even the HSV TK/GCV and the \textit{E. coli} CD/5-FC combinations, currently adopted in clinical trials, present a number of drawbacks. For example, since activated GCV and 5-FC interfere with DNA synthesis, they are not particularly suitable for the eradication of the slowly dividing hypoxic population in solid tumours. Although tumour cells transfected with a hypoxia-induced CD-encoding gene could be sensitised to 5-FC during subsequent drug exposure in air (Dachs et al., 1997), no cell kill could be detected when \textit{CD}-expressing cells were treated under anoxia (G.U. Dachs, unpublished observation). The potential of other newly developed combinations, such as CPG2/CMDA and CYP/CP to successfully eradicate radioresistant hypoxic tumour cells is yet to be reported. There is therefore scope for a novel GDEPT system, effective against normoxic as well as hypoxic cells.

The enzyme HRP and the plant hormone IAA represent a novel enzyme/prodrug combination for anti-cancer strategies (section 1.3). When activated by purified HRP, IAA inhibited colony formation in mammalian cells, while neither enzyme nor prodrug alone were cytotoxic at the same concentrations or duration of treatment (Folkes et al., 1998, 1999). Binding to DNA was observed in a cell-free system, indicating that cell proliferation may not be required for the induced toxicity (Folkes et al., 1999).
The experiments presented in this chapter aimed to determine the potential of utilising the HRP/IAA system for GDEPT. Three human tumour cell lines and one endothelial cell line were transfected in vitro with plasmid vectors containing HRP coding sequences, and their sensitisation to IAA was evaluated under normoxic and anoxic conditions. The ability of the HRP/IAA combination to induce a bystander effect was also studied.

3.2 Materials and methods

Further details on general methods and materials can be found in Chapter 2. Experimental methods specific to this chapter are described below.

3.2.1 Hypoxic conditions

For experiments under anoxic or hypoxic conditions, cells were incubated at 37°C in the anaerobic glove cabinet (anoxia), or in airtight perspex boxes flushed continuously with a humidified gas mixture containing 0.1% O₂ (hypoxia).

3.2.2 Plasmid DNA and cell transfection

The plasmids pRK34-HRP (containing the HRP cDNA; Figure 3.1.A; Connolly et al., 1994), pTK (bearing the HSV TK gene; Marples et al., 2000) and pCMV-CD4 (containing the gene for the marker CD4; Dachs et al., 2000) were kindly provided by Dr. D.F. Cutler (UCL), Dr. S.D. Scott (Gray Cancer Institute/Karmanos Cancer Institute) and Dr. G.U. Dachs, respectively.

To produce pEGFP-HRP (containing the HRP synthetic gene; Figure 3.1.B), pEGFP-N1 (Clontech, Basingstoke, UK) was linearised with Ssp BI, the termini blunted using Mung Bean endonuclease and then digested with Xba I (all enzymes from Life Technologies). The HRP synthetic gene (Smith et al., 1990) was excised from pBGS19-HRP (from Prof. A.T. Smith, University of Sussex, Brighton) by Hpa I/Xba I digestion and the resulting fragment ligated in frame to the 3’ end of the EGFP coding sequence. The sequence integrity of the construct was confirmed by automated sequencing (Cambridge Bioscience, Cambridge, UK).

The construct pEGFP-neo was made as follows, to contain an identical vector
backbone as pTK. The EGFP coding sequence was excised from pEGFP-N1 by *EcoR* I/*Not I* digestion and the resulting fragment inserted in the commercial vector pCI-neo (Promega, Southampton, UK), linearised with the same restriction enzymes.

In all plasmids, gene expression was controlled by the Cytomegalovirus (CMV) immediate early gene promoter.

![Figure 3.1. HRP-containing plasmids utilised in this chapter.](image)

A. The pRK34-HRP construct contained the HRP cDNA fused to the signal sequence from the human growth hormone (hGH) and the KDEL retention motif (Connolly et al., 1994).

B. The HRP synthetic gene was inserted at the C-terminus of the enhanced green fluorescent protein (EGFP) in the commercial plasmid pEGFP-N1.

Transient transfectants were obtained by exposing the cells to complexes of Lipofectin, integrin-targeted peptides and DNA (section 2.7.1), and were assayed for gene expression after 24 h. Transfection efficiency with the plasmid pRK34-HRP was analysed by immunofluorescence analysis (section 2.9). Transfection efficiency with the construct pTK was estimated by measuring the production of the marker protein EGFP in cells transfected under the same conditions with pEGFP-neo (section 2.8). Stable transfectants were isolated as described in section 2.7.1.
3.2.3 Detection of HRP expression

Procedures were carried out as described in Chapter 2. HRP activity was analysed using a TMB assay, and expressed as units of enzyme per μg of total cellular protein. For Western blots, whole cell extracts were subjected to SDS-PAGE using 12% polyacrylamide gels. Proteins in the gel were transferred to nitrocellulose membranes, and immunoblotting was performed with primary rabbit polyclonal anti-HRP antibodies (Dako) diluted 1:1000, and secondary HRP-conjugated goat anti-rabbit immunoglobulins (Dako), diluted 1:2000.

3.2.4 Clonogenic assays

Exponentially growing cells were plated at low density on Petri dishes and exposed to IAA (Sigma Aldrich) or GCV (from Dr. E. Littler, GlaxoWellcome, Stevenage, UK) in HBSS for 2 h or 24 h.

To evaluate the cytotoxicity of IAA activated by exogenous HRP, 1.2 μg/ml purified HRP enzyme (Sigma Aldrich) was added to untransfected cells incubated with IAA.

For experiments under anoxia/hypoxia, cells were pre-plated in the anaerobic cabinet and, after incubation for 5-6 h in the cabinet or under 0.1% O₂ to ensure anoxic/hypoxic conditions, they were exposed to the prodrugs.

To measure the bystander effect, cells transiently transfected with pRK34-HRP (HRP⁺) or pCMV-CD4 (HRP⁺) were mixed in different proportions and exposed to IAA for 24 h. After drug exposure, cells in the media and attached to the dishes were collected, counted using a haemocytometer and re-plated at low density for clonogenic survival.

In conditioned medium-switch experiments, HRP⁺ cells were exposed to HBSS containing a range of IAA concentrations for 2 h. This medium was then transferred to pre-plated HRP⁺ cells for a subsequent 2 h-incubation.

Surviving fractions were evaluated relative to HBSS-treated controls. The concentration of prodrug required to reduce cell survival by 50% (IC₅₀) was estimated from the survival curves.
3.3 Results

3.3.1 Cytotoxicity of IAA activated by purified HRP in human cells

To evaluate the cytotoxic potential of IAA activated by exogenous HRP in human cells, untransfected human bladder carcinoma T24 cells were exposed for time intervals from 15 min to 2 h to 0.1 mM IAA and 1.2 μg/ml purified HRP. Clonogenic assays showed a decrease in surviving fraction with increasing exposure time (Figure 3.2). The cells were also exposed to enzyme and prodrug independently and no significant cytotoxic effects were observed at the concentration/time indicated (Figure 3.2).

![Figure 3.2. Cytotoxicity of IAA after activation by purified HRP. T24 cells were incubated with IAA (0.1 mM) and purified HRP enzyme (1.2 μg/ml) for time intervals up to 2 h. The data are means of three independent clonogenic assay experiments (triplicate samples). Error bars are ± standard error (SE). ○: IAA; □: HRP; ▲: HRP + IAA.](image-url)
3.3.2 Transfection of human cells with HRP genes: HRP synthetic gene

For a gene therapy approach to be effective, selective cell kill should be observed when the prodrug is activated by the intracellularly produced enzyme. Two plasmid constructs containing either a synthetic HRP gene (pEGFP-HRP) or the HRP cDNA (pRK34-HRP) were used to test this strategy (Figure 3.1).

The HRP synthetic gene had been previously designed by Smith and co-workers (1990) to achieve high expression in *E. coli* and mammalian cells. However, studies in our laboratory showed that when the HRP synthetic gene was expressed in mammalian cells under the control of the CMV promoter in the construct pBGS19-HRP no peroxidase activity could be detected (G.U. Dachs, personal communication). Correct folding of the protein might have been impaired (Hartmann and Ortiz de Montellano, 1992; D.F. Cutler, personal communication), and extensions at either termini of the HRP gene required (Connolly et al., 1994; Stinchcombe et al., 1995; Norcott et al., 1996). Therefore, to stabilise the HRP and allow direct and easy detection of the protein in transfected cells, the synthetic gene was inserted at the C-terminus of EGFP in the plasmid pEGFP-N1. A correct in-frame fusion of EGFP and HRP coding regions was obtained in the construct pEGFP-HRP (Figure 3.1.B), as confirmed by sequence analysis. To demonstrate that the EGFP-HRP fusion protein was produced and retained the characteristics of the two components, T24 cells were transfected with pEGFP-HRP and stable lines were obtained by selecting clones with the neomycin analogue G418. Fluorescence microscopy and FACS analysis confirmed that the fluorescent properties of EGFP were preserved, albeit with an emission intensity for EGFP-HRP ~2 orders of magnitude lower than for EGFP alone (Figure 3.3.A). FACS analysis also showed that only 57 (±3)% of the antibiotic-selected population produced the fusion protein (Figure 3.3.A). HRP production was detected by Western blot analysis, enzyme activity assay and immunostaining (Figure 3.3.B, C, D). All assays confirmed that low levels of EGFP-HRP protein were synthesised in the stable line. This was not exclusive to the specific stable clone, since low EGFP-HRP production was also detected in transient pEGFP-HRP-transfectants and other stable EGFP-HRP lines (results not shown). When the EGFP-HRP-transfectants were exposed for 2 h to a range of IAA concentrations,
clonogenic assays revealed an increase in cellular sensitivity to the prodrug compared to EGFP-expressing controls (Figure 3.4). These results were encouraging, as they indicated that the prodrug could be activated to the toxic drug even at low levels of HRP. However, in order to analyse the effects and mechanisms of HRP-mediated GDEPT, more significant levels of protein synthesis and cell kill would have been preferable. We therefore tested the plasmid pRK34-HRP containing the HRP cDNA, which could be highly expressed in mammalian cells (Connolly et al., 1994). Moreover, transient rather than stable transfectants were used, since transient expression and lack of integration of the therapeutic gene are more likely to take place in vivo with current delivery systems (section 1.2.1). All subsequent experiments were therefore carried out using transiently transfected cells.
Figure 3.3. Production of the EGFP-HRP fusion protein in stably transfected T24 cells.

A. FACS profile (EGFP expression) of stable EGFP-HRP-transfectants (solid histogram), EGFP-transfectants (-), and mock-transfected controls (-).

B. Western blot of HRP protein extracts of stable EGFP- (lane 1) and EGFP-HRP-transfectants (lane 2).

C. Peroxidase activity detected in EGFP- and EGFP-HRP-transfectants using a TMB enzyme assay. The means of three independent experiments (triplicate samples) ± SE are shown.

D. HRP immunostaining of EGFP-HRP-stable transfectants. The cells were labelled with primary anti-HRP and secondary TRITC-conjugated antibodies. The slides were viewed on an Olympus BH-2 fluorescence microscope (300x magnification).
3.3.3 Transfection of human cells with HRP genes: HRP cDNA

To demonstrate feasibility of HRP-mediated GDEPT, four cell lines of human origin (T24 bladder carcinoma, MCF-7 breast adenocarcinoma, FaDu nasopharyngeal squamous carcinoma and HMEC-1 microvascular endothelial cells; Table 2.1) were transiently transfected with the pRK34-HRP construct (Figure 3.1.A). In this plasmid the HRP cDNA had previously been fused to the signal sequence from the human growth hormone (hGH) and the KDEL retention motif (Connolly et al., 1994). The KDEL-tag causes accumulation of the HRP in the endoplasmic reticulum and the nuclear envelope (Figure 3.5), preventing secretion of the enzyme. This may be an advantage in vivo, where non-specific prodrug activation at sites distant from the tumour would be undesirable.
Figure 3.5. Immunodetection of HRP in T24 cells transfected with the plasmid pRK34-HRP. Transient transfectants immunostained for HRP were examined on an Olympus BH-2 fluorescence microscope (300x magnification). The KDEL tetrapeptide fused to the C-terminus of the HRP cDNA causes intracellular accumulation in the endoplasmic reticulum and the nuclear envelope.

As assessed by immunostaining and FACS analysis, the transfection efficiencies were 20-25% in T24, 16-20% in MCF-7, 10-14% in FaDu and 18-20% in HMEC-1 cells (Table 3.1). As expected, untransfected cells and mock-transfected (HRP') cells did not stain positive for HRP (results not shown). Synthesis of an immunoreactive 52 kDa protein and competent peroxidase activity were confirmed in all HRP+ cells by Western blotting (Figure 3.6) and enzyme assay (Table 3.1). In T24, MCF-7 and FaDu cells HRP activity was $3.6\pm0.9$, $1.1\pm0.6$ and $0.8\pm0.2 \times 10^{-5}$ units/μg total protein, respectively. Compared with the other lines, a higher peroxidase activity was detected in HMEC-1 cells ($17\pm3 \times 10^{-5}$ units/μg total protein), which may indicate an increased number of HRP-containing plasmids per cell, compared with the other lines. No detectable HRP protein production or catalyst activity could be measured in HRP' cells (data not shown). Expression of HRP did not affect the phenotype of the transfectants, as judged by plating efficiency, growth rate or morphology (results not shown).
Table 3.1. Expression of HRP and sensitivity to IAA in human cell lines.
The transfection efficiency was determined by immunolabelling followed by FACS analysis; HRP activity was detected with a TMB assay. The selectivity index was expressed as the ratio of IAA concentration required to kill 50% (IC50) of HRP+ to HRP- cells (clonogenic survival assay, except *: MTS assay).

<table>
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<tr>
<th>Cell line</th>
<th>Transfection efficiency (%)</th>
<th>HRP activity (10^5 units/μg total protein)</th>
<th>IC50 (mM) HRP-</th>
<th>IC50 (mM) HRP+</th>
<th>Selectivity index (SI)</th>
</tr>
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<td>T24</td>
<td>20-25</td>
<td>3.6±0.9</td>
<td>1.8</td>
<td>0.05</td>
<td>36</td>
</tr>
<tr>
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<td></td>
<td>&gt;4*</td>
<td>1*</td>
<td>&gt;4*</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>16-20</td>
<td>1.1±0.6</td>
<td>0.85</td>
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<td>85</td>
</tr>
<tr>
<td>FaDu</td>
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<td>0.8±0.2</td>
<td>3.3</td>
<td>0.06</td>
<td>55</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>18-20</td>
<td>17±3</td>
<td>2.9*</td>
<td>0.96*</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 3.6. Production of HRP protein in transfected cells of human origin.
Western blot of extracts of HRP (lane 2) and of HRP+ T24 cells (lane 3); MCF-7 cells (lanes 4 and 5); FaDu cells (lanes 6 and 7); HMEC-1 cells (lanes 8 and 9). A protein marker is in lane 1.

3.3.4 In vitro efficacy of HRP/IAA GDEPT
Susceptibility of HRP-expressing T24, MCF-7 and FaDu cells to prodrug treatment was assessed by exposing the cells to increasing concentrations of IAA (Figure 3.7). After only 2 h-exposure, significant cell kill was induced in HRP-transfectants, while no toxicity was observed in HRP+ cells (Figure 3.7.A). At non-toxic doses of IAA, a 1-2
log cell kill was induced in HRP\(^+\) cells, even though less than 30% of the transient transfectants were shown to synthesise the foreign enzyme.

Above 4 mM IAA a rapid and dramatic decrease in survival was observed in both the HRP\(^-\) and HRP\(^+\) populations (data not shown). This effect was due to the acidification of the IAA-containing medium, since if the pH was adjusted to 7.4 no toxicity was detected in HRP\(^-\) cells exposed for 2 h to up to 20 mM IAA (results not shown). Doses above 4 mM were therefore not included in subsequent survival curves.

A higher efficacy of the HRP/IAA combination was observed after prolonged (24 h) incubation (Figure 3.7.C). In all the cell lines studied, expression of the HRP enzyme significantly enhanced the sensitivity to IAA, with levels of cell kill up to 4-5 log. Some toxicity was also observed in HRP\(^-\) cells at concentrations ≥1 mM (45% (T24), 24% (FaDu), 54% (MCF-7) cell kill after 1 mM IAA). Doses that reduced the surviving fraction to 50% (IC\(_{50}\)) for HRP\(^+\) cells were compared to the corresponding values for the HRP\(^-\) populations, and the ratio of IC\(_{50}\) for HRP\(^-\) cells to IC\(_{50}\) for HRP\(^+\) cells was defined as selectivity index (SI; Table 3.1). After 24 h incubation, SIs of 36, 55 and 85 were measured in T24, MCF-7 and FaDu cells, respectively. Inspection of the survival curves up to 1 mM IAA revealed no major differences in the response of these three tumour lines to HRP/IAA, although MCF-7 cells may be slightly more sensitive following both 2 h- and 24 h-exposure (Figure 3.7.A, C).

After 2 h, anoxic incubation did not significantly reduce the efficacy of the system, and, after 24 h, a 50-fold increase in cytotoxicity was estimated for HRP\(^+\) compared to HRP\(^-\) T24 cells (Figure 3.7.B, D). The shape of the anoxic survival curves and the IAA activation pathway (Dunford, 1999; Candeias et al., 1994) suggest that, compared to normoxia, different toxic metabolites may be involved in anoxic cell death, as discussed later (section 3.4).
Figure 3.7. Toxicity of the HRP/IAA combination in human tumour cells.

HRP\textsuperscript{*} and HRP\textsuperscript{+} cells were exposed to increasing concentrations of the prodrug IAA under normoxia (A, C) or anoxia (B, D) for 2 h (A, B) or 24 h (C, D).

The data are means of at least three independent clonogenic assay experiments (duplicate samples). Error bars are ± SE. The lines are interpolated. △: HRP\textsuperscript{*} T24 cells; ▲: HRP\textsuperscript{+} T24 cells; □: HRP\textsuperscript{*} MCF-7 cells; ■: HRP\textsuperscript{+} MCF-7 cells; ◊: HRP\textsuperscript{*} FaDu cells; ♦: HRP\textsuperscript{+} FaDu cells.
The potential of the HRP/IAA combination to target the tumour vasculature was evaluated in the HMEC-1 endothelial cell line. The growth of HMEC-1 cells was monitored using the MTS assay (section 2.14; Figure 3.8.A), since these cells do not form discrete colonies. For comparative purposes, the growth of T24 cells was analysed under the same experimental conditions (Figure 3.8.B). After 24 h IAA-treatment, inhibition of proliferation was detected in HRP⁺ HMEC-1 cells, following a dose response very similar to that of T24 HRP-transfectants (Figure 3.8). However, compared to HRP⁺ T24 cells, HRP⁺ HMEC-1 cells appeared more sensitive to IAA at concentrations >1 mM, resulting in a decrease in selectivity (SI (HMEC-1) = 3; SI (T24) >4, using the MTS assay; Table 3.1). This effect did not appear to depend on endogenous peroxidase levels, as assessed by TMB assay in HRP⁺ T24 and HMEC-1 cells (data not shown).

**Figure 3.8.** Effect of HRP/IAA in endothelial and bladder carcinoma cells.

Growth inhibition (MTS assay) of HRP⁺ and HRP⁻ human microvascular endothelial HMEC-1 (A) and T24 bladder carcinoma (B) cells after 24 h normoxic exposure to IAA. ○: HRP⁺ HMEC-1 cells; ●: HRP⁻ HMEC-1 cells; △: HRP⁺ T24 cells; ▲: HRP⁻ T24 cells. The means of three independent experiments ± SE are shown. The lines are interpolated.
3.3.5 Bystander effect of the HRP/IAA system

The bystander effect is defined here as the ability of HRP-expressing cells to kill the neighbouring ones in the presence of the prodrug IAA. Bystander killing is crucial for a successful GDEPT strategy, since, with the protocols currently adopted in clinical trials, the \textit{in vivo} transfection efficiency is still low (section 1.2.1).

The bystander effect induced \textit{in vitro} by the HRP/IAA system was examined in T24 cells. Populations of HRP\textsuperscript{−} and HRP\textsuperscript{+} cells mixed in varying proportions were exposed for 24 h to 0.5 mM IAA, which had little or no effect on cell survival in the mock-transfected population (less than one-half the IC\textsubscript{50} in HRP\textsuperscript{−} T24 cells; Table 3.1). The percentage of HRP\textsuperscript{+} cells was assessed by immunostaining. Figure 3.9.A shows that the IAA prodrug was able to induce significant bystander killing under normoxic as well as anoxic conditions. 70-80\% cell kill was achieved when only 5\% of the population expressed HRP. Killing levels of \~95\% under normoxia and \~99\% under anoxia were achieved when 20-25\% of the cells were transfected with the HRP cDNA, which was the maximum transfection efficiency achievable in these experiments (Figure 3.9.A). Under normoxia, this effect was not dependent on contact between HRP\textsuperscript{+} and HRP\textsuperscript{−} populations, since HRP\textsuperscript{−} cells were killed when exposed to IAA pre-activated by HRP\textsuperscript{+} cells (medium-switch; Figure 3.9.B). No such transferability was seen under anoxia (Figure 3.9.D), indicating that the anoxic toxicity is likely to be due to short-lived reactive species. However, as the oxygen concentration was raised to 0.1\%, transfer of the toxic product could be once again detected (Figure 3.9.C).
Figure 3.9. Bystander effect of the HRP/IAA combination in T24 cells.

A. HRP<sup>+</sup> and HRP<sup>+</sup> T24 cells were mixed in various proportions and treated with 0.5 mM IAA for 24 h under normoxia (●) or anoxia (○).

B. Conditioned medium-switch experiments were performed by transferring IAA-containing media pre-incubated for 2 h with HRP<sup>+</sup> cells to HRP<sup>+</sup> cells, for a subsequent 2 h exposure. Normoxia. △: HRP<sup>+</sup> cells + IAA; ▲: HRP<sup>+</sup> cells + IAA; ▼: HRP<sup>+</sup> cells + pre-activated IAA.

C. Hypoxia. □: HRP<sup>+</sup> cells + IAA under 0.1% O<sub>2</sub>; ■: HRP<sup>+</sup> cells + IAA under 0.1% O<sub>2</sub>; ▼: activation of IAA by HRP<sup>+</sup> cells under hypoxia, followed by media transfer to normoxic HRP cells; ◆: activation under hypoxia, followed by media transfer to hypoxic HRP cells.

D. Anoxia. ○: HRP<sup>+</sup> cells + IAA under anoxia; ●: HRP<sup>+</sup> cells + IAA under anoxia; ▼: activation of IAA with HRP<sup>+</sup> cells under anoxia, followed by media transfer to normoxic HRP cells; ◆: activation under anoxia, followed by media transfer to anoxic HRP cells.
3.3.6 Cytotoxicity of the HSV TK/GCV combination in T24 cells

In order to compare the novel HRP/IAA combination with an established enzyme/prodrug system, T24 cells were transiently transfected with a plasmid containing the HSV TK coding sequence and exposed to GCV. Parallel transfections with the plasmid pEGFP-neo, containing an identical vector backbone as pTK, indicated a maximum transfection efficiency of 60-70%. After a 2 h-incubation with GCV, no reduction in survival was detected in TK\(^+\) cells compared to the EGFP mock-transfected population (Figure 3.10.A). When the prodrug GCV was left to react for 24 h with the cell monolayers, a small increase in cytotoxicity (IC\(_{50}\)) was induced in normoxic TK-transfectants compared to TK\(^-\) cells (Figure 3.10.B). Under anoxic conditions, no selective cytotoxicity could be measured at any doses of GCV tested (up to 5 mM; Figure 3.10.B). Doses above 1 mM GCV appeared to be toxic in the mock-transfected cells under both normoxic and anoxic conditions.

![Figure 3.10](image-url)  
**Figure 3.10.** Cytotoxic activation of ganciclovir (GCV) in T24 cells.
T24 cells transiently transfected with the plasmid pTK and mock-transfected cells were exposed to GCV under normoxia or anoxia for 2 h (A) or 24 h (B). The data are means of three independent experiments (duplicate samples) ± SE. The lines are interpolated. ▽: TK\(^+\) cells, normoxia; ▼: TK\(^+\) cells, normoxia; ◊: TK\(^-\) cells, anoxia; ◆: TK\(^-\) cells, anoxia.
3.4 Discussion

The work presented in this chapter demonstrates the potential for utilising horseradish peroxidase (HRP) and indole-3-acetic acid (IAA) as a novel enzyme/prodrug combination for cancer gene therapy.

In initial studies, a synthetic HRP gene, constructed by using codons commonly found and highly expressed in both *E. coli* and mammalian genes (Smith et al., 1990), was used. The synthetic gene was cloned into a plasmid vector fused to EGFP to increase HRP stability and allow direct protein detection in transfected cells (Figure 3.1.B). Unfortunately, when the HRP was expressed in human cells the levels of enzyme produced, although high enough to induce selective cell kill in the presence of IAA, could not allow a detailed analysis of HRP-mediated GDEPT *in vitro* (Figures 3.3, 3.4). Therefore the plasmid pRK34-HRP (Connolly, et al. 1994; Figure 3.1.A) was utilised to transfect four cell lines of human origin. This plasmid construct, containing the HRP cDNA fused to the signal sequence from the hGH and the KDEL retention motif, has been previously used to monitor traffic through the Golgi apparatus, and shown to result in high HRP production levels in human cells (Connolly et al., 1994). Also, transient rather than stable transfectants were used, as they are more likely to mimic an *in vivo* scenario, where only a small fraction of the population expresses the therapeutic gene.

The response of the three tumour lines analysed, MCF-7 breast carcinoma, FaDu nasopharyngeal carcinoma and T24 bladder carcinoma cells, did not differ considerably (Figure 3.7; Table 3.1). After 24 h-incubation with IAA, at prodrug levels below 1 mM HRP expression conferred a slightly higher sensitivity to MCF-7 cells, whereas above 1 mM HRP T24 cells were markedly more affected by the treatment. The *p53* status did not appear to play a major role in the response of these tumour lines to HRP/IAA, since MCF-7 cells have a wild type *p53* gene (Wosikowski et al., 1995), while FaDu and T24 cells are characterised by *p53* non-sense and missense mutations at codons 248 and 126, respectively (Reiss et al., 1992; Kawasaki et al., 1996). HRP/IAA may therefore function efficiently in different tumours irrespective of *p53* status.
HRP/IAA GDEPT induced significant inhibition of proliferation also in HMEC-1 endothelial cells (Figure 3.8.A; Table 3.1). This may represent an advantage if the tumour vasculature was to be targeted. Selective killing of the endothelial cells forming the lining of tumour blood vessels may cause malignant cells to starve of nutrients, producing an amplification of the cytotoxic effects (Chaplin and Dougherty, 1999). Also, endothelial cells lack drug resistance characteristic of some neoplastic cells, requiring lower doses of cytotoxic agents. Additionally, the vicinity to the blood stream would allow direct and simplified agent delivery. For HRP/IAA, low prodrug doses would need to be used, as IAA alone showed some toxicity in HRP+ endothelial cells (Figure 3.8.A; Table 3.1). This may limit therapeutic efficacy in vivo, although in preliminary studies 250 mg/kg IAA i.p. in mice resulted in tumour peak prodrug levels of ~1 mM, and plasma levels in excess of ~3 mM, with no associated toxicity (J. Tupper, Gray Cancer Institute, personal communication).

In order to investigate the potential of the HRP/IAA combination to kill the hypoxic subpopulation in solid tumours, transfectants were exposed to IAA in an oxygen-free atmosphere and significant cell kill was measured (Figure 3.7.B, D). Different IAA-metabolites may be produced in oxic and anoxic cells. In the absence of oxygen, the peroxyl radical and its decay products cannot be formed, but the skatole radical can (Figure 1.12, 3; Candeias et al., 1994). Skatole-type radicals readily abstract hydrogen from donor molecules and have been shown to react with biomolecules such as DNA (Folkes et al., 1999). In the absence of oxygen, they could therefore lead to cell damage by the formation of secondary radicals in key biological targets. Involvement of relatively short-lived reactive species is also suggested by the lack of transferability of the toxic agent under anoxia (Figure 3.9.D). What remains to be clarified is how the peroxidase cycle is initiated under anoxia, since IAA did not reduce HRP under strict anaerobic conditions (Gazaryan et al., 1996). Organic peroxides present as impurities in biological media or produced by the cells may be involved.

In normoxic cells, on the other hand, skatole radicals are more likely to react with oxygen to form peroxyl radicals (Augusto, 1993). Prompt attack by radicals on cellular targets in air as the main cause of cell death may be ruled out, since conditioned
medium-switch experiments under normoxia and hypoxia (0.1% O₂; Figure 3.9.B, C) and incubation of mammalian cells with filtered products of IAA oxidation (Folkes et al., 1998) indicated that the toxic agent is a stable species. Of the stable products, indole-3-carbinol (Figure 1.12, 6) was shown to be non-toxic to V79 cells at experimentally produced concentrations, with or without HRP (Folkes and Wardman, 2001). 3-methylene-2-oxindole (MOI; Figure 1.12, 8) has been reported to be toxic in E. coli and some plants, to react with glutathione and to bind to sulphhydryl regions of histone DNA or RNA (Folkes and Wardman, 2001). The role of MOI in the HRP/IAA-induced toxicity is currently under investigation (Folkes et al., manuscript in preparation).

An essential requirement for GDEPT is that the activated drug should induce a bystander effect, whereby conversion of the prodrug to the active form in the enzyme-modified cells leads to the killing of adjacent untransfected ones. The killing of neighbouring cells can be due to the transfer of toxic metabolic products through gap junctions, via apoptotic vesicles, or through the diffusion of soluble toxic metabolites (section 1.2.5). Our studies suggest that the HRP/IAA system can produce a strong bystander effect. At neutral pH IAA is hydrophilic (polar and soluble) and can cross cell membranes within a few minutes (Pires de Melo et al., 1997; Folkes et al., 1999). In all experiments presented here, HRP-transfectants were estimated to represent at best a quarter of the cells exposed to IAA, but this mixed population could be almost completely eradicated (Figure 3.7). In mixing experiments, approximately 70% and 90% cell kill were observed under normoxia with only 5% and 20% of the cells expressing HRP, respectively (Figure 3.9.A). The effect does not appear to require contact between HRP⁻ and HRP⁺ cells, since incubation of HRP⁻ cells with pre-activated IAA resulted in cell death under both normoxia and hypoxia (0.1% O₂; Figure 3.9.B, C). This compares very favourably with in vitro data on the bystander cytotoxicity of other enzyme/prodrug systems. For example, 95% cell kill after GCV-treatment required expression of HSV TK in 50% of the exposed population (Freeman et al., 1993). Similarly, CD production in 5% of the cells resulted in 50% cell eradication after 5-FC (Lawrence et al., 1998), and 90% growth inhibition could be achieved when 34-50% of
the cells exposed to CB1954 produced the enzyme NTR (Bridgewater et al., 1997; Spooner et al., 2001). Importantly, an even more pronounced bystander effect was observed under anoxic conditions (Figure 3.9.A), which, as previously discussed, is likely to be due to short-lived reactive species.

Compared to HSV TK/GCV, in T24 cells in vitro HRP/IAA induced faster and more effective cell kill. HRP/IAA was selectively toxic after a brief 2 h-exposure (Figure 3.7.A, B), while HSV TK/GCV required a longer incubation to be effective (Figure 3.10). Moreover, after 24 h GCV-incubation, only a 4-fold increase in cytotoxicity (IC$_{50}$) was induced in TK$^+$ cells under normoxia, and no selective sensitisation could be detected under anoxia. In previous studies, 5-14 days growth in the presence of GCV was required to induce a 3 log increase in cell kill in transfected mammalian cells (e.g. Moolten, 1986; Freeman et al., 1993). It is important to note that the T24 cell line is known to be resistant to a number of chemotherapeutic agents (Mizutani et al., 1997). These cells were also more resistant to IAA when activated by purified HRP (Figure 3.2), compared to the mammalian cells used in previous studies (Folkes et al., 1998, 1999). For instance, after 2 h-exposure to 0.1 mM IAA activated by 1.2 $\mu$g/ml HRP 87% of T24 cells lost their clonogenic potential, while at the same concentration/time a 2 log cell kill was induced in V79 cells (Folkes et al., 1998, 1999). Overall, the HRP/IAA combination compared favourably with established systems.
Chapter 4
Mechanisms of HRP/IAA-mediated cell kill

4.1 Introduction
Understanding the processes induced by an anticancer agent may be important not only to deduce its mechanism of action but also to improve its therapeutic efficacy and targeting potential. So far, little is known about the cellular targets and the mechanisms of cell death involved in HRP/IAA-mediated toxicity.

Based on (not always distinct) differences in morphological, biochemical and molecular changes of the dying cell, at least two modes of cell death have been described: apoptosis and necrosis. Apoptosis, or programmed cell death, is an active regulatory process that occurs during the normal development of tissues and organisms. It can also be induced in response to anticancer agents, radiation and hyperthermia, when DNA repair is saturated, and after removal of growth factors (reviewed by Lockshin et al., 2000). The most characteristic feature of apoptosis is the active participation of the cell in its demise. The dying cell activates a cascade of molecular events that result in orderly degradation of its constituents, with minimal impact on the surrounding tissue. Shrinkage and blebbing of the cytoplasm, chromatin condensation, loss of distinct chromatin structure, nuclear fragmentation, formation of apoptotic bodies, intact mitochondria and lysosomes are some of the main characteristics of apoptosis (Wyllie, 1992). The membranes of cells undergoing apoptosis present structural and functional integrity, and the phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet. The activation of endonucleases leads to the degradation of the DNA at the internucleosomal (linker) sections, generating a characteristic ladder pattern during gel electrophoresis. Also, in cells undergoing apoptosis, caspases (cysteine aspartate proteases), synthesised as inactive proenzymes, are processed by self-proteolysis and/or cleavage by other proteases into two subunits, which associate to form an active catalyst (Lockshin et al., 2000). Active caspases
initiate a proteolytic cascade, which leads to the cleavage of numerous cytoplasmic and nuclear targets, including caspase-activated DNAase (CAD) and the DNA repair enzyme Poly (ADP-ribose) polymerase (PARP), which is inactivated by caspase cleavage (Kaufmann, 1989).

Necrosis is a passive and degenerative process, due to the general failure of cellular homeostasis and regulation following injury. In this case, swelling of mitochondria, followed by rupture of the plasma membrane and release of the cytoplasmic contents are among the early events (Lockshin et al., 2000). DNA degradation is not extensive, and the fragments generated are heterogeneous in size and do not form discrete bands during gel electrophoresis. Because of the release of many proteolytic enzymes, necrosis, in contrast to apoptosis, triggers an inflammatory reaction in vivo. However, on some occasions, depending on the cell type and the nature of the factor affecting cell viability, the mode of cell death may be typical of neither necrosis nor apoptosis, lacking the characteristics of either of these mechanisms, or exhibiting features of both (Collins et al., 1992).

The aim of this chapter was the analysis of some of the molecular and morphological features of the dying cells treated with HRP/IAA gene therapy. The mode of cell death was examined by testing several characteristics of apoptosis in HRP+ T24 cells exposed to IAA. The effect of drug treatment on cell cycle progression was also investigated.

4.2 Materials and methods

Drug exposure was carried out under the same experimental conditions as for the cytotoxicity assays reported in Chapter 3. General methodologies are described in Chapter 2.

4.2.1 Nuclear staining

To study nuclear integrity, chromatin condensation and the formation of micronuclei (MN), cells were treated for different time intervals on glass coverslips. After drug exposure, the media were removed and the cells fixed in 3.7% formaldehyde (Sigma
Aldrich)/PBS for 15-20 min at room temperature (rt). The samples were washed three times in PBS to remove the fixative and permeabilised in 0.1% Triton (Sigma Aldrich)/PBS for 5 min, rt. The coverslips were rinsed three times with PBS and mounted on a microscope slide using Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc.) and viewed using a NIKON TE200 fluorescence microscope and a custom designed image acquisition software (Gray Cancer Institute). To quantify cells with condensed chromatin and MN, at least 600 cells per sample were examined.

4.2.2 DNA fragmentation analysis by FACS
Following drug exposure, either the cells were assayed immediately or the drug-containing buffer was replaced with complete media and the cells were left to recover for 24 h or 48 h. To detect DNA fragmentation, cells and culture media were harvested, centrifuged and the pellet re-suspended in ice-cold 70% EtOH and incubated for 1 h on ice for fixation. After centrifugation, pellets were re-suspended in HBSS containing propidium iodide (PI) and RNase A at the final concentrations of 20 µg/ml, and incubated at rt for ~30 min. Samples were analysed using FACScan and gated to exclude cellular debris.

4.2.3 Annexin V binding
For Annexin V staining, following drug exposure cells and culture media were harvested, washed twice in ice-cold PBS and re-suspended in binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, 2.5 mM CaCl₂) at 10⁶ cells/ml. 100 µl cell suspension was transferred to a 5 ml polystyrene Falcon tube (Becton Dickinson) and incubated for 15 min with 0.5 µg/ml PI and 5 µl Annexin V-FITC (PharMingen, San Diego, CA) at rt, in the dark. Prior to FACS analysis (which was performed within 1 h), 400 µl binding buffer was added to the samples. Cells that showed increased FITC- or PI-related fluorescence compared to controls exposed to HBSS only were scored as positive.

4.2.4 Detection of PARP cleavage
Cell monolayers were harvested by scraping in lysis buffer. In addition, in order to also collect the cells that detached from the monolayer, media were collected, centrifuged,
washed in ice-cold PBS, and re-suspended in lysis buffer containing the corresponding lysed monolayer. Whole cell extracts were subjected to SDS-PAGE using 6% polyacrylamide gels, according to the protocol in section 2.11. Proteins in the gel were transferred to nitrocellulose membranes, and immunoblotting was performed with primary mouse monoclonal anti-PARP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000, and secondary HRP-conjugated goat anti-mouse immunoglobulins (Dako) diluted 1:2000.

4.2.5 Cell cycle analysis

Replacement of the methyl group of thymidine with halogen atoms of similar size, such as bromine, creates analogues like bromo-deoxyuridine (BrUdR), which are incorporated into elongating DNA via the same pathway as thymidine. Incorporated BrUdR can then be detected with specific antibodies, which recognise the small distortions caused in the DNA molecule (reviewed by Gray et al., 1986). For cell kinetic studies by flow cytometry, FITC-conjugated secondary antibodies and PI counterstaining for total DNA content are generally used. This way, the cells in S-phase at the time of BrUdR administration can be detected by increased green fluorescence and their progression through the cycle monitored with time.

Immediately before drug exposure, BrUdR was added to the culture medium at the final concentration of 2 μM and the cells were incubated for 25 min at 37°C. Following two washes in PBS, the cell monolayers were exposed to HBSS with or without IAA for up to 30 h. At 2 h-intervals, cells and media were harvested by scraping in 5 ml ice-cold PBS and centrifugation at 1200 rpm for 5 min. The pellets were re-suspended in 3 ml 70% EtOH and stored at 4°C for immunostaining. Antibody staining was performed by incubating the samples in 0.1 mg/ml pepsin (Sigma Aldrich) in 2 M HCl for 20 min at rt, followed by three washes in PBS. After centrifugation, the pellets were re-suspended and exposed to 1:25 mouse anti-BrUdR (Sigma Aldrich) in 1.25% normal goat serum and 5% Tween 20 (both from Sigma Aldrich) in PBS for at least 1 h at rt. The samples were washed in PBS and incubated with 1:25 FITC-conjugated goat anti-mouse immunoglobulins (Sigma Aldrich) in the dark for 45-60 min at rt. The cells were rinsed in PBS and re-suspended in 20 μg/ml PI in PBS for FACS analysis. Cell debris and
doublets were excluded from the analysis by gating. The data were displayed as two-parameter cytograms, green (FITC for BrUdR; FL1-H) versus red (PI; FL3-A) fluorescence intensities. Three subpopulations, representing G₁ cells (low green and low red fluorescence), G₂/M cells (low green and high red) and S-phase cells (high green), could readily be detected.

4.3 Results

4.3.1 Chromatin condensation

Apoptosis is characterised by a number of morphological changes. Due to rapid dehydration, the cells often become diminished in size, and elongated or convoluted in shape. Chromatin condensation and the loss of distinct chromatin structure occur in parallel with cell shrinkage, and they are usually followed by nuclear fragmentation (Wyllie, 1992). Distinct hyperchromicity and homogeneity characterise the ability of DNA in the fragmented nucleus to be stained with fluorochromes such as DAPI. Nuclear fragments, together with the constituents of the cytoplasm, are then packaged into apoptotic bodies, which, enveloped in plasma membrane, detach from the dying cell.

The nuclear morphology of T24 cells transiently transfected with the HRP cDNA and exposed to 3 mM IAA for 2, 4, 6, 8, 14, 18 and 24 h was monitored by fixation and staining with DAPI. After 2 h IAA-incubation, no major changes were observed (Figure 4.1.D). However, from 4 h on, fluorescence microscopy revealed the presence of condensed chromatin, especially at the nuclear periphery (Figure 4.1.E-I). Some of the cells treated with HRP/IAA presented highly damaged, shrunken, dehydrated nuclei (Figure 4.2.A). Similar damaged nuclei have been observed in T24 cells after co-administration of vitamins C and K₃ (Gilloteaux et al., 2001).

Condensed chromatin and shrunken nuclei were also observed in T24 cells exposed to 667 μM cisplatin (CDDP, Platamine, Pharmacia & Upjohn, Milan, Italy) and 0.2 μM staurosporine (Calbiochem, San Diego, Ca; Figure 4.1.B, C). No major changes were observed in the nuclei of HRP⁺ cells exposed to HBSS (Figure 4.1.A) or CD4-transfectants exposed to IAA (data not shown).
To test the involvement of caspases in HRP/IAA-induced apoptosis, the cell-permeable caspase inhibitor of broad specificity z-Val-Ala-Asp-CH\(_2\) (zVAD.fmk; Calbiochem) was administered during IAA treatment. Stained nuclei were analysed by fluorescence microscopy, the cells with condensed chromatin were counted and normalised to the total number of cells in the observation field. At least 600 cells were examined. After 3 mM IAA for 24 h, in the presence of 100 \(\mu\)M zVAD.fmk HRP\(^+\) T24 cells with condensed chromatin represented only 1.5% of the exposed population, versus 3.9% without caspase inhibitor. zVAD.fmk had no effect on HRP\(^+\) cells exposed to buffer or CD4\(^+\) cells incubated with IAA (results not shown).

Cells treated with HRP/IAA were also characterised by the presence of micronuclear structures, probably due to nuclear fragmentation and to the formation of apoptotic bodies (Figure 4.2.B, C). Compared to HBSS-treated controls, the frequency of micronuclei (MN) in HRP\(^+\) cells was markedly increased after 6 h-exposure to 3 mM IAA (Figure 4.3). After 24 h, 0.109 (±0.008) MN/cell were detected in HRP/IAA-treated cells, against a baseline of 0.039 (±0.008) MN/cell in untreated CD4\(^+\) cells. zVAD.fmk inhibited the formation of MN, reducing their frequency to baseline levels (0.041±0.005 MN/cell after 24 h IAA and 100 \(\mu\)M zVAD.fmk).

Condensed chromatin, MN and damaged nuclei were also selectively induced in HRP\(^+\) FaDu cells exposed to 3 mM IAA (data not shown).

**4.3.2 DNA fragmentation**

Activation of endonucleases that degrade DNA is another characteristic event of apoptosis. DNA cleavage could be detected by staining DNA in ethanol-fixed cells with the DNA fluorochrome propidium iodide (PI). Following FACS analysis, the cells showed different degrees of fluorescence depending on their DNA content. Figure 4.4 shows the FACS profile of permeabilised HRP\(^+\) T24 cells exposed to buffer alone, CDDP or IAA, fixed and stained with PI. Cells in the different phases of the division cycle at the time of assay can be detected. The appearance of cells with low DNA content, below that of G\(_1\) cells (sub-G\(_1\) peak), has been considered to be a marker of cell death by apoptosis (Afanas'ev et al., 1986).
Figure 4.1. Chromatin condensation in T24 cells.
Nuclei were fixed, stained with DAPI and viewed using a NIKON TE200 fluorescence microscope (100x objective). Representative images are shown. Arrows indicate condensed nuclei.
A. HRP' cells, no drug.
B. HRP' cells + 667 μM cisplatin (CDDP), 24 h.
C. HRP' cells + 0.2 μM staurosporine, 24 h.
D-I. HRP' cells + 3 mM IAA, 2 h (D), 4 h (E), 6 h (F), 8 h (G), 14 h (H), 24 h (I).
Figure 4.2. Examples of cells with damaged nuclei or micronuclei.
A. Damaged nuclei of HRP− T24 cells exposed to 3 mM IAA. The arrows indicate representative structures.
B, C. Micronucleated cells (indicated by arrows) after exposure to HRP/IAA GDEPT.

Figure 4.3. Induction of micronuclei (MN).
At different time intervals during treatment, T24 cells were fixed, the nuclei were stained with DAPI and visualised by fluorescent microscopy. At least 600 cells were examined per time point. The base line indicates the number of MN/cell in untreated CD4− cells.
To monitor the apoptotic subdiploid population, HRP\(^+\) and CD4\(^+\) T24 cells were exposed for 24 h to 0.5 mM or 3 mM IAA. The cells were either assayed immediately after drug treatment, or grown in fresh complete medium for 24 h or 48 h and subsequently analysed. In each experiment, the percentage of cells in the sub-G\(_1\) peak after IAA relative to buffer-treated controls (relative sub-G\(_1\) peak) was measured. Immediately after drug exposure, a significant increase in apoptotic HRP\(^+\) cells was detected at both concentrations of prodrug (Figure 4.5.A). When the cells were assayed 24 h or 48 h after HRP/IAA treatment, the relative sub-G\(_1\) peak was >1, but the increase was significant only after 3 mM IAA (Figure 4.5.A). In comparison, after 667 \(\mu\)M CDDP for 24 h, ~3-fold increase in sub-G\(_1\) cells was observed (data not shown).

After 3 mM IAA, the cells containing fragmented DNA represented 14% of the total population immediately after exposure, 58% at 24 h and 62% at 48 h. In the presence of 100 \(\mu\)M zVAD.fmk the percentage of sub-G\(_1\) cells was reduced by about one half, suggesting the involvement of caspases in the process. The induction of DNA fragmentation was also studied under anoxic conditions. When prodrug exposure was performed in the absence of oxygen, HRP\(^+\) T24 cells exposed to IAA presented a small (not significant) increase in the sub-G\(_1\) population compared to the HBSS-treated counterpart (Figure 4.5.B). This was due to high DNA fragmentation detected in the buffer-treated cells, whereas the percentage of sub-G\(_1\) cells exposed to IAA did not differ significantly compared to normoxia.

IAA-exposed CD4\(^+\) cells did not show any significant increase in the sub-G\(_1\) population under both normoxic and anoxic conditions.
Figure 4.4. DNA fragmentation in T24 cells.
HRP H24 cells exposed to buffer only, 667 μM cisplatin (CDDP), 0.5 mM or 3 mM IAA in air were fixed and stained with propidium iodide (PI). G1, G2 and sub-G1 cells are shown.
Figure 4.5. HRP/IAA-induced DNA fragmentation.

HRP⁺ and CD4⁺ T24 cells were exposed for 24 h to 0.5 mM or 3 mM IAA, under normoxic (A) or anoxic (B) conditions. The percentage of cells in the sub-G₁ peak after IAA relative to buffer-treated controls (relative sub-G₁ peak) was assessed immediately (0 h), 24 h or 48 h after exposure. The means of at least three independent experiments and SEs are indicated. *: significantly different from 1 (p<0.05).
4.3.3 Annexin V binding

One of the early events of apoptosis is the externalisation of the phospholipid phosphatidylserine (PS), normally present on the cytoplasmic moiety of the plasma membrane (Fadok et al., 1992). Annexin V is a protein with high affinity for PS that can bind to the cell surface of cells undergoing apoptosis (Schutte et al., 1998). If Annexin V is conjugated with a fluorochrome such as FITC, apoptotic cells can be detected by using microscopy and flow cytometry procedures. Moreover, at these early stages apoptotic cells present an intact membrane and, if not permeabilised, can exclude PI. The FACS profile of T24 cells exposed to CDDP or HRP/IAA and subsequently stained with FITC-conjugated Annexin V and PI is shown in Figure 4.6. Four populations can be observed: living cells negative for both dyes (bottom left quadrants, Figure 4.6), early apoptotic cells positive for Annexin V only (bottom right quadrants), necrotic cells positive for PI only (top left), and late apoptotic/necrotic cells positive for both dyes (top right).

A significant increase in Annexin V binding was observed in HRP⁺ T24 cells incubated with IAA for 24 h, compared to buffer-treated controls (Figures 4.6, 4.7.A). Induction levels (fraction of Annexin V-positive cells after IAA/fraction of Annexin V-positive cells after buffer only) of 1.9±0.2 (0.5 mM IAA) and 6.1±1.5 (3 mM) were measured, with average percentages of Annexin V-binding cells of 10% and 25%, respectively. Cells transfected with the gene for the marker protein CD4 did not show any increase in Annexin V binding at either prodrug concentration, while 24 h-incubation with CDDP (667 μM) induced a 5.4-fold increase in Annexin V binding (Figure 4.7.A). Compared to HBSS-treated controls, the increase in PI-positive cells in the drug-treated population was not significant (Figure 4.7.B). Moreover, the cells that were not able to exclude PI were also positive for Annexin V binding, suggesting that at the time of assay (and up to 24 h after drug exposure, data not shown), necrosis may not be the main death mechanism. The cells were also analysed after shorter incubation intervals with IAA (2 h or 6 h), but no significant increase either in Annexin V binding or in PI staining could be detected (results not shown).
Figure 4.6. Annexin V and PI staining of T24 cells.

HRP⁺ T24 cells were exposed for 24 h to HBSS, 667 μM CDDP, 0.5 mM or 3 mM IAA, and assayed immediately after incubation.
Figure 4.7. Annexin V binding and PI staining of T24 cells treated with HRP/IAA gene therapy. CD4+ and HRP+ cells were exposed for 24 h to 0.5 mM IAA, 3 mM IAA or 667 μM CDDP. Relative Annexin V binding (A) and PI staining (B) were expressed as the percentage of positive cells after IAA/percentage of positive cells after buffer only. The means of three independent experiments ± SE are indicated. *: significantly different from 1 (p<0.05).
4.3.4 PARP degradation

When the apoptotic process is initiated, one of the downstream events of caspase-3 activation is the cleavage of the DNA repair enzyme PARP into two inactive subunits of 27 kDa and 85 kDa (Pieper et al., 1999). In the last few years, PARP degradation has been used extensively as a marker of apoptosis.

PARP cleavage was monitored in HRP T24 cells treated with 3 mM IAA for up to 24 h by Western blotting. Only very little cleavage of PARP could be detected in the HRP/IAA-treated samples, as well as in cells exposed to 667 µM CDDP (Figure 4.8 shows a typical experiment). To make sure that the absence of PARP degradation was not due to technical faults, human umbilical vein endothelial cells (HUVECs) exposed for 12 h to 0.2 µM staurosporine were used as a positive control (from Dr. C. Kanthou, Gray Cancer Institute). In this case, the 85 kDa subunit could be detected (Figure 4.8).

The lack of PARP cleavage after HRP/IAA treatment was not limited to T24 cells, as it was also observed in the FaDu cell line (data not shown).

![Figure 4.8. Cleavage of PARP.](image)

HRP T24 cells were exposed to 3 mM IAA and cell extracts were analysed by Western blotting after 2, 6, 8, 14 and 18 h. Alternatively, the cells were incubated with 667 µM CDDP for 4 or 8 h. HUVECs treated with 0.2 µM staurosporine were used as positive controls (H). A protein marker (m) was run in parallel to the samples.
4.3.5 Cell cycle analysis

In order to evaluate the effect of HRP/IAA treatment on cell cycle progression, HRP$^+$ and CD4$^+$ T24 cells were pulse-labelled with BrUdR, and subsequently exposed to HBSS or 3 mM IAA for up to 30 h. This observation interval was chosen to monitor one whole division cycle, since analysis of the growth of cultured T24 cells indicated a doubling time of $\sim$24 h (data not shown). Cell samples were collected every 2 h, and FACS-analysed after antibody and PI staining.

While cells exposed to buffer only showed a normal cell cycle progression, HRP$^+$ cells incubated with IAA did not appear to progress through the cycle for the entire observation time (Figure 4.9). This cytostatic effect was confirmed by the quantification of the fraction of labelled cells in G$_1$ (Figure 4.10). Only cells exposed to HBSS appeared to enter a new division cycle.

The results were specifically dependent on the activated drug, as CD4$^+$ cells exposed to the same dose of IAA were not affected by prodrug treatment (data not shown).
Figure 4.9. Cell cycle analysis of HRP/IAA-treated T24 cells.

HRP<sup>+</sup> cells exposed to 3 mM IAA (+ IAA) or buffer only (- IAA) were pulse-labelled with BrUdR and analysed at 2 h intervals for 30 h. Selected profiles of a representative experiment are shown.
Figure 4.10. Cell cycle progression of T24 cells. The fraction of BrUdR-labelled cells in the G₁ phase in one representative experiment is shown. HRP⁺ T24 cells exposed to IAA (▲) or buffer only (△).

4.4 Discussion

HRP/IAA is a novel anticancer system, and the chemical and biological pathways leading from prodrug activation to cytotoxicity are yet to be elucidated. The experiments presented here were carried out in order to evaluate the death mechanisms induced in human tumour cells.

Several pathways leading to apoptosis have been reported in the literature. The apoptotic machinery can be broadly divided into “sensors” and “effectors”. Sentinels such as cell surface receptors that bind to survival or death factors (e.g. FAS ligand, TNF-α) are responsible for monitoring the extracellular environment for conditions that affect the decision of the cell to live or die. Intracellular sensors monitor abnormalities including DNA damage, oncogene activation, survival factor deficiency or hypoxia. For
instance, the p53 tumour suppressor protein, a key inducer of the apoptotic cascade, can elicit apoptosis by upregulating expression of Bax in response to sensing DNA damage (Hanahan and Weinberg, 2000). In turn proteins of the Bcl-2 family, whose members have either pro-apoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) function, act in part by governing mitochondrial death signalling through release of cytochrome c, a potent catalyst of apoptosis (Hanahan and Weinberg, 2000). Among the ultimate effectors of apoptosis are an array of intracellular proteases, including the set of caspases, which can activate other caspases or execute the death programme through selective degradation of subcellular structures, organelles and DNA.

Resistance to apoptosis can be acquired by cancer cells through a variety of strategies, the most common being mutations in the p53 gene, occurring in more than 50% of human cancers (Hanahan and Weinberg, 2000). It is currently accepted that even in these cases the cells do not lose their apoptosis potential, but activate the death machinery at a substantially higher threshold (Lockshin et al., 2000). The redundancy of the regulatory and effector components holds implication for the development of antitumour therapies targeting one of the many branches of the apoptotic signalling circuitry.

Identification of apoptotic cells is undoubtedly complex, and some of the features associated with apoptosis may also accompany cell necrosis (Collins et al., 1992). Therefore apoptosis may be identified with a higher degree of assurance only by using more than one assay.

Overall our results indicate that an apoptotic pathway may be activated by HRP/IAA gene therapy. Nuclear morphological features, DNA fragmentation and Annexin V binding could be selectively detected in the treated cells, and not in HRP or IAA controls (Figures 4.1-4.7). Caspases appear to be involved as effectors, since treatment with the general caspase inhibitor zVAD.fmk decreased the fraction of cells with MN by 30%, fragmented DNA by 50% and condensed chromatin by 60%. On the other hand, a functional p53 appears not to be required, since T24 and FaDu cells both express mutated forms of this protein (Reiss et al., 1992; Kawasaki et al., 1996).
However, very little degradation of one of the downstream targets of caspase-3, PARP, could be detected. The DNA repair enzyme PARP is selectively activated by DNA strand breaks to catalyse the addition of long branched chains of poly (ADP-ribose) from its substrate nicotinamide adenine dinucleotide (NAD; Pieper et al., 1999). Proteolytic cleavage of PARP may occur when the cell is no longer able to repair or replicate its DNA and initiates the disassembly that ensures its commitment to apoptosis. However, the complete role of PARP in the apoptotic process remains to be determined, since \( PARP^- \) cells display varying susceptibility towards apoptosis compared to their wild type counterparts (Pieper et al., 1999). Here very little PARP cleavage could be detected in FaDu and T24 cells treated with HRP/IAA as well as CDDP (Figure 4.8). This was surprising, as CDDP has been shown to induce apoptosis by a caspase 3-dependent pathway (Cenni et al., 2001), and degradation of PARP has been also observed in \( p53^- \) mutants (Hanai et al., 2001). Specific data on PARP cleavage in T24 and FaDu cells could not be found in the literature, but there is a growing body of evidence that in many cell systems apoptosis may be atypical, lacking one or more of the features that characterise classical apoptosis (Cohen et al., 1992). Moreover, a third type of cell death, autoshizis, characterised by features common to apoptosis (decrease in cell volume, chromatin condensation at the nuclear periphery, cell blebbing, DNA cleavage) has been identified in T24 cells presenting nuclear damage similar to that shown in Figure 4.2.A (Gilloteaux et al., 2001).

When exposure to the same doses of IAA was performed under anoxic conditions a small but not significant increase in apoptosis could be detected (Figure 4.5.B). This could indicate that not only different chemical but also biological mechanisms may be induced in the absence of oxygen, as discussed in Chapter 3. In particular, during the first 48 h after treatment, the percentage of normoxic apoptotic cells increased, while a decrease of the relative sub-\( G_1 \) peak was observed in the anoxic population (Figure 4.5). On the other hand, hypoxia itself can initiate apoptosis (section 1.1.1), making the detection of prodrug-induced effects over the background difficult. Indeed, the fraction of sub-\( G_1 \) cells exposed to buffer only under anoxia was almost doubled compared to
normoxic HBSS-treated cells. More detailed studies need to be carried out to verify these hypotheses.

Cells that initiate apoptosis cannot be clonogenic, but the percentages of apoptotic cells detected after HRP/IAA GDEPT cannot fully account for the killing levels measured with a clonogenic assay (Chapter 3). For example, 0.5 mM IAA induced up to 30% sub-G₁ and up to 10% Annexin V-positive cells in the HRP⁺ population, while clonogenicity revealed 94% cell kill after the same prodrug dose. Indeed PI staining of unfixed cells suggested that some cells underwent necrotic death, although the increase was not significant and restricted to the Annexin V-positive population (Figures 4.6, 4.7B). Several investigators have observed that cell sensitivity as assessed by clonogenic ability is greater than that measured by apoptosis assays (reviewed in Brown and Wouters, 1999). The relationship between apoptosis and clonogenic survival is complex and a correlation is yet to be demonstrated. Moreover, apoptosis is a dynamic process and could be underestimated by examining cells at a certain time point. In particular, at the time of detection the cells appeared not to have undergone mitosis yet, as suggested by the cell cycle profiles (Figures 4.9, 4.10). Delayed apoptosis, particularly important for p53-mutated cells (Brown and Wouters, 1999) may therefore be occurring.

Indeed, the signal to the cells to die could arise from their inability to progress through the cell cycle. HRP/IAA gene therapy caused a cytostatic effect, equally affecting all phases of the division cycle (Figures 4.9, 4.10). Interestingly, in accordance with the clonogenic assays, almost the entire exposed population was affected, even though only ~20% expressed the HRP and were therefore able to activate IAA (Chapter 3). This observation correlates with the survival data from the clonogenic assays that point towards a significant bystander effect of the HRP/IAA combination.

The initiating event triggering the apoptotic response is yet to be identified. Experiments carried out with the purified enzyme indicated binding and accumulation of [5-³H]IAA in the cell nucleus (Folkes and Wardman, 2001). Moreover, DNA adducts and strand breaks were observed in plasmid DNA incubated with activated IAA (Folkes
et al., 1999), and extensive DNA fragmentation was revealed with pulsed field gel electrophoresis in T24 cells (data not shown). The results presented here may help in understanding the mode of action of HRP/IAA-mediated toxicity, but further work is necessary to fully elucidate the initial damage and the pathways involved. Moreover, the impact of the observed apoptosis on tumour eradication will need to be assessed in in vivo models.
Chapter 5
Choice of IAA prodrugs under oxic and anoxic tumour conditions

5.1 Introduction
A number of criteria have been discussed for selecting enzyme/prodrug combinations in GDEPT, ADEPT and PDEPT approaches (section 1.2.5). Among the required properties are fast and efficient prodrug activation, even at low substrate concentrations, and high selectivity index. HRP is a robust and versatile enzyme, and, as for other enzyme/prodrug combinations, treatment efficacy of HRP/IAA could be improved by the use of prodrugs with higher affinity for HRP and lower basal toxicity in untransfected cells, compared to IAA.

The rate of oxidation of IAA analogues by HRP shows a marked dependence on components and substituent position (Candeias et al., 1997). In particular, the reaction velocity with the HRP form compound (Cpd) I (section 1.3) varies over four orders of magnitude within different indoles, being enhanced in the presence of electron-donating groups.

To evaluate the potential of optimising HRP-mediated gene therapy, the action of ten IAA derivatives was studied under normoxic as well as anoxic conditions. The data reported indicate that at least two analogues besides IAA induce efficient toxicity and bystander killing. Of these, 5-Br-IAA showed unexpected prompt and selective activation under anoxia.

5.2 Materials and methods
All methods are listed in Chapters 2 and 3.

The prodrugs utilised are illustrated in Figure 5.1. IAA, 1-Me-IAA, 2-Me-IAA, 5-MeO-IAA, 2-Me, 5-MeO-IAA, 5-BnO-IAA, 5-F-IAA and 5-Br-IAA were purchased
from Sigma Aldrich; 5-Ph-IAA, 6-F-IAA and 4-Cl-IAA were synthesised by Dr. S. Rossiter (Gray Cancer Institute; Rossiter et al., manuscript in preparation).

Figure 5.1. Compounds tested in combination with the enzyme HRP for GDEPT.

5.3 Results
To identify novel, more potent prodrugs for GDEPT, a panel of IAA derivatives (Figure 5.1) were studied in combination with HRP. Human T24 bladder carcinoma cells were transiently transfected with plasmid constructs containing either the HRP cDNA (pRK34-HRP; HRP⁺ cells) or the gene for the marker CD4 (pCMV-CD4; HRP⁻ cells).
HRP immunostaining revealed a transfection efficiency of 20-25% (section 3.3.3). The cells were subsequently exposed to IAA and analogues at the concentrations of 0.1 mM and 1 mM, for 2 h or 24 h under normoxia or anoxia. Cell survival was measured by clonogenic assay (Figures 5.2, 5.3).

Among the compounds studied, prodrugs characterised by prompt normoxic (1-Me-IAA; 2-Me-IAA) as well as anoxic (in particular 5-Br-IAA) cytotoxic activation, high HRP⁺ cell kill and selectivity (1-Me-IAA; 5-Br-IAA; 5-F-IAA) were identified (Figures 5.2, 5.3). Of the other analogues, 5-MeO-IAA, 6-F-IAA and 4-Cl-IAA showed effects very similar to IAA under normoxia (Figure 5.2), but almost no selective cytotoxicity could be detected in anoxic HRP⁺ cells (Figure 5.3). 2-Me,5MeO-IAA induced very little toxicity in both HRP⁻ and HRP⁺ cells under any of the exposure conditions analysed (Figures 5.2, 5.3). 5-BnO-IAA and 5-Ph-IAA showed prompt and effective HRP⁺ cell killing under both normoxia and anoxia, but they also induced high non-specific toxicity in HRP⁻ control cells (Figures 5.2, 5.3).

The toxicity of the different IAA derivatives under normoxia, as assessed by clonogenic survival assays in the experiments discussed above, was compared to the rate of reaction with HRP. The oxidation rate in the presence of HRP Cpd I in air had been previously measured by L.K. Folkes (Gray Cancer Institute; Folkes et al., manuscript in preparation). A simple relationship could not be found. Surprisingly, after 24 h-incubation with HRP⁺ cells, the IAA analogues that are not promptly oxidised by HRP (such as 5-F-IAA and 5-Br-IAA) resulted in the highest toxicity under normoxia (Figure 5.4). In particular, a significant positive correlation between reaction rate with Cpd I and surviving fraction was found, with correlation coefficients of 0.86 for 0.1 mM and 0.89 for 1 mM prodrug (Figure 5.4). On the other hand, no correlation could be measured between oxidation rate and surviving fraction after 2 h drug exposure (data not shown).
Figure 5.2. Use of IAA derivatives for GDEPT under normoxia.

T24 cells were exposed to a panel of IAA analogues (Figure 5.1) at the concentrations of 0.1 mM (A, C) or 1 mM (B, D), for 2 h (A, B) or 24 h (C, D). □: HRP' cells + 0.1 mM prodrug; ■: HRP' cells + 0.1 mM prodrug; ○: HRP' cells + 1 mM prodrug; ●: HRP' cells + 1 mM prodrug. The means of three independent clonogenic assay experiments ± SE are indicated. Prodrugs adopted in subsequent clonogenic survival studies are underlined.
**Figure 5.3.** Use of IAA derivatives for GDEPT under anoxia.

Clonogenic survival of T24 cells exposed to IAA derivatives under anoxia, at the concentrations of 0.1 mM (A, C) or 1 mM (B, D), for 2 h (A, B) or 24 h (C, D). □: HRP+ cells, 0.1 mM prodrug; ■: HRP+ cells, 0.1 mM prodrug; ○: HRP+ cells, 1 mM prodrug; ●: HRP+ cells, 1 mM prodrug. The data are means of three independent experiments ± SE. Prodrugs adopted in subsequent clonogenic survival studies are underlined.
Figure 5.4. Correlation between surviving fraction and oxidation rate of IAA and analogues. The clonogenic survival of HRP T24 cells exposed to 0.1 mM (A) or 1 mM (B) prodrug under normoxia showed a positive correlation with the reaction rate with HRP Cpd I in air (from Folkes et al., 2000).
1-Me-IAA, 2-Me-IAA, 5-F-IAA and 5-Br-IAA appeared particularly suitable for HRP-GDEPT, since they were characterised by high and prompt cytotoxicity as well as selectivity under normoxia and anoxia. Their action was further investigated in detailed clonogenic survival experiments using T24, MCF-7 and FaDu cells (Figures 5.5 and 5.6).

After 2 h, the response to the methylated compounds was similar to that of IAA (Figures 3.7, 5.5). On the other hand, little or no toxicity was detected in HRP^ cells, in particular in T24 cells, exposed for short intervals to 5-F-IAA or 5-Br-IAA (Figure 5.5). This was unexpected, since halogenated IAA derivatives showed increased toxicity compared to IAA when activated by purified HRP (Folkes et al., 2002). The drug batch, the number of cells plated and the transfection efficiency were therefore analysed, and found not to be responsible for the observed effect (results not shown). T24 cells were also more resistant than other tumour lines when exposed for up to 2 h to 5-F-IAA activated by purified HRP (Folkes et al., 2002). It is possible that under normoxia a longer activation interval is necessary in this cell line.

After prolonged incubation with the cellular monolayers (24 h), the prodrugs induced high and selective toxicity in HRP^ cells in all three tumour lines (Figure 5.6). In particular, 1-Me-IAA showed the highest selectivity indices (740 in T24, 71 in MCF-7 and 50 in FaDu cells; Table 5.1) and HRP^ cell death (Figure 5.6.A). 2-Me-IAA, on the other hand, induced surviving fractions of less than 10% only at doses above 1 mM (Figure 5.6.B). 5-F-IAA, although very effective in T24 (SI = 88) and FaDu (SI = 60) cells, was characterised by non-specific toxicity in HRP^ MCF-7 cells, resulting in a SI of only 1.6 in this line (Figure 5.6.C; Table 5.1). Finally, 5-Br-IAA was efficient and selective in all the lines analysed (SIs between 71 and 100; Figure 5.6.D; Table 5.1). In this case, prodrug levels up to 1 mM were studied, because of toxicity in HRP^ cells with higher concentrations.

1-Me-IAA and 5-Br-IAA were also effective in endothelial HMEC-1 cells (Figure 5.7). As observed with the model compound IAA, HRP^ HMEC-1 cells were more sensitive to the prodrugs than HRP^ T24 cells (data not shown).
Figure 5.5. Cytotoxicity of IAA analogues under normoxia (2 h-exposure).
Clonogenic survival of HRP and HRP' tumour cells exposed for 2 h to 1-Me-IAA (A), 2-Me-IAA (B), 5-F-IAA (C) and 5-Br-IAA (D). △: HRP T24 cells; ▲: HRP' T24 cells; □: HRP MCF-7 cells; ■: HRP' MCF-7 cells; ○: HRP FaDu cells; ♦: HRP' FaDu cells. Means and SEs of at least three independent experiments are indicated. The lines are interpolated.
Figure 5.6. Cytotoxicity of IAA analogues under normoxia (24 h-exposure).
Clonogenic survival of HRP\textsuperscript{+} and HRP\textsuperscript{-} tumour cells exposed for 24 h to 1-Me-IAA (A), 2-Me-IAA (B), 5-F-IAA (C) and 5-Br-IAA (D). \(\triangle\): HRP\textsuperscript{+} T24 cells; \(\blacktriangle\): HRP\textsuperscript{-} T24 cells; \(\square\): HRP\textsuperscript{+} MCF-7 cells; \(\blacksquare\): HRP\textsuperscript{-} MCF-7 cells; \(\diamondsuit\): HRP\textsuperscript{+} FaDu cells; \(\heartsuit\): HRP\textsuperscript{-} FaDu cells. Means ± SE of at least three independent experiments are indicated. The lines are interpolated.
<table>
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<th>Prodrug</th>
<th>T24 IC₅₀ (mM)</th>
<th>T24 IC₅₀ index</th>
<th>MCF-7 IC₅₀ (mM)</th>
<th>MCF-7 Selectivity index</th>
<th>FaDu IC₅₀ (mM)</th>
<th>FaDu IC₅₀ index</th>
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<td>0.01</td>
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<td>0.5</td>
<td>0.007</td>
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<td>100</td>
<td>0.5</td>
<td>0.007</td>
<td>71</td>
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Table 5.1. Cytotoxic effect of IAA and derivatives under normoxia.

HRP⁺ and HRP⁺ T24, MCF-7 and FaDu cells were treated for 24 h with IAA and analogues. The selectivity index is expressed as the ratio of IC₅₀ in HRP⁺ to HRP⁺ cells.

These four IAA derivatives were also tested in T24 cells under the extreme tumour conditions of anoxia (Figure 5.8, 5.9; Table 5.2). Compared to normoxic exposure, the effects of 1-Me-IAA, 2-Me-IAA and 5-F-IAA were not modified after either 2 h- (Figures 5.5, 5.8) or 24 h anoxic incubation (Figures 5.6, 5.9). Unexpectedly, 5-Br-IAA showed selective toxicity under anoxia at 2 h, with 2-3 log cell death induced at 3 mM (Figure 5.8.D), whereas normoxic 2 h-treatment did not induce any measurable effects in both HRP⁺ and HRP⁺ cells at the concentrations analysed (Figure 5.5.D). This anoxic selectivity was lost after prolonged exposure (24 h; Figures 5.6.D, 5.9.D). The 5-Br-IAA-mediated cell kill in HRP⁺ cells was higher than that observed after anoxic treatment with any of the other prodrugs tested.
Figure 5.7. Effect of IAA analogues in human microvascular endothelial HMEC-1 cells. HRP (○) and HRP⁺ (●) HMEC-1 cells were exposed to 1-Me-IAA (A) or 5-Br-IAA (B) for 24 h. The means of at least three independent experiments ± SE are shown. The lines are interpolated.

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>T24 IC₅₀ (mM) HRP⁺</th>
<th>T24 IC₅₀ (mM) HRP⁺</th>
<th>Selectivity index</th>
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</tr>
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Table 5.2. Cytotoxic effect of IAA and derivatives under anoxia. HRP⁺ and HRP⁺ T24 cells were exposed for 24 h in anoxia to IAA and analogues. The selectivity index is expressed as the ratio of IC₅₀ in HRP⁺ to HRP⁺ cells.
Figure 5.8. Cytotoxicity of IAA analogues under anoxia (2 h-exposure).

T24 cells transiently transfected with the HRP cDNA and mock-transfected cells were exposed to the prodrugs 1-Me-IAA (A), 2-Me-IAA (B), 5-F-IAA (C) and 5-Br-IAA (D) for 2 h. ○: HRP cells; ●: HRP’ cells. The means of three independent experiments ± SE are indicated. The lines are interpolated.
Figure 5.9. Cytotoxicity of IAA analogues under anoxia (24 h-exposure).

T24 cells transiently transfected with the HRP cDNA and mock-transfected cells were exposed to the prodrugs 1-Me-IAA (A), 2-Me-IAA (B), 5-F-IAA (C) and 5-Br-IAA (D) for 24 h. ○: HRP cells; ●: HRP’ cells. The means of three independent experiments ± SE are indicated. The lines are interpolated.
The ability of 1-Me-IAA and 5-Br-IAA to induce bystander killing was tested, as described in section 3.2.4, by exposing HRP⁻ and HRP⁺ T24 cells mixed in varying proportions to 0.5 mM 1-Me-IAA or 0.1 mM 5-Br-IAA (Figure 5.10). A bystander effect similar to that induced by activated IAA (Figure 3.9.A) was observed.

![Figure 5.10. Bystander effect of IAA analogues.](image)

HRP⁻ and HRP⁺ T24 cells were mixed in the proportions indicated and treated with 0.5 mM 1-Me-IAA (A) or 0.1 mM 5-Br-IAA (B) for 24 h under normoxia (●) or anoxia (○). Means ± SE are indicated. The lines are interpolated.

5.4 Discussion
The possibility of enhancing the antitumour effect of HRP-GDEPT was investigated using a panel of different IAA analogues. The screening of ten IAA derivatives in the
four cell lines adopted in this study allowed the identification of compounds characterised by prompt normoxic (1-Me-IAA; 2-Me-IAA; 5-MeO-IAA) or anoxic (5-Br-IAA) cytotoxic activation, high HRP$^+$ cell death (5-Br-IAA; 4-Cl-IAA; 5-BnO-IAA; 5-Ph-IAA; 5-F-IAA; 1-Me-IAA) or, most importantly, selectivity (1-Me-IAA; 5-Br-IAA; 5-F-IAA; Figures 5.2-5.9; Tables 5.1, 5.2). Some variations were observed in the response of cells of different origin, with IAA, 1-Me-IAA and 5-Br-IAA representing the most promising candidates for HRP-GDEPT. The choice of the appropriate prodrug and dose may therefore depend on the tumour type, the hypoxic fraction and the pharmacokinetics in vivo.

HRP-catalysed reactions with IAA derivatives exhibit dramatic variations in rate constants with changes in substituents (Candeias et al., 1997). The presence of electron-donating groups, such as methyl or methoxy, significantly enhanced the reaction rate constants with the intermediate Cpd I, commonly used as a measure of reactivity. For most substrates, oxidation by Cpd II is much slower and not considered relevant (Wardman, 2002). Ease of oxidation was thought to represent an important parameter in the choice of HRP-associated prodrugs, since in previous studies with the purified enzyme the analogues characterised by high reaction rate also induced high lipid peroxidation (Candeias et al., 1995). In this work, surviving fractions after different IAA analogues were shown to vary over 3 orders of magnitude (Figures 5.2, 5.3), and an inverse correlation between the rate of reaction of the prodrugs with HRP and the toxicity of the active drug was observed (Figure 5.4). Although survival after 2 h-exposure did not correlate with reaction rate, after 24 h the compounds with the highest affinity for HRP were also the least toxic (Figure 5.4). Factors other than the rates of oxidation or decarboxylation by HRP may control activity. Components and substituent position may both be relevant to the cytotoxic potential, as seen from the different efficacy of 1- and 2-Me-IAA, or 5- and 6-F-IAA (Figures 5.2, 5.3), for example. Similar results were observed when IAA analogues were activated by purified HRP (Folkes et al., 1999, 2002; Rossiter et al., manuscript in preparation). Nevertheless, care should be taken when comparing the GDEPT data with those obtained with the purified enzyme, as different levels of HRP are involved. For example, for GDEPT an average amount of
~2 x10^4 ng HRP/cell (2-100 ng/ml) was detected, while 1.2 µg HRP/ml buffer were used in the experiments carried out by Folkes et al. (1998, 1999, 2002). This difference could affect the cytotoxic activation of the prodrugs, since the reaction between HRP and IAA has been shown to depend on their relative concentration (Wardman, 2002).

The putative role of MOI (Figure 1.12, 8) in IAA-induced toxicity has been discussed previously (section 3.4). However, the toxicity of halogenated MOI analogues does not clearly correlate with that of the halogenated indole precursors (Folkes et al., manuscript in preparation). Also, the formation of MOI does not explain the toxicity of 2-Me-IAA, since methylation at the indole 2-position should prevent the formation of a carbonyl group.

Interestingly, 5-Br-IAA showed very prompt and selective anoxic activation (Figure 5.8.D). This may be due to an enhanced rate of one of the as yet undetermined reaction pathways, for this particular analogue only. It may be possible for an intermediate radical to abstract bromine from the aromatic ring of 5-Br-IAA to give an aryl radical species. Under anoxic conditions, this or other species may be long-lived enough to make a significant contribution to the enhanced cytotoxicity and bystander effect observed. Further conclusions cannot be easily drawn, as the toxic species and cellular targets involved in HRP-mediated toxicity are yet to be identified. A detailed analysis of the cytotoxic pathways activated may contribute to the design of the optimised prodrugs. Nevertheless, the in vitro analysis presented here allowed the identification of compounds with potential not only in gene therapy strategies, but also in ADEPT and PDEPT approaches. Particular attention will be drawn to the action of HRP in combination with IAA, 1-Me-IAA and 5-Br-IAA.
Chapter 6
Oxic and anoxic enhancement of radiation-mediated toxicity
by HRP/IAA gene therapy

6.1 Introduction
External beam radiation therapy remains one of the most important treatment modalities for human cancers. However, injury to normal tissues restricts the total dose that can be delivered to eradicate the solid tumour. Multiple factors contribute to the resistance of solid malignancies to radiation-induced cell death, including intrinsic genetic and extrinsic physiological determinants. The presence of hypoxic regions is one of the most significant predictive factors, affecting the response to radiotherapy in a range of human tumour sites (section 1.1.1). Resistance to ionising radiation (IR) via the classical oxygen effect is unlikely to be the only explanation. Hypoxia-induced modifications of gene expression may also contribute to poor outlook, giving rise to more aggressive locoregional disease and enhanced invasive capacity (section 1.1.1).

A number of biological strategies are currently under investigation to overcome the limitations of dose delivery in conventional radiotherapy. Of interest here is the combination of gene therapy approaches with IR (reviewed by Buchsbaum et al., 1996). In particular, GDEPT systems such as HSV TK/GCV and CD/5-FC induced selective enhancement of radiation-mediated toxicity, both in vitro and in vivo (section 1.2.5). Synergistic advantage has been demonstrated, and clinical trials are ongoing (Chikara et al., 2000; Freytag et al., 2000; Teh et al., 2001). Preliminary results indicate safety of combined HSV TK-GDEPT and radiotherapy in the treatment of prostate cancer, with no added toxicity compared to the single treatments (Teh et al., 2001).

HRP-mediated GDEPT holds potential to selectively enhance radiation cytotoxicity. HRP/IAA was shown to induce DNA strand breaks and adducts (Folkes et al., 1999), and thiol depletion (Folkes et al., 2002). These biological alterations, besides causing direct cytotoxicity, may provide sensitisation to IR concomitantly administered. Therefore, the interaction of HRP/IAA with therapeutically significant doses of IR was
evaluated under both oxic and anoxic conditions. Human tumour cells transfected with the HRP cDNA were exposed to IAA or the analogue 1-Me-IAA in conjunction with X-rays.

6.2 Materials and methods

General methods can be found in Chapter 2.

6.2.1 Irradiation

For radiation treatment (exposure to 1-7 Gy X-rays), cells were incubated in air-tight aluminium boxes. For anoxic experiments, the boxes were flushed continuously with a humidified gas mixture containing 5% CO₂ and 95% N₂ and irradiated under anoxia.

6.2.2 Statistical analysis

Radiation survival data were compared by performing a multiple regression analysis. Values of p<0.05 were considered as significant. The survival curves were analysed according to the single-hit multi-target (SHMT) model (SF = 1-[1-exp(-dose/D₀)]ⁿ), by using a non-linear fitting package (JMP, SAS Institute Inc., Marlow, UK). The SHMT rather than the linear-quadratic model was adopted, since it showed a better agreement with the experimental data. The sensitiser enhancement ratio (SER) was assessed from the survival curves by the ratio of doses of radiation that reduced cell survival by 50%. Surviving fractions were normalised for plating efficiency of mock-irradiated cells exposed to HBSS or prodrug. For each data point, at least three independent experiments (triplicate samples) were performed.
6.3. Results

Prior to combined radiation/GDEPT experiments, the effect of gene delivery on the radiation response of T24 cells was analysed. Untransfected cells and transient transfectants with plasmids containing either the HRP (HRP⁺) or the CD4 (CD4⁺) coding sequence were exposed to doses of X-rays ranging from 1 to 7 Gy. The intrinsic radiosensitivity of T24 cells was not significantly altered after transfection with either foreign gene (Figure 6.1).

Figure 6.1. Response of T24 cells to X-irradiation.

Untransfected T24 cells and transfectants with the HRP or the CD4 coding sequence were exposed to increasing doses of IR. Means of at least three independent experiments ± SE are indicated. The lines are best-fit curves (SHMT model). ◊: untransfected T24 cells; ▽: CD4⁺ cells; ▼: HRP⁺ cells.
In the presence of the prodrug IAA, a marked increase in sensitivity to radiation was selectively induced in HRP+ cells (Figure 6.2). HRP-expressing cells incubated for 24 h with 0.1 mM or 0.5 mM IAA prior to (Figure 6.2.B) or immediately after (Figure 6.2.D) irradiation demonstrated a significant enhancement of radiation-mediated toxicity. On the other hand, no significant difference in the response to radiation was observed in CD4+ exposed to IAA (Figure 6.2.A, C). The results shown in Figure 6.2 did not change whether the prodrug was present or not at the time of irradiation (data not shown).

The sensitiser enhancement ratio (SER) can be defined as the quotient of two radiation doses which cause the same cell kill (Lambin et al., 2000). In this work, 50% cell kill was considered as an endpoint. The survival curves were analysed according to the SHMT model, normalising for the plating efficiency of mock-irradiated cells exposed to the prodrug. In HRP+ T24 cells pre-incubated with IAA, SERs of 2.6 (0.1 mM IAA) and 5.4 (0.5 mM IAA) were measured. Very similar values (2.5 and 5.6) were estimated when prodrug incubation was performed after irradiation.

To confirm the observed effect in another tumour cell line, FaDu cells were exposed to a single radiation dose in combination with HRP/IAA GDEPT. Similarly to T24 cells, after pre-incubation with IAA, HRP+ FaDu cells showed a significant increase in IR-induced cell kill, while the response of control CD4+ FaDu cells was unaffected (Figure 6.3).
Figure 6.2. Effect of HRP/IAA GDEPT on the radiosensitivity of T24 cells under normoxia. CD4⁺ (A, C) and HRP⁺ (B, D) cells were exposed to 0.1 mM or 0.5 mM IAA for 24 h prior to (A, B) or after (C, D) irradiation. The data are means ± SE. Lines are best-fit curves (SHMT model). Cell survival curves were normalised for the plating efficiency of mock-irradiated cells exposed to IAA, to account for drug toxicity. –∇–: CD4⁺ T24 cells, no prodrug; –○–: CD4⁺ cells + 0.1 mM IAA; –■–: CD4⁺ cells + 0.5 mM IAA; –▼–: HRP⁺ cells, no prodrug; –◆–: HRP⁺ cells + 0.1 mM IAA; –□–: HRP⁺ cells + 0.5 mM IAA.
Figure 6.3. Effect of HRP/IAA GDEPT on the radiosensitivity of FaDu cells in normoxia. CD4' and HRP' cells were exposed to 0.1 mM or 0.5 mM IAA for 24 h prior to irradiation (5 Gy). Means ± SE are shown. The surviving fraction at 5 Gy was normalised to exposure to IAA only. Hashed bars: CD4' cells; solid bars: HRP' cells. *: significantly different from cells exposed to radiation only; **: significantly different from CD4' cells after identical treatment (p<0.05).

The efficacy and selectivity of HRP/IAA GDEPT may be improved by adopting different IAA derivatives (Chapter 5). Therefore, the use of 1-Me-IAA in combination with IR was studied. As illustrated in Figure 6.4, HRP' T24 cells exposed to 0.1 mM or 0.5 mM 1-Me-IAA before or after radiation showed increased cell kill compared with cells exposed to buffer only (Figure 6.4.B, D), while no sensitisation was induced in control CD4-transfectants (Figure 6.4.A, C). SER values were calculated to be 2.8 (0.1 mM 1-Me-IAA) and 4.6 (0.5 mM 1-Me-IAA), when the prodrug was administered for 24 h before X-rays, and 2.1 and 3.0, respectively, if prodrug incubation took place immediately after irradiation. Therefore, no major differences were detected in the radiosensitisation potential of the two prodrugs tested (IAA and 1-Me-IAA), with IAA being slightly more potent.
Figure 6.4. Combination of HRP/1-Me-IAA GDEPT with radiation.

CD4⁺ (A, C) and HRP⁺ (B, D) cells were exposed to 0.1 mM or 0.5 mM 1-Me-IAA for 24 h prior to (A, B) or after (C, D) X-rays. The data are means of at least three independent experiments ± SE. Lines are best-fit curves. Cell survival curves were normalised to exposure to prodrug only. − ▽ − : CD4⁺ T24 cells, no prodrug; - ◊ : CD4⁺ cells + 0.1 mM 1-Me-IAA; - □ − : CD4⁺ cells + 0.5 mM 1-Me-IAA; - ▼ − : HRP⁺ cells, no prodrug; - ● : HRP⁺ cells + 0.1 mM 1-Me-IAA; - ■ : HRP⁺ cells + 0.5 mM 1-Me-IAA.
A synergistic effect of HRP/IAA GDEPT in combination with radiation could be of even higher therapeutic impact if effective in hypoxic cells, since the presence of hypoxic areas in solid tumours has been shown to correlate with poor outcome after radiotherapy. T24 cells irradiated under anoxia showed an oxygen enhancement ratio (OER) of ~2, i.e. double the dose of radiation was necessary to induce 50% cell kill compared with oxic cells. When pre-incubated with 0.1 mM IAA, HRP⁺ cells showed a 3.6-fold increase in sensitivity to IR (Figure 6.5.B), and the OER was reduced to 1.2. The response of control cells transfected with the marker CD4 was not significantly different whether the monolayers were pre-exposed to IAA or buffer only (Figure 6.5.A).

6.4. Discussion

The use of gene therapy protocols to enhance the effect of IR holds promise for anticancer therapy. The results presented here show that human tumour cells engineered to express the plant enzyme HRP, which selectively activates the prodrug IAA into a cytotoxic agent, show a substantial increase in radiation-induced toxicity under normoxic as well as anoxic conditions.

Statistical evaluation of combined radiation and GDEPT protocols had previously shown that in vitro SER values of 1.2 can significantly increase local control after radiotherapy (Lambin et al., 2000). In our studies, values between 2.1 and 5.6 were measured under oxic conditions, depending on prodrug concentration. These values compare favourably with in vitro published data on other GDEPT approaches, as incubation with 5-FC induced a SER of 2.8 in CD⁺ cells (Khil et al., 1996), exposure to GCV resulted in a SER of ~2 in HSV TK⁺ cells (Kim et al., 1994) and the combined prodrugs induced an SER equal to 4.5 in cells producing a CD-HSV TK fusion protein (Blackburn et al., 1999). Moreover, HRP/IAA-mediated sensitisation could be detected at doses of radiation as low as 1 Gy, indicating that doses used in a multifractionated treatment (usually 2 Gy/fraction over 6-8 weeks) in a clinical setting may be effective.

Significant synergy of HRP/IAA GDEPT and X-ray exposure was also detected under the extreme condition of anoxia. Anoxic HRP⁺ cells pre-exposed to 0.1 mM IAA
were 3.6-fold more sensitive to radiation than buffer-treated controls, and the OER was reduced from 2 to 1.2.

**Figure 6.5.** Effect of HRP/IAA GDEPT on the radiosensitivity of T24 cells under anoxia. CD4+ (A) and HRP+ (B) cells were exposed to 0.1 mM IAA for 24 h prior to irradiation. Means of three independent experiments (triplicate samples) ± SE are shown. Lines are best-fit curves. Cell survival was normalised to exposure to IAA, to account for drug toxicity. Survival data for oxic cells without IAA is shown for comparative purposes. — ▽—: CD4+ T24 cells, no prodrug, normoxia; — △—: CD4+ cells, no prodrug, anoxia; — ◊—: CD4+ cells + 0.1 mM IAA, anoxia; — ▼—: HRP+ cells, no prodrug, normoxia; — ▲—: HRP+ cells, no prodrug, anoxia; — ◆—: HRP+ cells + 0.1 mM IAA, anoxia. Inset: survival of CD4+ cells irradiated in normoxia and anoxia replotted for clarity.

Delivery and expression of a foreign gene alone (HRP or the marker CD4) did not alter cellular radiosensitivity. Similar findings were reported by other investigators who
utilised retroviral (Kim et al., 1994; Khil et al., 1996) or adenoviral vectors (Hanna et al., 1997; Blackburn et al., 1999) for GDEPT. Moreover, Mansur et al. (2001) observed that neither retroviral nor liposome-mediated delivery of the glutathione peroxidase gene resulted in an effect on radiosensitivity of human T cells and Chinese hamster ovary (CHO) fibroblasts. On the other hand, an increase in sensitivity to IR was observed in cells transduced with Ads containing the CD gene even in the absence of 5-FC (Pederson et al., 1997; Stackhouse et al., 2000).

True radiation sensitisers have no lethal effect of their own. The prodrugs tested here reduced the survival of HRP\(^+\) cells to ~30% at 0.1 mM and ~0.6% at 0.5 mM after 24 h (Chapter 3). Further selective toxicity could be achieved with the addition of IR. These concentrations of prodrug were found to kill less than 20% of HRP\(^-\) T24 and FaDu cells, they are likely to be achievable in vivo (tumour prodrug levels of ~1 mM can be obtained in mice, with no associated toxicity; J. Tupper, unpublished data), and therefore may represent a possible clinical scenario.

Limited transfection efficiency (10-14% in FaDu and 20-25% in T24 cells) compared with the levels of cell kill measured (Figures 6.2-6.5) indicated the induction of a bystander effect, whereby the activated prodrug induced radiosensitisation also in surrounding untransfected tumour cells.

Similar SERs were measured when prodrug incubation was performed prior to or after X-rays, suggesting that activated IAA may increase susceptibility to IR and also interfere with post-irradiation repair processes. To date, the activated drugs and the cellular targets involved in HRP/IAA-mediated cytotoxicity and radiosensitisation have not been identified. Exposure of hamster fibroblast V79 cells to IAA or 5-F-IAA activated by purified HRP resulted in a decrease of cellular glutathione (GSH) up to ~70% of control levels (Folkes et al., 2002). Moreover, the addition of excess GSH to HRP/IAA-generated oxidation products significantly reduced cytotoxicity (Folkes et al., 1998), suggesting that potentially cytotoxic products had reacted with this thiol. Depletion of intracellular thiols has been shown to have a radiosensitising effect in vitro

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(Clark et al., 1984; Varnes et al., 1984; Biaglow et al., 1986) and in vivo (Bump et al., 1982; Stevens et al., 1995). The effect has been observed under both oxic and anoxic conditions (Varnes et al., 1984; Debieu et al., 1985), with preferential sensitisation of cells at low or intermediate oxygen levels (Bump et al., 1982; Clark et al., 1984; Debieu et al., 1985; Scott et al., 1993; Stevens et al., 1995). GSH depletion did not induce any significant alteration of the OER in normal mouse skin (Stevens et al., 1995), V79 (Mitchell et al., 1983) and A549 human lung carcinoma cells (Biaglow et al., 1983 a; Mitchell et al., 1983). A decrease in OER has been observed in cells that show no oxic response to thiol depletion (Biaglow et al., 1983 b).

However, GSH depletion is unlikely to be solely responsible for the effects shown in this chapter. The reduction in GSH levels observed by Folkes et al. (2002) is probably insufficient to account for the SERs achieved here, as a reduction of >90% was shown to be necessary to achieve full radiosensitisation (Clark et al., 1984). Moreover, GSH depletion would only be expected to radiosensitise prior to irradiation, while HRP/IAA was shown to induce sensitisation also after IR. Additional biochemical alterations, probably involving the DNA macromolecule, are likely to take place. Further studies are warranted to shed light on the possible mechanisms associated with HRP/IAA-mediated enhancement of IR toxicity.

With 50% of all human cancer patients treated with radiation, improvement of the efficacy of a radiotherapy schedule remains a major issue in cancer research and treatment. Numerous clinical trials have focused on combining IR with conventional chemotherapeutic agents, such as doxorubicin, methotrexate, 5-FU and CDDP (Stewart and Saunders, 1997). However, normal tissue toxicity remains a major limiting factor with these protocols. The results presented here demonstrate that IAA and 1-Me-IAA selectively enhance radiation-induced lethality in human tumour cells expressing the enzyme HRP at doses of prodrug achievable in vivo. In order to achieve a significant advantage by using combined gene therapy and radiotherapy protocols, prodrug activation should take place at the tumour site only. This can be obtained by selective HRP gene delivery and/or selective gene expression at the target, as suggested in Chapter 7.
Chapter 7
Development of chimeric gene promoters responsive to hypoxia and ionising radiation for HRP/IAA GDEPT

7.1 Introduction

Tumour hypoxia, despite being an independent prognostic marker of poor treatment outcome, represents a physiological difference that may be exploited for selective cancer treatment. In particular, hypoxia-targeted GDEPT has the potential to overcome the radioresistance of poorly oxygenated cells, by taking advantage of the tumour-specificity of the hypoxic microenvironment for gene delivery, gene expression and prodrug activation.

Hypoxia regulatory elements (HREs) are cis-acting sequences containing the core motif 5’-(A/G)CGT(G/C)(G/C)-3’ (section 1.2.3). They are found in the enhancer region of several hypoxia-regulated genes and contain the binding site of the transcription factor HIF-1. The high frequency of HIF-1 expression across human tumours of diverse tissue origin (Zhong et al., 1999; Talks et al., 2000) represents a possible therapeutic target for HRE-directed gene therapy of the hypoxic environment (Dachs et al., 1997). Indeed, promoters containing HREs have been used to selectively control therapeutic gene expression in hypoxic cells (section 1.2.3).

Hypoxia-targeted gene therapy is likely to be part of a combination therapy, and, with a view to targeting the oxic tumour population using conventional radiotherapy, increased therapeutic gain could be achieved both at the promoter and at the therapeutic gene level. The limitations of dose delivery in radiotherapy might be overcome by gene therapy systems that can enhance IR-mediated cytotoxicity, such as HRP/IAA (Chapter 6). Furthermore, radiation itself may be exploited for selective transgene expression, as it has been shown to activate a number of genes involved in cell cycle progression, DNA damage sensing and repair (section 1.2.4). IR-mediated transcriptional activation of the Egr-1 gene was demonstrated, and the essential sequences were identified as 10 nucleotide motifs of the consensus sequence CC(A/T)nGG, also known as CArG.
elements (Datta et al., 1992). ROS were recognised to be involved in CArG-mediated induction, since a similar activation pathway was observed after cell exposure to H$_2$O$_2$ or IR, which was abrogated by the concurrent use of antioxidants (Datta et al., 1993). Efficacy and selectivity of CArG-based promoters for cancer gene therapy has been demonstrated \textit{in vitro} and \textit{in vivo} (section 1.2.4).

As radiotherapy is likely to remain one of the primary cancer treatment modalities, approaches to increase tumour cell kill for a given clinical dose have potential to improve patient treatment. Furthermore, new therapeutic systems that can also address the inherent problems of hypoxia may be particularly advantageous. The aim of the work presented in this chapter was the development of vectors responsive to these tumour conditions, via the use of novel, chimeric gene promoters containing HREs and CArG elements. The potential of such promoters to successfully control HRP-mediated GDEPT was investigated.

7.2 Materials and methods

General methods are in Chapter 2.

7.2.1 Vector construction

The plasmids used in this chapter were constructed in collaboration with Dr. S.D. Scott. The backbone vector pCI-puro (Figure 7.1) was made by replacing the neomycin resistance cassette from pCI-neo (Clontech, Basingstoke, UK) with the puromycin resistance gene from pPUR (Clontech) as a \textit{Bam} HI/\textit{Pvu} II fragment (all enzymes from Life Technologies and Promega).

Chimeric promoters were produced by replacing the enhancer region of the CMV gene promoter in pCI-puro with double-stranded linker molecules, derived from single-stranded oligo-deoxyribonucleotides (ODNs; from MWG-Biotech, Milton Keynes, UK; Table 7.1). Complementary ODNs were annealed by mixing 50 pmole of each ODN in a 20 µl volume, heating to 55°C for 5 min, then cooling to rt. 0.5 pmole double-stranded linkers were inserted into 200 ng vector digested with the \textit{Bgl} II and \textit{Sgf}I enzymes.

The plasmids pPGK1-puro, pEpo-puro and pnVEGF-puro were constructed by inserting synthetic enhancers containing five copies of the respective HRE units (Table
The EGFP coding sequence was excised from pEGFP-N1 (Clontech) by EcoRI/NotI digestion, and the resulting fragment inserted into each vector linearised with the same restriction enzymes (Figure 7.2). The plasmid containing nine CArG elements (pE9-EGFP; Figure 7.2) was donated by Dr. S.D. Scott (Scott et al., manuscript in preparation). For the constructs containing both HREs and CArG elements, pE9-EGFP was digested with BglII, treated with calf intestinal alkaline phosphatase, and the HREs were inserted directly upstream of the CArG elements. For pVEGF/E9-EGFP, this meant the addition of a single linker containing five HRE units (Table 7.1; Figure 7.2). For pPGK1/E9-EGFP and pEpo/E9-EGFP, two separate BglII cloning stages were needed, because of unit rearrangements. First, three HRE units were inserted upstream of E9, followed by a further two HRE units making a total of five in the final enhancer (Table 7.1; Figure 7.2). The first sets of ODNs were designed to produce BglII ‘locks’, in order to allow the second linkers to be added without losing the first BglII recognition sequence. Finally, for the construct containing ten PGK-1 HRE units plus E9 (pPGK110/E9), two five-unit ODNs were cloned, in one ligation reaction, adjacently and immediately upstream of E9 (Table 7.1).

**Figure 7.1.** The backbone vector pCI-puro.

The restriction sites utilised for the cloning procedures described in this chapter are indicated.
HRP-containing plasmids were made by a two-stage process. First, the HRP cDNA from pRK34-HRP (Connolly et al., 1994) was subcloned into pBluescript KS (Stratagene Ltd., Cambridge, UK) using Bam HI. This construct was then digested with Sma I/Not I, and the HRP fragment inserted into the series of recipient vectors which had been linearised with EcoR I, blunt-ended with DNA Pol I Large Klenow fragment, and then Not I-digested.

Finally, to produce the pCD4 negative control plasmid, the CD4 gene was excised from pMACS 4.1 (Miltenyi Biotech, Bergisch Gladbach, Germany) by Xho I/Pst I digestion and inserted in pBluescript KS. The gene was then subcloned by EcoR I/Not I digestion into pCI-puro. The sequence integrity of all plasmids was confirmed by DNA sequencing (section 2.6).

7.2.2 Cell transfection
Transfectants with the EGFP-constructs were obtained by using Lipofectamine (Life Technologies; section 2.7.2). Transient transfectants with the HRP-constructs were obtained by exposing the cells to complexes of Lipofectin (Life Technologies), integrin-targeted peptides and DNA (section 2.7.1) for more efficient transfection. In all cases, the cells were exposed to hypoxia and/or IR 24 h after transfection.

7.2.3 Irradiation procedure
Cells were irradiated using a Pantak X-ray generator. For hypoxic irradiation, cells transfected in air were pre-equilibrated at 0.1% O₂ for 24 h and then exposed to X-rays in airtight aluminium boxes. Gene expression was assayed 24 h post-irradiation.
<table>
<thead>
<tr>
<th>Plasmid vector</th>
<th>ODN sequence</th>
</tr>
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| pPGK1          | 5' - GATCTGGCGCGCCC(TGTCACGTCTGCACGAC)₃TTAATTAAGCGAT-3'  
|                | 5' - CGCTTAATTAA(GTCGTGCAGGACGTGACA)₃GGCGCGCCA-3' |
| pEpo           | 5' - GATCT(GGCCCTACGTGCTGTCTCACACA)₂GCGAT-3'  
|                | 5' - CGC(TGTCGTACAGCACGTAGGGCC)₃A-3' |
| pnVEGF         | 5' - GATC(TTGATACGTCTGCACGAC)₂GCGAT-3'  
|                | 5' - CGC(TGTCGTACAGCACGTAGGGCC)₃A-3' |
| pE9            | 5' - GATCT(CCTTATTTGG)₃CGAT-3'  
| 1st step       | 5' - CGC(GGAATAAGG)₃A-3' |
| pE9            | 5' - CGC(CCTTATTTGG)₃GCGAT-3'  
| 2nd step       | 5' - CGC(CCAAAATAAGG)₃GCGAT-3' |
| pPGK1/E9       | 5' - GATC(TGTCACGTCTGCACGAC)₃-3'  
| 1st step       | 5' - GATC(GTCGTGCAGGACGTGACA)₃-3' |
| pPGK1/E9       | 5' - GATC(TGTCACGTCTGCACGAC)₂-3'  
| 2nd step       | 5' - GATC(GTCGTGCAGGACGTGACA)₂-3' |
| pEpo/E9        | 5' - GATCTGGCGCGCCC(TGTCACGTCTGCACGAC)₂GCGAT-3'  
| 1st step       | 5' - GATC(TGTCGTACAGCACGTAGGGCC)₃A-3' |
| pEpo/E9        | 5' - GATCTACGTCTGCACGAC)₂GCGAT-3'  
| 2nd step       | 5' - GATC(TGTCGTACAGCACGTAGGGCC)₃A-3' |
| pnVEGF/E9      | 5' - GATC(TTGATACGTCTGCACGAC)₂GCGAT-3'  
|                | 5' - GATC(TGTCGTACAGCACGTAGGGCC)₃A-3' |
| pPGK1/E9       | 5' - GATC(TGTCACGTCTGCACGAC)₂GCGAT-3'  
| 2 steps        | 5' - GATC(TGTCGTACAGCACGTAGGGCC)₃A-3' |

**Table 7.1.** Single-stranded oligo-deoxyribonucleotides (ODNs) used in this study.

The sequence of complementary ODNs for each plasmid vector is shown. For pE9, pPGK1/E9 and pEpo/E9, two separate cloning stages were needed. The proposed HIF-1 binding sites are shown in bold, CArG elements are underlined. E9: 9 CArG elements; Epo: erythropoietin; PGK1: phosphoglycerate kinase-1; nVEGF: novel sequence based on the vascular endothelial growth factor (VEGF) HRE.
Figure 7.2. Schematic representation of plasmid constructs containing the EGFP coding sequence used in this chapter.

All plasmids were based on the backbone vector pCI-puro (Figure 7.1). Restriction sites in brackets were deliberately deleted during the cloning procedure (Bgl II “locks”).
7.2.4 Analysis of gene expression

Transfectants were harvested, washed in PBS and re-suspended in HBSS. Relative EGFP fluorescence was measured by FACS analysis. Dead cells were gated out of the analysis by forward and side scatter. Cells were scored as positive if they showed an increased fluorescence with respect to control cells transfected with pCD4. The level of hypoxia- and/or IR-induced EGFP expression was indicated as the percentage of EGFP-expressing cells in the treated sample, compared with the corresponding oxic/sham-irradiated sample transfected with the same plasmid. HRP activity was analysed as described previously (section 2.10).

7.2.5 Clonogenic assays

Following hypoxic and/or radiation exposure, HRP\(^{-}\) and HRP\(^{+}\) cells were plated at low density, left to settle for 5-6 h and exposed to IAA in HBSS for 24 h in the 37°C incubator. Surviving fractions were normalised for plating efficiency of cells exposed to HBSS only, after equal trigger treatment.

7.3 Results

7.3.1 Construction of synthetic promoters

Hypoxia- and radiation-responsive enhancer components were placed immediately upstream of the basal CMV gene promoter in all vector constructs (Figure 7.2). The synthetic promoters contained either five directly repeated HREs, derived from three different genes, or nine directly repeated CArG elements (E9) derived from the Egr-1 gene, or five HREs upstream of E9 (Figure 7.2). The three different HREs tested were from the human Epo gene, from the murine PGK-1 gene, and a novel sequence based on the human VEGF HRE (nVEGF; designed by Dr. A.V. Patterson, University of Manchester, Manchester, UK/University of Auckland, Auckland, New Zealand). The Epo enhancer was composed of 24 bp units, including a second 5 bp element 3’ to the HIF-1 binding site, previously shown to be essential for hypoxia-inducibility (Wang and Semenza, 1993). The HRE from PGK-1 consisted of an 18 bp sequence that could fully retain its enhancer activity (Firth et al., 1994). In the nVEGF enhancer, the core of the HIF-1 binding site in the PGK-1 promoter, found to induce robust expression levels
(Boast et al., 1999), was fused to the 5' sequences from the VEGF HRE, characterised by high inducibility (Shibata et al., 2000).

### 7.3.2 Induction of HRE and CArG enhancers by separate hypoxia or radiation treatments

The activity of the promoters was analysed in transient transfection experiments using human MCF-7 (breast adenocarcinoma) and T24 (bladder carcinoma) cells. Both cell lines were shown to express HIF-1α after hypoxic induction (An et al., 1998; C. Coralli, personal communication).

Even outside their natural promoter context, all synthetic HRE enhancers were activated after 24 h hypoxic (0.1% O₂) exposure, followed by 7 h reoxygenation, in both cell types (Figure 7.3). The fold induction of EGFP expression levels in hypoxic T24 cells was 2.7 (±0.5), 3.0 (±0.4) and 2.1 (±0.2), using the PGK1, Epo and nVEGF enhancers, respectively. Similarly, 2.1- (±0.3), 2.5- (±0.4) and 2.6- (±0.6) fold inductions were seen in MCF-7 cells. Net expression levels under hypoxia from all HREs were equal to or greater than those of the strong, constitutive CMV promoter under normoxia. Interestingly, besides being activated by IR, the E9 enhancer also produced a 2.5- (±0.2) fold greater level of EGFP expression in hypoxic compared with normoxic T24 cells (Figure 7.3). Only a marginal induction (1.3±0.1) was detected in hypoxic MCF-7 cells (Figure 7.3). Similarly, radiation was able to activate the PGK1 and nVEGF HRE enhancers, but not Epo (Figure 7.4), with induction levels after 5 Gy of 2.4±0.9 (PGK1) and 1.7±0.1 (nVEGF) in T24 cells, and 1.7±0.3, and 1.3±0.1 in MCF-7, respectively. Radiation-inducibility of the HREs has been confirmed in independent work (K.J. Williams, N. Chadderton and S. Robinson, University of Manchester, personal communication). Finally, the CMV promoter was slightly down-regulated by both treatments (Figures 7.3, 7.4).
**Figure 7.3.** Induction of the synthetic promoters under hypoxia.

T24 (A) and MCF-7 (B) tumour cells transiently transfected with the constructs in Figure 7.2 were incubated under 0.1% O₂ for 24 h, followed by 7 h reoxygenation. The fold induction was defined as the ratio of EGFP expression after hypoxia compared with normoxic treatment. Results of at least three independent experiments (duplicate samples) ± SE are shown. *: significantly different from E9; **: significantly different from the corresponding HRE (p<0.05).
Figure 7.4. Induction of the synthetic promoters following ionising radiation (IR).

T24 (A) and MCF-7 (B) transient transfectants were exposed to a trigger dose of 5 Gy. The fold induction was defined as the ratio of EGFP expression after IR compared with sham-treatment. Results are means of at least three independent experiments (duplicate samples) ± SE. **: significantly different from the corresponding HRE (p<0.05).
Similar results were obtained by HRP activity assay in T24 cells transfected with the constructs in Figure 7.2, in which the EGFP coding sequence was replaced with the HRP cDNA (Figure 7.5). Unfortunately, due to the low sensitivity of the assay, a high variation within experiments resulted in standard errors that did not allow a further analysis of the results. It was therefore decided to proceed to measuring promoter activity by analysing the cytotoxic effect of the HRP/IAA combination using clonogenic assays (section 7.3.5).

7.3.3 Induction of the HRE/CArG combinations by hypoxia or radiation

To obtain combined hypoxia- and radiation-inducible transcription from the same enhancer, chimeric promoters were constructed containing combinations of HREs and CArG elements. Following hypoxia, all the enhancers tested showed some degree of induction, and, except for the PGK-1 HRE in T24 cells, the presence of the E9 did not inhibit the hypoxic response of the HREs. After 24 h 0.1% O₂ treatment (Figure 7.3), PGK1/E9, Epo/E9 and nVEGF/E9 enhancers showed induction levels of 1.4 (±0.2), 3.0 (±0.6) and 2.0 (±0.4) in T24 cells, and of 1.9 (±0.4), 3.4 (±0.2) and 1.3 (±0.2) in the MCF-7 cell line. The addition of a further five copies of the PGK-1 HRE in the construct pPGK1₁₀/E9 did not significantly increase the hypoxia-inducibility in T24 cells (fold induction of 1.7±0.3), and slightly diminished it in MCF-7 cells (1.4±0.5).

Analogously, all the HRE/E9 chimeric enhancers were activated to some extent by radiation. In T24 cells (Figure 7.4.A), fold-inductions were 1.9 (±0.6), 1.9 (±0.2), 1.5 (±0.3) for PGK1/E9, Epo/E9 and nVEGF/E9 respectively. In MCF-7 cells (Figure 7.4.B), induction levels of 1.2 (±0.1), 1.4 (±0.1) and 1.6 (±0.1) were observed. This compared with 1.8- (±0.1) and 1.7-fold (±0.2) inductions when nine isolated CArG elements were similarly tested in T24 and MCF-7 cells, respectively.

The Epo/E9 combination was the most robust, both in terms of absolute induction values and consistency of response in individual experiments.
Figure 7.5. Induction of HRP activity using synthetic promoters.

T24 transfectants with plasmids containing HRE or CArG enhancers controlling the expression of HRP were exposed to 0.1% O2 for 24 h followed by 7 h reoxygenation (A), or 5 Gy X-rays (B). The fold induction was defined as the ratio of HRP activity after hypoxia or radiation compared with mock treatment. Results of at least three independent experiments (duplicate samples) ± SE are shown.
7.3.4 Induction of enhancers by dual hypoxia and radiation treatment

The induction of the series of enhancers was examined following hypoxic irradiation of transfected cells, in order to mimic treatment conditions within a solid tumour. All promoters tested showed activation after this dual treatment, in both cell lines (Figure 7.6). The greatest induction was seen with the Epo/E9 hybrid enhancer (3.0±0.4 in T24 and 3.5±0.3 in MCF-7 cells), notably higher than with the individual components tested separately (2.0±0.2 (Epo) and 2.4±0.4 (E9) in T24 cells, 2.4±0.5 (Epo) and 1.7±0.3 (E9) in MCF-7 cells). However, for the other two HREs there appeared to be no advantage gained by combination with CArG elements. When the PGK110/E9 enhancer was tested after hypoxic irradiation, the presence of the extra five HREs did not change induction levels in MCF-7 cells, and caused a reduction in response in T24 cells. As expected, the CMV promoter showed no induction in either cell type (0.8±0.2 in T24 cells and 1.0±0.2 in MCF-7 cells).

The Epo/E9 combination again proved to be the most responsive and provided the most useful profile in terms of low basal levels, high responsiveness and experimental reproducibility. It was thus chosen for subsequent GDEPT assays and compared with the model single enhancer components.

7.3.5 Hypoxia- and radiation-regulated HRP/IAA GDEPT

Once the responsiveness of the HRE/CArG promoters was assessed with the EGFP reporter, their potential to effectively control HRP/IAA GDEPT was assessed in vitro. For this purpose, the EGFP coding sequence was replaced with the HRP cDNA in the vectors containing either the full-length CMV promoter or the Epo, E9 or Epo/E9 enhancers. The pCD4 plasmid encoding the marker CD4 was used as a negative control. After promoter induction by hypoxia and/or IR, transfected T24 cells were exposed to IAA for 24 h under normoxia. The IAA dose used (0.5 mM) chosen killed less than 20% HRP-negative cells (Chapter 3).

As expected, when the strong CMV promoter was used to control HRP expression, significant toxicity was seen in the presence of IAA, although reduced after hypoxic pre-induction (Figure 7.7). When either the Epo or the Epo/E9 enhancers controlled HRP, 75% and 80% cell death, respectively, was demonstrated after hypoxic promoter
induction, while little toxicity was detected in uninduced oxic cells (Figure 7.7.A). Similarly, transfectants containing the pE9-HRP construct were more sensitive to IAA after a 5 Gy trigger dose as compared with sham irradiation (15% vs. 84% surviving fraction; Figure 7.7.B). The Epo/E9 promoter had a comparable effect to E9 after the same treatment (Figure 7.7.B). Finally, efficient and selective prodrug-mediated toxicity was observed when the Epo/E9 promoter controlled the expression of HRP after hypoxic irradiation, reducing the survival fraction to 20% (Figure 7.7.C). In all cases, the induced synthetic promoters resulted in a surviving fraction comparable to that of CMV in air. When T24 cells were transfected with the CMV-controlled pCD4 plasmid, little or no toxicity was detected under all treatment conditions (Figure 7.7).
Figure 7.6. Induction of the synthetic promoters after dual hypoxia and radiation treatment.

T24 (A) and MCF-7 (B) transfectants were exposed to 0.1% O₂ for 24 h, followed by 5 Gy X-rays administered under hypoxia. Levels of EGFP expression were assessed after 7 h reoxygenation. Results of at least three independent experiments (duplicate samples) ± SE are shown. *: significantly different from E9 (p<0.05).
Figure 7.7. Hypoxia- and radiation-mediated HRP/IAA GDEPT.
T24 cells transfected with plasmid constructs containing the HRP cDNA controlled by HRE- and/or CArG-containing promoters were exposed to the inducing stimuli of hypoxia (A), radiation (B) or both (C). The cells were subsequently re-seeded at low density and incubated under normoxia for 24 h with 0.5 mM IAA. The control plasmid contains the CD4 coding sequence. Open symbols: oxic/sham-treated cells; solid symbols: hypoxic/irradiated cells. The means of three independent clonogenic survival experiments (triplicate samples) ± SE are shown.
7.4 Discussion

Combining gene therapy and radiotherapy protocols has the potential to overcome many of the limitations of adverse tumour biology on cancer treatment. The combination of HREs and radiation-responsive CArG elements was found to induce selective and robust transgene expression under separate or concomitant stimuli, and also to effectively control HRP-mediated GDEPT.

One of the main goals of gene therapy is to selectively express a therapeutic gene at the tumour site. Tumour-specific gene expression may increase anti-cancer efficacy and avoid side effects, even if the foreign DNA is delivered to normal tissues. In particular, artificial hybrid promoters can potentially target a wide range of cancer types, without losing specificity. They are also characterised by flexibility, because several different elements can be arranged in various orders, numbers and relative orientations. Dual-specificity promoters have been described in the literature containing HREs in combination with oestrogen responsive promoters (Hernandez-Alcoceba et al., 2001), cytokine-inducible (Modlich et al., 2000) and hepatoma-associated enhancers (Ido et al., 2001). The results shown here indicate that combinations of HREs and CArG elements (also known as serum response elements or SREs) can act as gene enhancers, responding to hypoxic and radiation stimuli. In particular, the Epo/E9 enhancer showed good inducibility under all conditions, and the highest levels of gene expression when both stimuli were present (Figures 7.3, 7.4, 7.6).

An example of endogenous transcriptional co-operation between an HRE and a CArG element has been observed in the 5’ enhancer of the murine glucose transporter (Glut)-1 gene (Ebert et al., 1995). The same CArG sequence was shown to be responsible for HIF-1-independent hypoxic activation of the c-fos gene, via a mitogen-activated protein kinase (MAPK) pathway, involving the ternary complex factor Elk1 (Müller et al., 1997). However, when isolated from its natural promoter context, this specific CArG element was found to respond poorly to IR (Scott et al., manuscript in preparation), and was therefore not adopted in the present study. The CArG elements used here, previously shown to be induced by X-ray doses as low as 1 Gy (Marples et
al., 2000), were also responsive to hypoxia in T24 cells and, to a lesser extent, in MCF-7 cells (Figure 7.3). Fast transcriptional inducibility of the Egr-1 gene after hypoxia has been observed in vivo in the lung of mice breathing ~6% O₂ (Yan et al., 1999), and in vitro in endothelial cells (Lo et al., 2001) and mononuclear phagocytes (Yan et al., 1999, 2000). Mutational analysis indicated that the distal Ets binding site (EBS)-SRE elements were critical for hypoxia-responsiveness (Yan et al., 1999). The Egr-1 hypoxic activation, via an Elk1/serum response factor (SRF) complex, was analogous to that of c-fos (Müller et al., 1997; Yan et al., 1999). The effect reported here is most likely to be the result of a hypoxic stress-associated pathway rather than a direct response to a change in ambient oxygen levels, the latter a specific characteristic of HIF-1. ROS contribute to the induction of the CArG elements, as transcriptional activation was observed after IR as well as H₂O₂ exposure (Datta et al., 1992, 1993). Reoxygenation (González-Flecha et al., 1993) and hypoxia itself (Chandel et al., 1998, 2000) increase the intracellular levels of ROS, and indeed Egr-1 mRNA accumulated in mouse and rat kidney after ischemia/reperfusion (Bonventre et al., 1991).

Three different HRE sequences were analysed, from the Epo and PGK-1 genes, and a novel element (nVEGF) based on a consensus derived from the VEGF and PGK-1 HRE sequences (designed by Dr. A.V. Patterson). Although, nVEGF was more responsive than the other HREs under some circumstances, it didn’t exhibit an overall advantage (Figures 7.3, 7.4, 7.6). Also, no increase in gene expression was observed when five extra HREs were added in the PGK1₁₀/E9 promoter, compared to PGK1/E9 (Figures 7.3, 7.4, 7.6). A similar saturation effect has been observed for promoters containing more than five copies of the VEGF HRE (Shibata et al., 2000), or duplicating an LDH-A HRE dimer or Eno-1 and PGK-1 trimers (Boast et al., 1999). On the other hand, a steep increase in hypoxia inducibility has been reported when the number of copies of Epo HREs was increased from six to nine (Ruan et al., 2001).

All HREs were significantly induced by hypoxia (Figure 7.3), with hypoxic gene expression as high as the strong CMV promoter. The PGK1 and nVEGF enhancers were also activated by exposure to 5 Gy X-rays, while no radiation response was observed in the cells transfected with pEpo-EGFP (Figure 7.4). A cell line-dependent IR-inducibility
of PGK-1, LDH-A and Epo HREs has been observed in another laboratory (Chadderton, Robinson et al., manuscript in preparation). Moreover, when the PGK HRE utilised in this chapter was inserted in the SV40 promoter, a 40-60-fold increase in luciferase activity was measured after 5 Gy administered under 1% O₂ (N. Chadderton, K.J. Williams, personal communication). Enhanced VEGF production after IR has been noted in tumour cells in vitro and in vivo, with a ~2-fold increase in VEGF levels 24 h after 5 Gy (Gorski et al., 1999). ROS have been shown to play a role in the expression of Epo, PGK-1 and VEGF in response to hypoxia (Chandel et al., 1998), by stabilising HIF-1α (Chandel et al., 2000). Differentiated C₂C₁₂ myoblasts and endothelial cells exposed to ROS-producing agents exhibited a concentration dependent increase in VEGF production in vitro and in vivo (Kuroki et al., 1996; Chua et al., 1998; Kosmidou et al., 2001). Also, the flavoprotein diphenylene iodonium, which blocks ROS generation by NAD(P) oxidase, abolished the hypoxic induction of HIF-1-regulated genes (Gleadle et al., 1995). On the other hand, H₂O₂ inhibited HIF-1 binding and Epo induction under hypoxia (Huang et al., 1996). This could be due to cellular damage by overwhelming antioxidant defences, since low H₂O₂ levels did allow induction of the Epo and Glut-1 HREs (Fandrey et al., 1997; Chandel et al., 2000). Further studies are required to elucidate the mechanisms involved in IR-mediated HRE induction.

Although responsive to the separate stimuli of hypoxia and radiation, isolated HREs and CArG elements did not show any further induction when both stimuli were concomitantly administered (Figures 7.3, 7.4, 7.6). However, some significant cooperation was observed when the combination enhancers were utilised (Figures 7.3, 7.4, 7.6).

The full-length CMV promoter was marginally down-regulated in T24 cells after hypoxic treatment (Figures 7.3, 7.6), which may explain the slightly reduced efficacy of HRP/IAA GDEPT under hypoxia and anoxia in the T24 cell line compared to normoxia (Figure 7.7.A; Chapter 3). This effect appears to depend on the cell type, since the CMV promoter was not affected by hypoxia in MCF-7 cells. In previous studies, positive, negative and negligible responses have been observed in a number of different cell lines (Binley et al., 1999; Boast et al., 1999; Shibata et al., 2000; Cao et al., 2001). Also, post-
transcriptional and post-translational modifications of the reporter EGFP were shown not to be significantly influenced by exposure to an atmosphere of 0.1% O₂, and, even under anoxia, were fully recovered after 5 h reoxygenation (Coralli et al., 2001).

To determine whether hypoxia- and IR-mediated gene expression was applicable to HRP/IAA gene therapy, T24 cells were transiently transfected with constructs in which the inducible promoters controlled the production of HRP. After a trigger hypoxic incubation and/or X-ray dose, their sensitivity to the prodrug IAA was examined. As predicted, under normoxic conditions only cells transfected with pCMV-HRP were killed by IAA (Figure 7.7). However, prodrug incubation resulted in selective toxicity in transfectants when the inducible promoters were stimulated by hypoxia and/or IR (Figure 7.7). After induction of the Epo/E9 enhancer, cell kill levels equivalent to those observed with the CMV promoter were observed, indicating the potential of this system. Even if only a fraction (about 20%) of the transient transfectants expressed the HRP, almost 90% cell death was measured. This observation is consistent with the bystander effect demonstrated previously with the HRP/IAA system (Chapter 3). IAA incubation was performed under normoxia for ease of experimental procedures, but the HRP/IAA system can induce cell death and bystander killing under hypoxia and anoxia (Chapter 3). Moreover, HRP/IAA could significantly enhance the cytotoxic effects of IR, when X-irradiation took place immediately before or after prodrug incubation (Chapter 6). This system may therefore be a promising candidate for use in a combined radiotherapy and gene therapy approach.
Anticancer strategies, in order to be of any significant advantage, should be characterised by high specificity as well as efficacy. On this basis, gene-directed enzyme/prodrug therapy (GDEPT) was introduced, whereby the target cells are genetically modified to synthesise an enzyme able to convert a prodrug into a cytotoxin. In the present work, a novel system consisting of horseradish peroxidase (HRP) and indole-3-acetic acid (IAA) is proposed as an enzyme/prodrug combination for cancer gene therapy. In particular, this GDEPT approach was aimed at targeting the hypoxic areas of solid tumours resistant to conventional treatment. The efficacy of HRP/IAA gene therapy and the induction of a bystander effect were demonstrated in vitro under normoxic as well as hypoxic conditions (Chapter 3). To date, the chemical agents and the cellular targets involved in HRP/IAA-induced toxicity are unknown, but the results presented here indicate that an apoptotic pathway may be activated (Chapter 4). An enhancement of the therapeutic potential of HRP-mediated GDEPT was demonstrated by adopting novel IAA analogues characterised by fast normoxic and hypoxic cytotoxic activation, high HRP<sup>+</sup> cell kill or selectivity (Chapter 5). Moreover, with a view to combining GDEPT and radiotherapy protocols, the interaction with therapeutically significant doses of ionising radiation (IR) was evaluated, and oxic and anoxic enhancement of IR toxicity was observed (Chapter 6). Finally, selective transgene expression and prodrug activation in hypoxic and/or irradiated cells was demonstrated by the use of synthetic promoters containing hypoxia regulatory elements (HREs) and IR-responsive CArG elements (Chapter 7).

For the choice of effective enzyme/prodrug combinations for GDEPT, a number of criteria have been listed (Connors, 1995; Knox, 1999). As also done in an ADEPT context (Wardman, 2002), these requirements, which are not fulfilled by most of the current systems, are compared here with the properties of the HRP/IAA combination:
Monomeric enzyme with no equivalent in humans. HRP is a monomeric glycoprotein, and glycosylation is not required for its activity (Welinder, 1979; Smith et al., 1990). Human peroxidases are much less effective than HRP in oxidising IAA and analogues. IAA was moderately toxic to rodent neutrophils (~40% loss of viability after 1 mM IAA for 24 h), while lymphocytes were not affected by IAA treatment (Pires de Melo et al., 1997, 1998). This effect was attributed to endogenous myeloperoxidase (MPO) activity, which in lymphocytes is 10-fold lower than in neutrophils (Pires de Melo et al., 1998). Analogously, human pro-myelocytic leukaemia lymphocytes (HL60) were moderately affected by IAA treatment, as 10 mM IAA for 1 h induced 25% cell kill only (Folkes et al., 1998). The formation of the skatole radical was detected, but the MPO/IAA reaction was inhibited after a few minutes (Folkes et al., 1998). Some toxicity was measured in IAA-treated endothelial HMEC-1 cells, but it did not appear to depend on endogenous peroxidase levels (section 3.3.4). Therefore the pathways leading to cytotoxic prodrug activation appear to be specific for HRP, and not for endogenous peroxidases.

Active at physiological pH. Although HRP activity may be higher at pH ~5 (Dunford, 1999), the experiments described in this work demonstrated significant cytotoxic prodrug activation at neutral pH.

Low $K_m$ and high $k_{cat}$ for the prodrug. IAA and derivatives are notably better substrates for HRP than for other biological indoles, such as tryptophan (Wardman, 2002). Comparison of the $K_m$ with other enzymes may be misleading, as there is no evidence of typical Michaelis and Menten kinetics for the HRP/IAA reaction (Candeias et al., 1997). However, one study provided a value of $K_m = 19$ μM for IAA with 23 nM HRP at pH 5 and 25°C (Smith et al., 1982). For therapeutic purposes, prodrug activation should occur rapidly and at low concentrations of the substrate. Indeed, it was shown here that significant toxicity was induced in HRP-expressing cells after only 2 h-incubation, at levels of prodrug in the 0.1-1 mM range.

No co-substrate requirements. To activate IAA and analogues neither H$_2$O$_2$ nor other cofactors are required (Dunford, 1999).

Freely diffusible prodrug. At pH 7.4 IAA is hydrophilic and can cross cell membranes within a few minutes (Pires de Melo et al., 1997; Folkes et al., 1999).
**Bystander effect.** A significant bystander effect was demonstrated with IAA and analogues, under oxic as well as hypoxic conditions, not dependent on cell-to-cell contact (Chapter 3). The half-life of the active drug will need to be investigated in vivo, to ensure that no toxic species escape into the circulation to damage normal tissue.

**High differential toxicity.** After brief incubation intervals (2 h) with up to 20 mM IAA, no toxicity could be detected in HRP− cells, while 3-4 mM induced 1-2 log cell kill in HRP-transfectants. Longer exposure times induced differential toxicities of 36-85 (IC₅₀), depending on the tumour cell line. Higher differentials were measured using some IAA derivatives, such as 1-Me-IAA (selectivity index = 740 in T24 cells). However, in microvascular endothelial HMEC-1 cells lower selectivity was observed, compared to the tumour cell lines analysed. This may limit therapeutic efficacy.

**Active drug neither phase specific nor proliferation dependent.** HRP/IAA induced a cytostatic effect that appeared to be independent on cell cycle phase at the time of exposure (Chapter 4). Further studies may be necessary using cells synchronised in different phases. Also, cytotoxicity in quiescent cells may be specifically measured by blocking cell proliferation during treatment.

Thus HRP/IAA appears to fulfil most requirements, justifying further work and its evaluation in in vivo models. A number of factors need to be taken into account, such as prodrug levels and kinetics, tumour selectivity, normal tissue toxicity, local and distant bystander effect and host immunicity. The compounds IAA, 1-Me-IAA and 5-Br-IAA will be tested in animal models, on their own and, based on the radiosensitisation observed in vitro, in combination with IR. When X-irradiation took place immediately before or after prodrug incubation, sensitivity enhancement ratios (SERs) of 2.1-5.6 under normoxia, and of 3.6 under anoxic conditions were observed (Chapter 6). Statistical evaluation of combined radiation and GDEPT protocols has shown that in vitro SER values of 1.2 can significantly increase local control after radiotherapy (Lambin et al., 2000). Therefore, it is possible that the HRP/IAA system may not only eradicate the hypoxic radioresistant tumour cells, but also directly enhance the cytotoxic effects of IR. The scheduling of a combination protocol should also be devised, in order to achieve maximum efficacy in vivo. Multiple dosing of radiation and prodrug may be
needed to maximise gene expression, direct cytotoxicity and radiosensitisation in hypoxic and/or irradiated areas.

Future work is recommended to assess the chemical agents and the cellular mechanisms involved in HRP/IAA-induced cell death and radiosensitisation. In particular it would be of interest to investigate if the DNA macromolecule is damaged in the exposed cells, as observed in a cell-free system (Folkes et al., 1999). High sensitivity in measuring DNA strand breaks within a cell population may be achieved with single-cell electrophoresis, or comet assay (Olive, 1999). Immunostaining the single cell preparations (Dachs et al., 1997) with anti-HRP antibodies may allow the identification of HRP-expressing and non-expressing damaged cells, giving further insights into the bystander effect of the HRP/IAA combination. The induction of base damage and base loss may be evaluated by utilising purified repair enzymes such as AP-nucleases and DNA glycosylases (endonuclease III, endonuclease IV) and formamidopyrimidine-DNA glycosylase (Fpg protein), which are able to recognise specific lesions and convert them into strand breaks, detectable by comet assay (Chaudhry and Weinfeld, 1995; Banath et al., 1999).

HRP localisation within the transfected cell may play a role in IAA activation and cytotoxicity, due to proximity to the target molecules. The plasmids used in this work contained the HRP cDNA previously fused to the KDEL tetrapeptide, which caused its accumulation in the cytoplasm and the nuclear membrane. If DNA is the critical target, localisation of the HRP in the nucleus via nuclear localisation sequences may result in enhanced toxicity. Nuclear accumulation and enhancement of the bystander effect may also be achieved by fusing the HRP to the HSV virion protein VP22, which has been shown to export fusion transgene products from transfected to surrounding untransfected cells, where it specifically accumulates in the nucleus (Elliott and O’Hare, 1999). These genetic manipulations should not affect the catalytic properties of HRP, as it is a robust and efficient enzyme, able to retain at least 50% of its peroxidase activity when conjugated to polymers and antibodies (Folkes and Wardman, 2001).

To minimise IR- and drug-mediated toxicity to normal tissues, synthesis of the prodrug activating enzyme has to be targeted to the tumour. Selective gene expression at
the tumour site may be achieved by using promoters that respond to the tumour-specific stimulus of hypoxia. HREs are DNA sequences found in the regulatory regions of a number of genes responsive to tissue hypoxia, such as erythropoietin (Epo), phosphoglycerate kinase (PGK)-1 and vascular endothelial growth factor (VEGF), and they have been successfully utilised to target therapeutic genes to the hypoxic tumour environment (Dachs et al., 1997). Moreover, IR itself may be exploited for selective transgene expression, by induction of CArG elements from the early growth response (Egr)-1 gene. Chimeric promoters containing pentamers of HREs from the human Epo and the murine PGK-1 genes, and a novel sequence based on the human VEGF HRE (nVEGF) were constructed (Chapter 7). Surprisingly, the PGK1 and nVEGF enhancers were responsive not only to hypoxia (0.1% O₂) but also to IR (5 Gy). In order to clarify this effect, future work will need to be carried out. For instance, to establish if the induction is due to transcriptional activation, reporter mRNA levels after IR will need to be analysed, and, to detect if an HIF-1-associated pathway is activated, HIF-1α protein levels and HRE binding activity measured. In a similar fashion, when nine CArG elements controlled transgene expression, they were independently induced by IR as well as by hypoxia. Whether reactive oxygen species (ROS) generated during hypoxia/reoxygenation rather than a direct response to a decrease in oxygen levels are involved is yet to be elucidated.

In order to combine and enhance the response to the transcriptional stimuli of hypoxia and IR, the HREs were inserted upstream to the CArG elements. These chimeric promoters containing combinations of HREs and CArG elements retained the ability to respond to individual and dual trigger treatments, with the Epo HRE/CArG combination proving to be the most responsive and robust. The Epo and CArG enhancers, on their own or in combinations, could selectively sensitise hypoxic and/or irradiated cells to IAA, when regulating the HRP cDNA. Such chimeric promoters may therefore be an effective tool to control therapeutic gene expression within the tumour microenvironment in GDEPT approaches aimed at addressing the problem of hypoxia in radiotherapy.

Although selective gene expression is of paramount importance to minimise normal tissue toxicity, the use of inducible promoters may result in reduced therapeutic
protein levels compared to those under the control of strong constitutive promoters. Further improvements to the system may be achieved by optimising the spacing between the elements, their orientation and position from the transcription start site. Moreover, in order to amplify suicide gene expression further, a molecular switch (Scott et al., 2000) could be utilised. The resultant high-level, stimuli-controlled expression system may provide sufficient therapeutic product in the tumour environment for future clinical application.

For a successful GDEPT, the prodrug should be able to reach the transfected cells. This may pose a particular problem in targeting cells under chronic hypoxia, since IAA will need to diffuse efficiently in the extravascular compartment to reach the HRP-expressing cells, located 100-200 μm away from the blood vessels. It is likely that IAA would reach populations at intermediate oxygen concentrations, closer to functional vessels, which appear to be critical in tumour response to fractionated radiotherapy (Wouters and Brown, 1997). Also, dynamic changes in microregional perfusion have been related to the formation of areas of acute, intermittent hypoxia, which may initially allow the delivery of the prodrug in the tumour mass, and, subsequently, activate a hypoxia-responsive promoter.

The diffusion properties of IAA and analogues in tumour conditions will have to be analysed. Tumour penetration may be improved by encapsulating the drug in carrier molecules, such as liposomes, hydrogels or polymers (reviewed by Langer, 1998). After prodrug delivery, tumour hypoxia may be increased by concomitant use of antivascular agents such as combretastatin A-4-P, which has been shown to induce a rapid shut-down of tumour blood flow leading to significant hypoxia (Tozer et al., 1999, 2001).

Finally, as for all gene therapy strategies, gene delivery remains the main concern. To date, viral vectors are characterised by the highest transfection rates in vivo, and major clinical experience has been gained with adenoviruses. Modification of the capsid proteins and fibre may allow increased selectivity via the recognition of cell-specific receptors, but biosafety is at present still disputable. Non-viral systems, although remarkably safe and easy to produce, are limited by poor gene transfer. Hypoxia-
specific therapeutic strategies, such as bacteria and macrophages, may represent interesting approaches for hypoxia-targeted gene therapy. The analysis of their pathogenesis and the induced inflammation in humans are the main problems that will need to be answered in future clinical studies.

The need for local control in the cure of cancer is a matter of crucial importance. Therapeutic strategies aimed at delivering high and localised concentrations of cytotoxic agents to clinically resistant solid tumour populations may provide a fundamental clinical gain, improving not only the efficacy of standard treatments, without concurrent systemic complications, but overall survival and patient quality of life.

Gene therapy is a promising approach, and 12 years after the approval of the first clinical trial, it is still in the early stages of development. Some major problems remain to be solved before these new strategies become routinely adopted in the clinic. One of the main challenges is the improvement of gene delivery, and therefore therapeutic efficacy. Nevertheless, the results collected so far and their potential clinical application are encouraging, and illustrate both feasibility and future promise for cancer treatment. Clinical trials have already addressed the issues of safety. As vector technology fulfils the requirement of efficient delivery, it can be anticipated that the results observed in the pre-clinical studies will more quickly translate into clinical benefit.

These in vitro studies showed that the horseradish peroxidase/indole-3-acetic acid system has the potential for use in cancer gene therapy, particularly in cases currently refractory to treatment as a result of inherent or hypoxia-mediated radioresistance. Taken collectively, these observations suggest that the use of HRE- and CArG-mediated HRP-GDEPT may be an effective tool to target the microenvironment of solid malignancies.
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Publications
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Reviews


Book chapters
Development of a novel enzyme/prodrug combination for gene therapy of cancer: horseradish peroxidase/indole-3-acetic acid

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This paper demonstrates the potential for utilizing the plant enzyme, horseradish peroxidase (HRP), in a gene-directed enzyme prodrug therapy context. Human T24 bladder carcinoma cells transfected with a mammalian expression vector containing the HRP cDNA were selectively sensitized to the nontoxic plant hormone, indole-3-acetic acid (IAA). The HRP/IAA-induced cell kill was effective in normoxic and anoxic conditions. The activated drug is a long-lived species able to cross cell membranes, and cell contact appears not to be required for a bystander effect to take place. These preliminary results suggest that the delivery of the HRP gene to human tumors followed by IAA treatment may provide a novel cancer gene-directed enzyme prodrug therapy approach, with potential to target hypoxic cells.

Key words: Gene-directed enzyme/prodrug therapy; HRP; IAA; hypoxia; bladder carcinoma; ganciclovir.

Gene-directed enzyme prodrug therapy (GDEPT) consists of a two-phase molecular chemotherapy. Firstly, a gene encoding a foreign enzyme is delivered to the target cells; secondly, a prodrug is administered and selectively converted to a cytotoxin by the enzyme synthesized at the target. To obtain specificity, therapeutic gene expression can be regulated by tumor-specific or tumor condition-specific promoters (reviewed in Ref.1).

The most well-known example of enzyme/prodrug combination in cancer GDEPT is the herpes simplex virus thymidine kinase (HSV TK)/ganciclovir (GCV)2 currently adopted in clinical trials.3 Because activated GCV interferes with DNA synthesis, the HSV TK/GCV system is particularly suitable for the eradication of rapidly dividing tumor cells invading nonproliferating tissue. However, it is not the combination of choice to target the slowly dividing hypoxic population in solid tumors,4,5 which has been shown to contribute to resistance of human tumors to chemotherapy and radiotherapy (reviewed in Ref.6).

The horseradish peroxidase (HRP) and the plant hormone, indole-3-acetic acid (IAA), represent a novel enzyme/prodrug combination, with potential for hypoxia-regulated gene therapy.7 When reacting with IAA, HRP does not require hydrogen peroxide and is characterized by a different substrate specificity and reaction pathway from the mammalian myeloperoxidase.8-9 At neutral pH, IAA is oxidized by HRP compound I to a radical cation, which undergoes scission of the exocyclic carbon–carbon bond to yield the carbon-centered skatolyl radical10 (Fig 1:2,3). In the presence of oxygen, the skatolyl radical rapidly forms a peroxyl radical (Fig 1:4), which then decays to a number of products, the major ones being indole-3-carbinol, oxindole-3-carbinol, and 3-methylene-2-oxindole11 (Fig 1:5). In anoxic solution, decarbonylation of the radical cation can still take place and the carbon-centered radical preferentially reacts with hydrogen donors12,13 (Fig 1).

When activated by purified HRP, IAA was shown to inhibit colony formation in mammalian cells, whereas, neither enzyme nor prodrug alone was cytotoxic at the same concentration or times.14,15 Endogenous peroxidases in human tumor cells13 and rat leukocytes and phagocytes14 were significantly less efficient in converting IAA into a cytotoxin. Moreover, no major toxicity has been observed in patients after oral administration of 100 mg/kg IAA.15

The aim of the current study was to determine the potential of utilizing the HRP-encoding gene for cancer GDEPT. Human tumor cells were transfected in vitro with the HRP cDNA and their sensitization to IAA was evaluated in normoxic and anoxic conditions. The ability of the HRP/IAA combination to induce a bystander effect was also studied.

MATERIALS AND METHODS

Cell culture

Human bladder carcinoma T24 cells (European Collection of Cell Cultures, Salisbury, UK) were maintained in Dulbecco's...
modified Eagle's medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (Sigma, Gillingham, UK), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), and incubated in a humidified incubator at 37°C and 5% CO₂/air. For experiments in anoxic conditions, cells were incubated in an anaerobic glove cabinet (Don Whitley Scientific, Shipley, UK) with 5% CO₂, 5% H₂, 90% N₂ and palladium catalyst at 37°C. Only cells which tested negative for mycoplasma infection were utilized.

**Plasmid DNA and cell transfection**

The plasmids, pRK34-HRP and pCI-TK, were kindly provided by Dr. D.F. Cutler (UCL, London) and Dr. S.D. Scott (Gray Laboratory). The constructs of pCI-EGFP were made as follows to contain an identical vector backbone as pCI-TK: the enhanced green fluorescent protein (EGFP) gene was excised from pEGFP-N1 (Clontech, Basingstoke, UK) by EcoRI/NorI digestion (enzymes from Life Technologies) and the resulting fragment inserted in the commercial vector pCI-neo (Promega, Southampton, UK), linearized with the same restriction enzymes. The sequence integrity of the plasmids was confirmed using a Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, Amersham, UK) and Gene Readir DNA Analyzer (LI-COR, Lincoln, NE).

Transient transfectants were obtained by exposing T24 cells to complexes of integrin-targeted peptides (Dr. S.L. Hart, ICH, London, U.K.), Lipofectin (Life Technologies) and DNA as described previously, and were assayed for gene expression after 24 hours.

Transfection efficiency with the plasmid pRK34-HRP was analyzed by immunofluorescence analysis (see **Immunofluorescence staining**). Transfection efficiency with the construct pCI-TK was estimated by measuring the production of the marker protein EGFP in cells transfected under the same conditions with pCI-EGFP. Intracellular EGFP (excitation maximum, 488 nm; emission maximum, 507 nm) was detected by fluorescence-activated cell sorting (FACS) on a Becton Dickinson FACSscan. Cells were scored as positive if they showed an increased fluorescence with respect to untransfected cells.

**Immunofluorescence staining**

Antibody staining on paraformaldehyde-fixed transfected and untransfected T24 cells was carried out as reported previously. Rabbit polyclonal anti-HRP (Dako, Ely, UK) diluted 1:200 was used as the primary antibody; TRITC-conjugated swine antirabbit immunoglobulins (Dako) were diluted 1:200 as secondary antibodies. Slides were viewed using an Olympus BH-2 fluorescence microscope. Alternatively, to measure the transfection efficiency with the plasmid pRK34-HRP, cell suspensions were FACS-analyzed after immunostaining.

**HRP activity assay**

The HRP activity was analyzed using a modified 3,3',5,5'-tetramethylbenzidine dihydrochloride assay. Transfected and untransfected cells were harvested and resuspended in 0.5% hexadecyltrimethylammonium bromide (Sigma) in 50 mM phosphate buffer (pH 6.0). Cells were lysed by
Figure 3. Production of HRP in transfected T24 cells. A: T24 cells transiently transfected with the plasmid pRK34-HRP containing the HRP cDNA were stained with primary anti-HRP and secondary TRITC-labeled antibodies, and examined on an Olympus BH-2 fluorescence microscope (magnification: x300). The KDEL tetrapeptide fused to the C-terminus of the HRP cDNA causes intracellular accumulation in the endoplasmic reticulum and the nuclear envelope. B: Western blotting of extracts of HRP-transfected T24 cells (lane 1) and of untransfected cells (lane 2). Two protein markers are indicated. C: HRP activity per cellular protein content (A652/μg total cellular protein) detected in transfected and untransfected cells using a modified TMB enzyme assay. The means of three experiments (triplicate samples) ± SE are shown.
freezing/thawing three times in liquid nitrogen at 37°C and by centrifugation in a microfuge for 15 minutes at 13,000 rpm. Enzyme reactions were carried out at room temperature by diluting 20 µL supernatant in 80 mM phosphate buffer (pH 5.4), 3.2 mM 3',3',5,5'-tetramethyl-benzidine dihydrochloride (Sigma), 3.0 mM H2O2 in a total volume of 2 mL. Absorbance at 652 nm (A652) was read at room temperature over 60 minutes at 1-minute intervals on a Hewlett Packard model 8452A diode array spectrophotometer. The total cellular protein content in the samples was determined by using a commercial protein assay kit (Lowry procedure, Sigma). The HRP activity was expressed as the absorbance measured after 60 min/µg of total cellular protein in the sample.

The HRP content in transfected cells was also evaluated. The A652 rate of increase up to 10 minutes (linear least square regression) and the A652 at 60 minutes were estimated and compared to calibration curves obtained with purified HRP (results not shown). The HRP content was then normalized to the total protein content in the sample.

Western blotting
Western blotting analysis of HRP protein was conducted as previously described.2o Briefly, cell monolayers were resuspended in Laemmli sample buffer and whole cell extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% polyacrylamide gels, ProtoGel, National Diagnostic, Atlanta, GA). Proteins in the gel were transferred to nitrocellulose membranes (Genetic Research Instrumentation, Rayne, UK) using an LKB-Pharmacia semidry blotter. Immunoblotting was performed with primary rabbit polyclonal anti-HRP antibodies and secondary HRP-conjugated goat antirabbit immunoglobulins (Dako). Detection of immunoreactive bands was performed using the enhanced chemiluminescence technique (ECL kit, Amersham Pharmacia Biotech).

Clonogenic assays
Exponentially growing transfected and untransfected cells were plated at low density on Petri dishes and exposed to IAA (Aldrich, Gillingham, UK), with or without 1.2 µg/mL HRP, or GCV (from Dr. E. Littler, GlaxoWellcome, Stevenage, UK) for 2 or 24 hours in phenol red–free Hank’s balanced salt solution (HBSS, LifeTechnologies) in the 37°C incubator. Alternatively, cells were preplated in the anaerobic cabinet and, after incubation for 5–6 hours, were exposed to the prodrug for 2 or 24 hours in anoxic conditions. All plastics and media were preincubated in anoxia for 48 hours before use to remove residual oxygen.

In conditioned medium-switch experiments, HBSS containing a range of concentrations of IAA was transferred after a 2-hour incubation with transient transfectants to preplated untransfected cells and left to react for 2 hours with the cell monolayers.

Following drug exposure, cells were rinsed with PBS and grown for 10 days in complete DMEM supplemented with feeder cells (V79 cells exposed to 250 Gy 40Co irradiation). After fixation and staining with 2.5% wt/vol crystal violet (Sigma) in isomethylated spirit, colonies of >50 cells were scored. Surviving fractions were evaluated relative to HBSS-treated controls. The concentration of prodrug required to reduce cell survival by 50% (IC50) was estimated from the survival curves.

RESULTS
Cytotoxicity of IAA activated by purified HRP in human cells
In our GDEPT strategy, we aim to sensitize the target cells to the prodrug IAA activated by the enzyme HRP. Consequently, human bladder carcinoma T24 cells were exposed for time intervals from 15 minutes to 8 hours to 0.1 mM IAA and 1.2 µg/mL purified HRP. Clonogenic assays showed a decrease in surviving fraction with increased exposure time (Fig 2). The cells were also exposed to the enzyme and prodrug independently and no significant cytotoxic effects were observed at the concentration/time indicated (Fig 2).

Transfection of human cells with the HRP-encoding gene
The T24 cells were transiently transfected with the pRK34-HRP plasmid and 20–26% transfection efficiency was observed from immunostaining followed by FACS analysis. Immunolabeling for HRP protein in transfecteds is shown in Figure 3A. HRP is evident in the cytoplasm and the nuclear envelope. This is consistent with the fact that in the pRK34-HRP construct, the HRP cDNA had been previously fused to the signal sequence from the human growth hormone and the KDEL retention motif, allowing the HRP to be accumulated in the endoplasmic reticulum and the nuclear envelope. Untransfected cells and EGFP-expressing cells did not stain positive for HRP (results not shown). Synthesis of an immunoreactive protein of correct size (about 52 kDa) was confirmed by Western blotting (Fig 3B).

Increase in peroxidase activity of 60-fold was observed in HRP transfecteds compared to untransfected T24 cells (Fig 3C). An HRP content of 18 ± 1 pg/µg total cellular protein was estimated in transfecteds.

In vitro efficacy of the HRP/IAA system
Figure 4 shows the effect of increasing IAA concentration on clonogenicity of transfected and untransfected T24 cells. After only 2-hour prodrug incubation under normoxic or anoxic conditions, increased cytotoxicity in HRP-expressing cells was detected (Fig 4A). At nontoxic doses of IAA, one- to two-log cell kill was induced in HRP-positive cells, even though only 20–26% of the transient transfecteds was shown to synthesize the foreign enzyme. Expression of the HRP gene per se did not affect the transfecteds, as judged by plateauing efficiency, proliferation rate, or microscopic appearance in the absence of IAA (results not shown). Cells transiently transfected with the plasmid, pEGFP-N1, incubated with IAA gave the same response as the untransfected population (data not shown).

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Figure 4. Toxicity of the HRP/IAA combination in T24 cells. Transfected and untransfected cells were exposed to increasing concentrations of the prodrug IAA in air and in an oxygen-free atmosphere (catalyst-induced anoxia) for 2 (A) or 24 hours (B). Only 20–26% of the cells was estimated to synthesize the HRP protein. Conditioned medium-switch experiments (A) were performed by transferring IAA-containing medium preincubated for 2 hours with HRP transfectants to untransfected cells for a subsequent 2-hour exposure. The data are means of at least three independent experiments (duplicate samples). Error bars are ± SE. The lines are interpolated. △: untransfected cells in air; ▲: HRP-transfected cells in air; ●: untransfected cells + preconditioned medium in air; ○: untransfected cells in anoxia; ■: transfected cells in anoxia.

IAA doses above 4 mM produced a significant decrease of the medium pH affecting cell survival, and therefore were not included in the survival curves. When the medium pH was adjusted to 7.4, no toxicity was detected in untransfected T24 cells exposed for 2 hours to up to 20 mM IAA (results not shown).

A higher efficacy of the HRP/IAA system was observed after 24-hour incubation (Fig 4B). In HRP-expressing cells, 95% colony inhibition was induced at doses of IAA around 1 mM. A 28-fold increase in cytotoxicity in air and a 50-fold increase in anoxia were estimated for transfected compared to untransfected cells, as assessed by equivalent doses to reduce surviving fraction to 50% (IC50).

Anoxic incubation did not reduce the efficacy of the system. The shape of the anoxic survival curves and the IAA activation pathway suggest that different toxic metabolites may be involved in the induced cell death.

IAA-containing medium, preconditioned by transfected cells, reduced the clonogenicity of untransfected cells (Fig 5).

Figure 5. Cytotoxic activation of GCV in T24 cells. T24 cells transiently transfected with the plasmid pCI-TK containing the HSV TK gene and untransfected cells were exposed to GCV in air and in anoxia for 2 (A) or 24 hours (B). The means of three independent experiments (duplicate samples) ± SE are indicated. The lines are interpolated. ○: Untransfected cells in air; ▼: TK-transfected cells in air; ◻: untransfected cells in anoxia; ♦: transfected cells in anoxia.
Cytoxicity of the HSV TK/GCV combination in T24 cells

In order to compare the novel HRP/IAA combination with an established enzyme/prodrug system, T24 cells were transiently transfected with the HSV TK gene and exposed to GCV. From parallel transfections with the plasmid pCI-EGFP, a transfection efficiency of 60–70% was estimated. After a 2-hour incubation with GCV, no reduced survival was detected in TK-expressing cells compared to the untransfected population (Fig 5A). When the prodrug GCV was left to react for 24 hours with the cell monolayers, a 4-fold increase in cytototoxicity (IC$_{50}$) was induced inoxic TK transfectants (Fig 5B). In anoxic conditions, no selective cytotoxicity could be measured at any dose of GCV tested (up to 5 mM) (Fig 5B). Doses above 1 mM GCV appeared to be toxic in the untransfected cells under both oxic and anoxic conditions.

DISCUSSION

This preliminary report demonstrates the potential for utilizing HRP and IAA as a novel enzyme/prodrug combination for cancer gene therapy. The efficacy of the HRP/IAA system was evaluated in vitro by exposing human tumor T24 cells transiently transfected with an expression vector containing the HRP cDNA to the prodrug IAA. Significant cytotoxicity could be evoked after 2-hour exposure and was further increased after 24-hour incubation. Up to two-log cell kill was induced at nontoxic doses of IAA. In order to investigate the potential of the HRP/IAA combination to kill the hypoxic subpopulation in solid tumors, transfected were exposed to IAA in an oxygen-free atmosphere. These extreme tumor conditions did not reduce the efficacy of the system and significant cell kill was measured.

Different IAA metabolites may be produced in oxic and anoxic cells. In the absence of oxygen, the peroxyl radical and its decay products cannot be formed, but the skatolyl radical can (Fig 1:3). Skatolyl-type radicals readily abstract hydrogen from donor molecules and have been shown to react with biomolecules such as DNA. They could therefore lead to cell damage by the formation of secondary radicals in key biological targets. In normoxic cells, however, skatolyl radicals are more likely to react with oxygen to form peroxy radicals. Prompt attack by radicals on cellular targets in air may be ruled out because conditioned medium-switch experiments (Fig 4A) and incubation of mammalian cells with filtered products of LAA oxidation indicate that the toxic agent is a stable long-lived species. Of the stable products, indole-3-carbinol is one of the likely candidates. After 24-hour incubation, only a 50% decrease in survival was observed. A three-log increase in cytotoxicity (IC$_{50}$) was induced in air, and no selective sensitization could be detected in anoxic TK-expressing T24 cells. In previous studies, the T24 cells may also explain their poor response to HSV TK/GCV, which had been demonstrated to induce programmed cell death. After 24-hour incubation, only a 4-fold increase in cytototoxicity (IC$_{50}$) was induced in air, and no selective sensitization could be detected in anoxic TK-expressing T24 cells. In previous studies, at least a three-log increase in cytotoxicity has been detected when transfected mammalian cells were grown for 5–14 days in the presence of GCV.

This preliminary in vitro study showed that the HRP/IAA system has the potential for use in cancer gene therapy and that in vivo testing is now warranted. Taken collectively, these observations support the further development of this GDEPT approach.

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Horseradish Peroxidase-mediated Gene Therapy: Choice of Prodrugs in Oxic and Anoxic Tumor Conditions

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Abstract

We have previously proposed the plant enzyme horseradish peroxidase (HRP) and the plant hormone indole-3-acetic acid (IAA) as an enzyme/prodrug combination for cancer gene therapy. In the current study, we evaluated the potential of HRP/IAA for gene-directed enzyme/prodrug therapy in three human tumor cell lines (T24 bladder carcinoma, MCF-7 breast adenocarcinoma, and FaDu nasopharyngeal squamous carcinoma) and one endothelial cell line (HMEC-1). The action of 10 IAA analogues in combination with HRP was studied in vitro in normoxic conditions as well as in the extreme tumor conditions of anoxia. Compounds characterized by prompt normoxic or anoxic cytotoxic activation and high HRP transfectant killing or selectivity were identified. Some variations were observed in the response of cells of different origin, with IAA, 1-Me-IAA, and 5-Br-IAA representing the most promising candidates for HRP gene therapy. In particular, 5-Br-IAA showed a very prompt and selective activation in anoxia. A strong bystander effect was produced by activated IAA and analogues because 70–90% cell kill was obtained when only 5% of the cells expressed the HRP enzyme. These results indicate that HRP/IAA represents an effective system for enzyme/prodrug-based anticancer approaches, and further improvements could be achieved by the use of novel IAA derivatives.

Introduction

One of the major goals of antitumor therapies is to target toxic agents to tumor cells selectively and specifically while sparing normal tissue from damage. This may be achieved by gene therapy that can combine highly specific gene delivery and gene expression. To date, more than 400 gene therapy clinical trials have been undertaken worldwide, more than half of which relate to cancer (1). A promising approach in the design of therapeutic genes for cancer gene therapy is suicide gene therapy or GDEPT (2). GDEPT is a two-step strategy: initially, a foreign gene encoding a nontoxic enzyme is delivered to the tumor. In a second step, a prodrug is administered, which is converted into a potent cytotoxin by the enzyme expressed at the target (reviewed in Ref. 2).

In the choice of the appropriate combination for GDEPT, a number of properties should be considered. The enzyme should have high catalytic activity under physiological conditions and fast and efficient prodrug activation even at low concentrations of the substrate (high Ke and low Kcat), without dependence on further catalysis by other cellular enzymes. The induced cytotoxicity should be cell cycle phase or proliferation independent to kill a wide range of tumor cell populations. The toxic agent should also have a half-life that allows transport to the surrounding untransfected cells (bystander effect) but ensures that any drug escaping into the circulation will be inactive. The bystander phenomenon, initially described by Moolten (3), can be defined as an extension of the killing effects of the active drug to untransfected neighboring cells. This implies that even if only a fraction of the target cells are genetically modified and express the therapeutic gene, tumor eradication may still be achieved.

We have developed a novel GDEPT system consisting of HRP and the nontoxic plant hormone IAA (4). Using this system, we demonstrated fast and efficient in vitro prodrug activation in a preliminary report (4). When compared with the well-established herpes simplex virus-1 thymidine kinase/ganciclovir combination, HRP/IAA showed increased toxicity in normoxia and, more significantly, in the extreme tumor conditions of anoxia. This could imply a therapeutic advantage because hypoxia is common to solid tumors and presents an adverse prognostic indicator (reviewed in Ref. 5). The HRP/IAA system has the potential to be used in a variety of anticancer strategies (6). Besides GDEPT, specific HRP targeting to the tumor could be achieved with HRP-conjugated antibodies [antibody-directed enzyme/prodrug therapy (7)] or polymers [polymer-directed enzyme/prodrug therapy (8)]. IAA is well tolerated in humans (9), and nonspecific activation in normal tissue is unlikely to take place because mammalian peroxidases failed to convert it into a cytotoxin at therapeutically significant prodrug doses (10–12).

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The abbreviations used are: GDEPT, gene-directed enzyme/prodrug therapy; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; CMV, cytomegalovirus; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; SI, selectivity index.
In the present work, we evaluate the potential of HRP/IAA for GDEPT in three human tumor cell lines and one endothelial cell line. The action of 10 IAA derivatives in combination with HRP was studied in normoxic and anoxic conditions. The data reported indicate that at least two analogues besides IAA induce efficient toxicity and bystander killing, suggesting the efficacy and selectivity of the HRP/IAA system for enzyme/prodrug-based anticancer approaches.

Materials and Methods

Cell Culture. T24 bladder carcinoma and MCF-7 mammary adenocarcinoma (both from the European Collection of Cell Cultures, Salisbury, United Kingdom), FaDu nasopharyngeal squamous carcinoma (American Type Culture Collection, Manassas, VA), and HMEC-1 dermal microvascular endothelial (13) cell lines were maintained in DMEM (Life Technologies, Inc., Paisley, United Kingdom), and 2 mM L-glutamine (Life Technologies, Inc.) in a humidified incubator at 37°C and 5% CO\textsubscript{2}/air. Only cells that tested negative for Mycoplasma infection were used.

Hypoxic Conditions. For experiments in anoxic and hypoxic conditions, cells were incubated at 37°C in an anaerobic glove cabinet (DON Whitley Scientific Limited, Shipley, United Kingdom) with 5% CO\textsubscript{2}, 5% H\textsubscript{2}, 90% N\textsubscript{2}, and palladium catalyst (anoxia) or in air-tight Perspex boxes flushed continuously with a humidified gas mixture containing 0.1% O\textsubscript{2}, 5% CO\textsubscript{2}, and 94.9% N\textsubscript{2} (hypoxia). For all anoxic/hypoxic cultures, experiments were conducted in the anaerobic cabinet, and plastics and fluids were reincubated in the cabinet for 24–48 h before use to remove residual oxygen.

Plasmid DNA and Cell Transfection. The plasmid pRK34-HRP (14) was kindly provided by Dr. D. F. Cutler (University College London, London, United Kingdom); the control plasmid pCMV-CD4 was constructed as described previously (15). In both cases, gene expression was driven by the CMV early promoter. Transient transfectants were obtained by exposing the cells to complexes of DNA, Lipofectin (Life Technologies, Inc.), and integrin-targeted peptides (16) and assayed for gene expression after 24 h.

Detection of HRP Expression. Antibody staining was carried out by fixing cell suspensions in 3% paraformaldehyde (Sigma Chemical Co. Aldrich)/PBS for 20 min at room temperature. After centrifugation, the pellets were rinsed in PBS and incubated for 15 min at room temperature in 15 μM glycine (Sigma Chemical Co. Aldrich)/PBS. Nonspecific binding was blocked by the addition of wash buffer [5% FCS/1% Tween 20 (Sigma Chemical Co. Aldrich)/PBS]. The samples were centrifuged and incubated for 1 h at room temperature with rabbit polyclonal anti-HRP (Dako, Ely, United Kingdom) diluted 1:200 in 10% FCS/wash buffer. After extensive rinsing in wash buffer (at least three times), the samples were resuspended in TRITC-conjugated swine antirabbit immunoglobulins (Dako) diluted 1:200 in 10% FCS/wash buffer (1 h, room temperature). The cells were rinsed in wash buffer and resuspended in HBSS (Life Technologies, Inc.) for fluorescence-activated cell-sorting analysis on a Becton Dickinson FACScan. Cells were scored as positive if they showed an increase in fluorescence with respect to CD4-expressing cells.

HRP activity was analyzed using a modified 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma Chemical Co. Aldrich) assay, as described previously (4). The total cellular protein content in the samples was determined by using a commercial protein assay kit (Bio-Rad, Hemel Hempstead, United Kingdom). The HRP activity was expressed as units of enzyme per micromgram of total cellular protein. A unit is defined as the amount of enzyme that produces an increase of 1 unit of absorbance at 652 nm (A\textsubscript{652,mm}) per minute.

For Western blots, cell extracts were subjected to SDS-PAGE as described previously (4). Detection of immunoreactive bands was performed using the enhanced chemiluminescence technique (ECL kit; Amersham Pharmacia Biotech, Amersham, United Kingdom).

Compounds. The IAA analogues used are illustrated in Fig. 1. IAA, 1-Me-IAA, 2-Me-IAA, 5-MeO-IAA, 2-Me,5-MeO-IAA, 5-BrO-IAA, 5-F-IAA, and 5-Br-IAA were purchased from Sigma Chemical Co. Aldrich.

Fig. 1. IAA, 1-Me-IAA, 2-Me-IAA, 5-MeO-IAA, 2-Me,5-MeO-IAA, 5-BrO-IAA, 5-F-IAA, and 5-Br-IAA were prepared as follows, using adaptations of procedures described previously in the literature (17–19). A solution of the indole (2–10 mmol) in tetrahydrofuran was cooled to 0°C, and n-butyllithium (1.1 equivalents) was added. The yellow solution was stirred at 0°C for 20 min, zinc chloride (1.1 eq. of a 1 M solution in diethyl ether) was added, and the mixture was stirred for 2 h at room temperature. Ethyl bromoacetate (1.1 eq.) was added, and the mixture was stirred overnight. After the addition of water, the mixture was partitioned into ethyl acetate and 1 M HCl. The organic layer was dried (MgSO\textsubscript{4}), the solvent was removed in vacuo, and the crude product was purified by flash column chromatography (3,1 hexane:ethyl acetate) to furnish the substituted ethyl indole-3-acetate. The ester was heated under reflux in a solution of NaOH in 1:1 methanol:water for 4 h. The indole was precipitated by acid-
ifcation (HCl) and purified by recrystallization from chloroform or methanol-water. All air- and water-sensitive reactions were carried out under nitrogen. Glassware was oven-dried and cooled in an anhydrous atmosphere before use. Products were analyzed for purity using a Waters Integrity high-performance liquid chromatography mass spectrometer system, nuclear magnetic resonance spectra were recorded on a JEOL JMS-800 spectrometer, and melting points (uncorrected) were measured using a Gallenkamp hot plate apparatus. Elemental analyses were carried out by Medac (Egham, United Kingdom) and were in agreement with calculated values.

Clonogenic Assay. Exponentially growing cells were plated at low density and exposed to IAA or derivatives for 2 or 24 h in phenol-red-free HBSS in a 5% CO2 incubator at 37°C. For experiments under anoxia/hypoxia, cells were pre-plated in the anaerobic cabinet and, after incubation for 5–6 h in the cabinet or under 0.1% O2 to ensure anaerobic/hypoxic conditions, exposed to the prodrugs.

In conditioned medium-switch experiments, cells transduced with pRK34-HRP (HRP⁺) were exposed to HBSS containing a range of IAAs concentrations for 2 h. This medium was subsequently transferred to preplated cells transduced with the control plasmid pCMV-CD4 (HRP⁻) for a subsequent 2-h incubation.

To measure the bystander effect, HRP⁺ and HRP⁻ populations were mixed in different proportions and exposed to IAA for 24 h. After drug exposure, cells were counted using a hemocytometer and replated at low density for clonogenic survival.

After treatment, cells were rinsed with PBS and grown for 8–20 days in complete DMEM supplemented with feeder cells (V79 cells exposed to 250 Gy of 60Co irradiation). After fixation and staining with 2.5% (w/v) crystal violet (Sigma Chemical Co, Aldrich) in isomethylated spirit, colonies of >50 cells were scored. Surviving fractions were evaluated relative to HBSS-treated controls. The concentration of prodrug required to reduce cell survival by 50% (IC50) was estimated from the survival curves. At least three independent experiments were conducted (triplicate samples).

Growth Inhibition. Cells were preplated in 96-well plates (2000 cells/well, 8 wells/drug concentration) and exposed to the prodrugs as described in the previous section. After drug exposure, the cells were grown until control plates reached confluence (3–5 days) and assayed using the CellTiter 96 AQueous Assay (Promega, Southampton, United Kingdom) according to manufacturer’s instructions. Briefly, the culture medium was supplemented with MTS and phenazine methosulfate at final concentrations of 166 μg/ml and 12.5 μM, respectively, and left to react for 2 h. The plates were agitated to ensure complete mixing and scanned on a multivariate reader (Labsystems Multiskan MCC/340) at 492 nm. Cell density was evaluated relative to HBSS-treated controls. The IC50 was estimated from the curves.

Results
HRP/IAA Therapy of Human Cells. To demonstrate wide applicability of HRP-mediated GDEPT, four cell lines of human origin (T24 bladder carcinoma, MCF-7 breast adenocarcinoma, FaDu nasopharyngeal squamous carcinoma, and microvascular endothelial cell line HMEC-1) were evaluated. Cells were transiently transduced with the pRK34-HRP construct, in which the HRP cDNA was previously fused to the signal sequence from the human growth hormone and the KDEL retention motif (14). The KDEL tag causes accumulation of the HRP in the endoplasmic reticulum and the nuclear envelope (4, 14), preventing secretion of the enzyme. This would be an advantage in vivo, where nonspecific prodrug activation at sites distant from the tumor is undesirable. As assessed by immunostaining, the transfection efficiencies were 20–25% in T24 cells, 16–20% in MCF-7 cells, 10–14% in FaDu cells, and 18–20% in HMEC-1 cells. As expected, untransfected cells and mock-transfected (HRP⁻) cells expressing the reporter CD4 did not stain positive for HRP (results not shown). Synthesis of an immunoreactive M, 52,000 protein and competent peroxidase activity were confirmed in all HRP⁺ cells by Western blotting (results not shown) and HRP assay. In T24, MCF-7, and FaDu cells, HRP activity was 3.6 ± 0.9, 1.1 ± 0.6, and 0.8 ± 0.2 × 10⁶ units/μg total protein, respectively. Compared with the other cell lines, a higher peroxidase activity was detected in HMEC-1 cells (17 ± 3 × 10⁻⁵ units/μg total protein), which was consistent with an increased number of HRP-containing plasmids/cell, compared with the other cell lines. No detectable HRP protein production or catalyst activity could be measured in HRP⁻ cells (data not shown).

Susceptibility of HRP-expressing T24, MCF-7, and FaDu cells to prodruk treatment was assessed by exposing the cells to increasing concentrations of IAA (Fig. 2). Results on T24 cells confirmed findings reported previously (4). After only a 2-h exposure, significant cell killing was induced in HRP transfectants, whereas no toxicity was observed in HRP⁻ cells (Fig. 2A). However, above 4 μM, a rapid and dramatic decrease in survival was observed both in the HRP⁺ and HRP⁻ populations. This effect was due to a significant acidification of the IAA-containing medium because when the pH was adjusted to 7.4, no toxicity was detected in HRP⁻ cells exposed for 2 h to up to 20 μM IAA (results not shown). Doses above 4 μM were therefore not included in the survival curves.

A higher efficacy of the HRP/IAA combination was observed after prolonged (24 h) incubation (Fig. 2B). In all of the cell lines studied, expression of the HRP enzyme significantly enhanced sensitivity to IAA, with levels of cell kill of up to 4–5 logs. Some toxicity was also observed in HRP⁻ cells at concentrations ≥ 1 μM. Equivalent doses that reduced the surviving fraction to 50% (IC50) for HRP⁺ cells were compared with the corresponding HRP⁻ populations. The ratios of IC50 for HRP⁻ cells/IC50 for HRP⁺ cells were defined as SI. After a 24-h incubation, SIs of 36, 55, and 85 were measured in T24, MCF-7, and FaDu cells, respectively. After analysis of the full survival curves, the response of these three tumor cell lines to HRP/IAA was not found to differ considerably at doses of prodruk below 1 μM (Fig. 2B), with MCF-7 cells being slightly more sensitive. At higher concentrations of IAA, HRP⁺ T24 cells showed an increase in cell kill of 1–2 logs compared with HRP⁺ FaDu and MCF-7 cells.
The potential of the HRP/IAA combination to target the tumor vasculature was evaluated in the HMEC-1 endothelial cell line. The growth of HMEC-1 cells was monitored using the MTS assay (Fig. 3A) because these cells do not form discrete colonies. For comparative purposes, the growth of T24 cells was analyzed under the same experimental conditions (Fig. 3B). After 24 h of IAA treatment, inhibition of proliferation was detected in HRP”-HMEC-1 cells, following a dose response very similar to that of T24 HRP transfectants (Fig. 3). However, compared with HRP”-T24 cells, HRP”-HMEC-1 cells appeared to be more sensitive to IAA, resulting in a decrease in selectivity (HMEC-1 cells, SI ~ 3; T24 cells, SI > 4, as assessed using the MTS assay).

Use of IAA Derivatives for GDEPT. To increase the antitumor potential of the HRP/IAA system, a panel of IAA derivatives (Fig. 1) were studied in combination with HRP. 5-MeO-IAA, 6-F-IAA, and 4-CI-IAA showed effects very similar to IAA in normoxia (Fig. 4), but almost no selective cytotoxicity could be detected in HRP” cells in anoxia (Fig. 5). 2-Me,5MeO-IAA induced nearly no toxicity in both HRP” and HRP” cells under any of the exposure conditions analyzed (Figs. 4 and 5). 5-BrO-IAA and 5-Ph-IAA showed fast and effective HRP” cell killing in both normoxia and anoxia, but they also induced high nonspecific toxicity in HRP” control cells (Figs. 4 and 5).

The action of the compounds characterized by prompt normoxic (1-Me-IAA and 2-Me-IAA) as well as anoxic (in particular, 5-Br-IAA) cytotoxic activation and high HRP” cell kill and selectivity (1-Me-IAA, 5-Br-IAA, and 5-F-IAA) was further investigated in detailed survival experiments using different cell types. T24, MCF-7, and FaDu cells were exposed for 2 or 24 h to the analogues 1-Me-IAA, 2-Me-IAA, 5-F-IAA, and 5-Br-IAA in normoxia.

After prolonged incubation with the cellular monolayers (24 h), the prodrugs studied induced high and selective sensitization in HRP” cells in all three tumor lines (Fig. 6) and in HMEC-1 cells (data not shown). In particular, 1-Me-IAA showed the highest SIs (SI = 740 in T24, 71 in MCF-7, and 50 in FaDu cells) and HRP” cell kill, especially at low doses (Fig. 6A). 2-Me-IAA, on the other hand, induced surviving...
fractions of less than 10% only at doses above 1 mM (Fig. 6B). 5-F-IAA, although very effective in T24 (SI = 88) and FaDu (SI = 60) cells, was characterized by nonspecific toxicity in HRP. MCF-7 cells, resulting in a SI of only 1.6 in this cell line (Fig. 6C). Finally, 5-Br-IAA was efficient and selective in all of the cell lines analyzed (SIs were between 71 and 100; Fig. 6D) In this case, prodrg levels of up to 1 mM were studied because of toxicity in HRP+ cells.

The above-mentioned IAA derivatives were also tested in combination with HRP in T24 cells in the extreme tumor conditions of anoxia (Fig. 7). Compared with normoxic exposure, the effects of IAA, 1-Me-IAA, 2-Me-IAA, and 5-F-IAA were not modified after either a 2-h (data not shown) or a 24-h incubation (Figs. 2B, 5, and 7, A–D). Similar findings were obtained after hypoxic (0.1% O2) IAA treatment (data not shown). Unexpectedly, 5-Br-IAA showed toxicity in anoxic at 2 h, with 2–3 log cell kill induced at 3 mM (Fig. 7E), whereas normoxic 2-h treatment did not induce any measurable toxicity effects in both HRP− and HRP+ cells at the concentrations analyzed (data not shown). This anoxic selectivity was lost after prolonged exposure (24 h; Figs. 6D and 7F), although the 5-Br-IAA-mediated cell kill in HRP+ cells was higher than that observed after anoxic treatment with any of the other prodrgs tested.

**Bystander Effect of the HRP/IAA System.** The bystander effect can be defined as the ability of HRP-expressing cells to kill neighboring HRP+ cells in the presence of the prodrg IAA. Bystander killing is crucial for a successful GDEPT strategy because with the protocols currently adopted in clinical trials, the in vivo transfection efficiency is unlikely to be equal to 100%.

The bystander effect induced in vitro by the HRP/IAA system was examined in T24 cells by exposing populations of HRP− and HRP+ cells mixed in varying proportions to IAA, 1-Me-IAA, or 5-Br-IAA for 24 h. The concentration of prodrgs used in these experiments was chosen to be less than one-half the IC50 in HRP− T24 cells, i.e., 0.5 mM IAA, 0.5 mM 1-Me-IAA, and 0.1 mM 5-Br-IAA, which had little or no effect on cell survival in the mock-transfected population. The percentage of HRP+ cells was assessed by immunostaining. Fig. 8 shows that the three prodrgs were able to induce significant bystander killing in normoxic as well as in anoxic conditions. A total of 60–70% of the cells in the normoxic culture mixture could be killed when only 5% expressed HRP. A cell kill of 80–95% was achieved when 20–25% of cells were transfected with the HRP gene, which was the maximum transfection efficiency achievable in these experiments (Fig. 8).
This effect was not dependent on contact between HRP⁺ and HRP⁻ populations because HRP⁺ cells were killed when exposed to IAA preactivated by HRP⁻ cells (medium-switch experiments), as we have shown previously (4).

An even greater bystander effect was observed in anoxia: 80% (for IAA) to 96% (for 5-Br-IAA) cell kill occurred when only 5% of the exposed population were HRP⁺ (Fig. 8), and 2-log killing was induced at the highest concentration of HRP⁺ cells (20%). In contrast to what was observed in normoxia, the toxic product generated in anoxia was not transferable in medium-switch experiments (results not shown). However, transfer of cytotoxicity could be detected when the oxygen concentration was raised to 0.1% (data not shown).

Discussion

We have previously shown the potential of the HRP/IAA combination for GDEPT of cancer (4). In the present study, the action of HRP GDEPT was validated in four cell lines of human origin. The response of the three tumor cell lines analyzed, MCF-7 breast adenocarcinoma, FaDu nasopharyngeal carcinoma, and T24 bladder carcinoma cells, did not differ considerably (Fig. 2). After a 24-h incubation with IAA at prodrug levels below 1 mM, HRP expression conferred a slightly higher sensitivity to MCF-7 cells, whereas at prodrug levels above 1 mM, HRP⁺ T24 cells were markedly more affected by the treatment. The p53 status does not appear to play a major role in the response of these tumor cell lines to HRP/IAA because MCF-7 cells have a wild-type p53 gene (20), whereas FaDu and T24 cells are characterized by p53 nonsense and missense mutations at codons 248 and 126, respectively (21, 22). HRP/IAA may therefore function efficiently in the many tumors exhibiting mutant p53 phenotypes.

HRP/IAA GDEPT also induced significant inhibition of proliferation in HMEC-1 endothelial cells (Fig. 3A). This could represent an advantage if the tumor vasculature was to be targeted. Selective killing of the endothelial cells forming the lining of tumor blood vessels may cause malignant cells to starve for lack of nutrients, producing an amplification of the cytotoxic effects. Also, endothelial cells lack the drug resistance characteristic of neoplastic cells, requiring lower doses of cytotoxic agents, and the vicinity to the blood stream allows direct and simplified agent delivery. For HRP/IAA, low prodrug doses would be used because IAA alone showed some toxicity in HRP⁻ cells (Fig. 3A). Thus far, the effects of
HRP/IAA have not been tested in normal cells other than HMEC-1 cells, but preliminary in vivo studies indicate very low toxicity of the prodrug and the activated drug in non-cancer tissue. The possibility of enhancing the antitumor effect of HRP GDEPT was investigated in a panel of different IAA analogues. The screening of 10 IAA derivatives in the four cell lines adopted in this study allowed the identification of compounds characterized by fast normoxic (1-Me-IAA, 2-Me-IAA, and 5-MeO-IAA) or anoxic (5-Br-IAA) cytotoxic activation, high HRP+ cell kill (5-Br-IAA, 4-CI-IAA, 5-BrO-IAA, 5-Ph-IAA, 5-F-IAA, and 1-Me-IAA), or selectivity [1-Me-IAA, 5-Br-IAA, and 5-F-IAA (Figs. 4–7)]. Some variations were observed in the response of cells of different origin, with IAA, 1-Me-IAA, and 5-Br-IAA representing the most promising candidates for HRP GDEPT. In particular, 5-Br-IAA showed very prompt and selective anoxic activation (Fig. 7, E and F). The choice of the appropriate prodrug and dose may therefore depend on the tumor type, the hypoxic fraction, and the pharmacokinetics in vivo. HRP+ activation of the IAA prodrug in the presence of oxygen leads to formation of a stable toxic product, and the toxicity is transferable to HRP+ cells under normoxia (4, 10) or hypoxia (0.1% O2; results not shown). However, no such transferability was seen under anoxia (data not shown), indicating that the anoxic toxicity is caused by relatively short-lived reactive species. The initial activation of IAA by HRP generates a radical species (skatole radical), which is followed by a complex series of reactions with several possible pathways involving short-lived radical intermediates and longer-lived species (6). In normoxia, 3-methylene-2-oxindole is likely to be involved in HRP-mediated cell death. Under anoxic conditions, the pathway leading to 3-methylene-2-oxindole would not be available, and other toxic species are likely to be involved, as discussed in Ref. 4. In the case of 5-Br-IAA, the early toxicity after 2 h of incubation in anoxia may be due to an enhanced rate of one of these as yet undetermined reaction pathways, for this particular analogue only. Further work will concentrate on the analysis of the cytotoxic pathways at different oxygen concentrations.

The variation in toxicity of the compounds analyzed does not appear to depend on the rate of oxidation in the presence of HRP because no correlation could be measured between

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Footnotes:
4 L. K. Folkes, unpublished data.
5 S. Rosalier, unpublished data.
oxidation rate (6) and surviving fraction after a 2-h drug exposure. Unexpectedly, after a 24-h incubation with HRP+ cells, the IAA analogues that are not promptly oxidized by HRP (such as 5-F-IAA and 5-Br-IAA) resulted in the highest toxicity. Components and substituent position may both be relevant to the cytotoxic potential, as seen from the different efficacies of 1-Me-IAA and 2-Me-IAA or 5-F-IAA and 6-F-IAA (Figs. 4–7), for example.

Gene delivery to tumors in vivo is unlikely to lead to the modification of 100% of the target cells. Therefore, an essential requirement for GDEPT is that the activated drug should induce a bystander effect, whereby conversion of the prodrug to the active form in the enzyme-modified cells leads to the killing of adjacent untransfected ones. The killing of neighboring cells can be due to the transfer of toxic metabolic products through gap junctions (23), via apoptotic vesicles (24), or through the diffusion of soluble toxic metabolites (25, 26). Our studies suggest that the HRP/IAA system can produce a strong bystander effect. In all experiments, HRP transfectants were estimated to represent at best a quarter of the cells exposed to IAA, but this
mixed population could be almost completely eradicated (Fig. 2). Transient rather than stable lines were used because transient expression and lack of integration of the therapeutic gene are more likely to take place in vivo with current viral or nonviral delivery systems (e.g., adenoviruses and cationic liposomes). In mixing experiments, approximately 70% and 90% cell kill was observed, with only 5% and 20% of the cells expressing HRP, respectively (Fig. 8). The effect does not appear to require contact between HRP and HRP′ populations because incubation of HRP′ cells with preactivated IAA resulted in cell death under both normoxia (4) and hypoxia (0.1% O2). This compares very favorably with in vivo data on the bystander cytotoxicity of other enzyme/prodrug systems. For example, 95% cell kill after ganciclovir treatment required exposure of herpes simplex virus-1 thymidine kinase in 50% of the exposed population (24). Similarly, expression of cytosine deaminase in 5% of the cells resulted in 50% cell eradication after 5-fluorocytosine (27), and 90% growth inhibition could be achieved when 34–50% of the cells exposed to CB1954 produced the enzyme nitroreductase (26,28). Importantly, an even more pronounced bystander effect was observed in anoxic conditions (Fig. 8), which, as discussed previously, is likely to be due to short-lived reactive species.

The prodrug concentrations used in the in vitro bystander cytotoxicity experiments fall within the range of concentrations that could be achieved in vivo. In preliminary in vivo studies, 250 mg/kg IAA and 50 mg/kg 5-F-IAA i.p. in mice resulted in tumor prodrug levels of −1 and −0.2 mM, respectively, and plasma levels in excess of −1–3 mM.6 No adverse effects were recorded in normal tissues. Nevertheless, to achieve prodrug activation at the target only, current work is focused on placing the HRP gene under the control of hypoxia-responsive promoters,7 which may ensure selective therapeutic gene expression at the tumor site (29).

In summary, HRP represents an effective enzyme for use in combination with the IAA prodrug and its analogues. We are currently investigating the use of this combination for GDEPT in vivo in HRP-modified tumor xenografts of the bladder and nasopharyngeal carcinoma cell lines described. The in vitro analysis presented here allowed the identification of compounds with potential not only in gene therapy strategies, but also in antibody-directed enzyme/prodrug therapy and polymer-directed enzyme/prodrug therapy approaches. Particular attention will be drawn to the action of HRP in combination with IAA, 1-Me-IAA, and 5-Br-IAA.

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References

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6 Unpublished observations.


Oxic and anoxic enhancement of radiation-mediated toxicity by horseradish peroxidase/indole-3-acetic acid gene therapy

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Abstract.

Purpose: To evaluate the interaction of horseradish peroxidase (HRP)/indole-3-acetic acid (IAA) gene therapy with therapeutically relevant doses of radiation.

Materials and methods: Human T24 bladder and FaDu nasopharyngeal carcinoma cells were transiently transfected with the HRP cDNA using a non-viral delivery method. HRP expression was confirmed by immunostain and enzyme activity assay. The cells were exposed to IAA or the analogue 1-Me-IAA in conjunction with X-rays in air or in anoxic conditions, and cytotoxicity was determined by clonogenic assay.

Results: A significant and selective enhancement of radiation-mediated cytotoxicity was observed. Pre-incubation with the prodrugs induced sensitizer enhancement ratios (SER) ranging from 2.6 (0.1 mM IAA) to 5.4 (0.5 mM IAA). Radiosensitization was also observed when prodrug exposure was performed immediately after irradiation (SER = 2.1–5.6), or in anoxic conditions (SER = 3.6).

Conclusions: The use of gene therapy protocols to enhance the effect of ionizing radiation holds promise for anticancer therapy. The data suggest that the combination of HRP/IAA gene therapy with ionizing radiation could present therapeutic advantages in the treatment of solid malignancies, in particular to target the hypoxic population, which has been shown to correlate with poor outcome after radiotherapy.

1. Introduction

The use of suicide genes for cancer treatment is a promising approach that has shown selectivity and efficacy in experimental systems as well as clinical trials (for a recent review, see Greco and Dachs 2001). Suicide gene therapy, or gene-directed enzyme/prodrug therapy (GDEPT), is a two-step strategy in which a therapeutic gene encoding a non-toxic enzyme is delivered to the tumour. In the second step, a prodrug is administered and converted into a potent cytotoxin by the enzyme synthesized at the target. An important feature of GDEPT is the bystander effect (Pope et al., 1997), which can be defined as an extension of the killing effects of the active drug to untransfected neighbouring cells. This implies that even if only a fraction of the target cells are genetically modified and express the therapeutic gene, tumour eradication may still be achieved.

External beam radiotherapy remains one of the most important treatment modalities for human cancers. The tolerance of normal tissues to treatment-induced injury restricts the total dose that can be delivered to eradicate the solid tumour. Multiple factors contribute to the resistance of solid malignancies to radiation-induced cell death, including intrinsic genetic and extrinsic physiological determinants. The presence of hypoxic regions is the most significant adverse factors, affecting the response to radiotherapy in a range of human tumour sites (Brizel et al., 1997, 1999, Fyles et al. 1998, Nordmark et al. 1998, Knocke et al. 1999). However, resistance to radiation via the classical oxygen effect is unlikely to be the only explanation (Höckel et al. 1999). Hypoxia-induced modifications of gene expression may also contribute to poor outlook, giving rise to more aggressive locoregional disease and enhanced invasive capacity (Alarcon et al. 1996, Graeber et al. 1996, Sundfor et al. 1998).

A number of biological strategies are currently under investigation to overcome the limitations of dose delivery in conventional radiotherapy. For example, the combination of gene therapy approaches with ionizing radiation has been analysed (reviewed in Buchsbaum et al. 1996). In particular, the widely used GDEPT systems of herpes simplex virus-1 thymidine kinase (HSV TK)/ganciclovir (GCV) and cytosine deaminase (CD)/5-fluorocytosine (5-FC) induced selective enhancement of radiation-mediated toxicity, both in vitro (Kim et al. 1994, Khil et al. 1996) and in vivo (Kim et al. 1995, Hanna et al. 1997, Pederson et al. 1997, Rogulski et al. 1997). Synergistic advantage was demonstrated, and clinical trials are ongoing, where doses of prodrugs can be delivered in combination with radiotherapy without severe toxicity to the patients (Chikara et al. 2000, Freytag et al. 2000). Furthermore, exploiting the tumour-specificity of the hypoxic microenvironment for gene delivery, gene expression and prodrug activation, hypoxia-targeted GDEPT has the potential to contribute to overcoming the
radioresistance of poorly oxygenated tumour areas (Greco et al. 2000a).

A novel GDEPT system is currently being developed, which consists of the peroxidase from horseradish (HRP) and the plant hormone indole-3-acetic acid (IAA; Greco et al. 2000b). In vitro studies demonstrated that in HRP-transfected human tumour cells prodrug activation was prompt and efficient (Greco et al. 2000b). Cytotoxicity could be evoked within a 2 h exposure, and it was further increased after 24 h incubation. The HRP/IAA combination resulted in faster and more effective cell kill than HSV TK/GCV, in particular in the extreme tumour conditions of anoxia (Greco et al. 2000b). The toxic agents and the cellular targets involved in HRP/IAA-mediated toxicity are yet to be identified. At neutral pH, IAA is oxidized by HRP-compound I (Cpd I, figure 1) to a radical cation, which undergoes scission of the exocyclic carbon–carbon bond to yield the carbon-centred skatolyl radical (Dunford 1999, figure 1). In the presence of oxygen, the skatolyl radical rapidly forms a peroxyl radical, which then decays to a number of products, the major ones being indole-3-carbinol, oxindole-3-carbinol and 3-methylene-2-oxindole (MOI). In anoxic solution, decarboxylation of the radical cation can still take place and the carbon-centred radical preferentially reacts with hydrogen donors (Candeias et al. 1994). The skatolyl radical is very reactive towards DNA (Folkes et al. 1999) and lipids. Inoxic conditions, MOI has been reported to react with cellular nucleophiles such as thiols, DNA and sulphhydryl groups in enzymes or histone (Folkes and Wardman 2001). These pathways appear to be specific for HRP, as mammalian peroxidases failed to convert IAA into a cytotoxic at therapeutically significant prodrug doses (Pires de Melo et al. 1997, 1998, Folkes et al. 1998). Also, IAA is well tolerated in humans, as after oral administration of 100 mg/kg IAA (0.57 mmol/kg) no significant side effects were reported (Mirsyky and Diergott 1956).

Besides causing direct cytotoxicity, HRP/IAA-induced DNA strand breaks and adducts (Folkes et al. 1999) and/or thiol depletion (Folkes et al., in press) may provide sensitization to other treatments, such as radiation, concomitantly administered. Therefore, the potential of the HRP/IAA combination to selectively enhance radiation cytotoxicity was investigated in vitro. In the present study, human tumour cells transfected with the HRP cDNA were exposed to IAA or the analogue 1-Me-IAA in conjunction with X-rays. The interaction of the GDEPT protocol with therapeutically significant doses of ionizing radiation was evaluated inoxic and anoxic conditions.

2. Materials and methods

2.1. Cell culture

T24 bladder carcinoma cells (European Collection of Cell Cultures, Salisbury, UK) and FaDu, nasopharyngeal squamous carcinoma cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (Sigma Aldrich, Gillingham, UK), 2 mM l-glutamine (Life Technologies), 100 U/ml penicillin (Sigma Aldrich), 100 µg/ml streptomycin (Sigma Aldrich), and incubated in a humidified incubator at 37°C and 5% CO₂/air. Only cells that tested negative for mycoplasma infection were utilized.

2.2. Anoxic conditions

For experiments in anoxic conditions, cells were plated in 6 cm oxygen-impermeable dishes (Permanox, Nalge Nunc International, Loughborough, UK) and incubated at 37°C in an anaerobic glove cabinet (DON Whitley Scientific Limited, Shipley, UK) with 5% CO₂, 5% H₂, 90% N₂ and palladium catalyst, or, for radiation treatment, in airtight aluminium boxes flushed continuously with a
humidified gas mixture containing 5% CO₂ and 95% N₂. All sample manipulations were carried out in the anaerobic cabinet. Plastics and media were pre-incubated in anoxia for 48 h before use to remove residual oxygen.

2.3. Plasmid DNA and cell transfection

The plasmid pRK34-HRP (Connolly et al. 1994) was kindly provided by Dr. D. F. Cutler, UCL, London. The control plasmid pCMV-CD4, containing the gene for the marker protein CD4, was constructed as described previously (Dachs et al. 2000). In both cases, gene expression was driven by the strong cytomegalovirus (CMV) early promoter.

Transient transfectants were obtained by exposing the cells in air to complexes of integrin-targeted peptides, lipofectin (Life Technologies) and DNA (Hart et al. 1998), and were assayed for gene expression after 24 h. Transfection efficiency with the plasmid pRK34-HRP was analysed by immunofluorescence analysis (§2.4).

2.4. Immunofluorescence staining

Antibody staining was carried out by fixing cell suspensions in 3% paraformaldehyde (Sigma Aldrich)/PBS for 20 min at room temperature (RT). After centrifugation the pellets were rinsed in PBS and incubated for 15 min at RT in 15 mM glycine (Sigma Aldrich)/PBS. Non-specific binding was blocked by addition of the wash buffer (5% FCS/1% Tween 20 (Sigma Aldrich)/PBS). Specific binding was blocked by addition of the wash buffer (5% FCS/1% Tween 20 (Sigma Aldrich)/PBS). The samples were centrifuged and incubated for 1 h at RT in rabbit polyclonal anti-HRP (Dako, Ely, UK) diluted 1:200 in 10% FCS/wash buffer. Following extensive rinsing in wash buffer (at least three times), the samples were re-suspended in TRITC-conjugated swine anti-rabbit immunoglobulins (Dako) diluted 1:200 in 10% FCS/wash buffer and incubated for 1 h at RT. The cells were rinsed in wash buffer and re-suspended in Hanks' balanced salt solution (HBSS; Life Technologies) for FACS analysis on a Becton Dickinson FACScan. Cells were scored as positive if they showed an increased fluorescence with respect to control cells transfected with pCMV-CD4.

2.5. HRP activity assay

HRP activity was analysed using a modified 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB; Sigma Aldrich) assay, as described previously (Greco et al. 2000b). The total cellular protein content in the samples was determined by using a commercial protein assay kit (Bio-rad, Hemel Hempstead, UK).

2.6. Prodrug exposure

Exponentially growing cells were plated at low density and exposed to IAA or 1-Me-IAA (Sigma Aldrich) in phenol-red-free HBSS for 24 h in the 37°C incubator. Alternatively, cells were pre-plated in the anaerobic cabinet and, after allowing the cells to settle for 5–6 h, they were exposed to the prodrugs for 24 h in anoxic conditions.

2.7. Irradiation procedure

Cells were irradiated at 37°C using a Pantak X-ray generator, operated at 240 kVp (HVL 1.3 mm Cu), at a dose rate of 1.6 Gy/min. For irradiation in anoxic conditions, the cells were exposed to X-rays in air-tight aluminium boxes flushed with the humidified anoxic gas mixture.

2.8. Clonogenic assay

Following drug exposure and/or irradiation, cells were rinsed with PBS and grown for 7–10 days in complete DMEM supplemented with feeder cells (V79 cells exposed to 250 Gy ⁶⁰Co irradiation) at 37°C in water-saturated environment. After fixation and staining with 2.5% crystal violet (Sigma Aldrich) w/v in isomethylated spirit (IMS), colonies of >50 cells were scored. Surviving fractions were normalized for plating efficiency of mock-irradiated cells exposed to HBSS or prodrug. For each data point, at least three independent experiments (triplicate samples) were performed.

2.9. Statistical analysis

Radiation survival data were compared by performing a multiple regression analysis. Values of \( p < 0.05 \) were considered as significant. The survival curves were analysed according to the single-hit multi-target (SHMT) model \( \left( SF=1-\left[1-exp\left(-dose/D_{50}\right)\right]^n \right) \), by using a non-linear fitting package (JMP; SAS Institute Inc, Marlow, UK). The SHMT rather than the linear–quadratic model was adopted, since it showed a better agreement with the experimental data. The sensitizer enhancement ratio (SER) was assessed from the survival curves by the ratio of doses of radiation that reduce cell survival by 50%.

3. Results

Immunolabelling of HRP followed by FACS analysis indicated that human T24 bladder carcinoma cells and FaDu nasopharyngeal squamous carcinoma
cells exposed to complexes of integrin-targeted peptides, lipofectin and plasmid DNA (pRK33-HRP; Connolly et al. 1994) could be transiently transfected with the HRP cDNA. Transfection efficiencies of 20–25% for T24 cells and 10–14% for FaDu cells were measured. Synthesis of a competent peroxidase was confirmed in the HRP-expressing (HRP⁺) cells by enzyme activity assay (data not shown).

Figure 2 shows the survival of HRP⁺ cells and of control cells, transfected with the marker CD4 (CD4⁺), exposed for 24 h to two doses of the prodrugs IAA and 1-Me-IAA. Incubation of T24 cells with IAA was performed in air as well as in the extreme tumour conditions of anoxia. At the doses of 0.1 and 0.5 mM no cytotoxic effects were measured in control CD4⁺ cells. On the other hand, HRP⁺ cells could be selectively sensitized to IAA and its analogue in a dose-dependent fashion (figure 2). No major differences were detected in the response of the two lines under either oxygenation condition, and 70–80% (0.1 mM) or 86–94% (0.5 mM) cell kill were measured. Detailed evaluation of the response of T24 and FaDu cells to HRP/IAA GDEPT has been reported elsewhere (Greco et al. 2000b, Greco et al., in press).

Prior to combined radiation/GDEPT experiments, the effect of gene delivery on the radiation response of T24 cells was analysed. Untransfected cells and transient transfectants with either the HRP or the CD4 gene were exposed to doses of X-rays ranging from 1 to 7 Gy. The intrinsic radiosensitivity of untransfected T24 cells was not significantly altered after transfection with either foreign gene (data not shown). Only CD4⁺ transfectants were used in further experiments as controls.

In the presence of the prodrug IAA, a marked increase in sensitivity to radiation was selectively induced in HRP⁺ cells (figure 3). No significant difference in the response to radiation was observed in CD4⁺ cells in combination with IAA (figure 3A, C). On the other hand, HRP⁺ cells incubated for 24 h with 0.1 or 0.5 mM IAA prior to (figure 3B) or immediately after (figure 3D) irradiation demonstrated a significant enhancement of radiation-mediated toxicity. The results shown in figure 3 did not change whether the prodrug was present or not at the time of irradiation (data not shown).

To confirm the observed effect in another tumour cell line, FaDu cells were exposed to a single radiation dose in combination with HRP/IAA. Similarly to T24 cells, after pre-incubation with IAA, HRP⁺
FaDu cells showed a significant increase in radiation-induced cell kill, while the response of control CD4+ cells was unaffected (figure 4).

SER can be defined as the quotient of two radiation doses which cause the same cell kill (Lambin et al. 2000). In this work, 50% cell kill was considered as an endpoint. The survival curves were analysed according to the SHMT model, normalizing for the plating efficiency of mock-irradiated cells exposed to the prodrug. In HRP+ T24 cells pre-incubated with IAA, SER values of 2.6 (0.1 mM IAA) and 3.4 (0.5 mM IAA) were measured. Very similar values (2.5 and 5.6) were estimated when prodrug incubation was performed after irradiation.

In a previous study, it was shown that the efficacy and selectivity of HRP/IAA GDEPT could be improved by adopting different IAA derivatives (Greco et al., in press). Our results indicated that in T24 cells 1-Me-IAA could induce a higher selectivity index and bystander killing than IAA, both in air and anoxia. The use of the 1-Me-IAA in combination with ionizing radiation was therefore studied. As illustrated in figure 5, HRP+ T24 cells exposed to 0.1 or 0.5 mM 1-Me-IAA before or after irradiation showed increased cell kill compared with cells exposed to buffer only (figure 5B,D), while no sensitization was induced in control cells (figure 5A,C). SER values were calculated to be 2.8 (0.1 mM 1-Me-IAA) and 4.6 (0.5 mM 1-Me-IAA), when the prodrug was administered for 24 h before X-rays, and 2.1 and 3.0, respectively, if prodrug incubation took place immediately after irradiation.

A synergistic effect of HRP/IAA GDEPT in combination with radiation could be of even higher therapeutic impact if effective in hypoxic cells, since the presence of hypoxic areas in solid tumours has been shown to correlate with poor outcome after radiotherapy.

T24 cells irradiated in anoxia showed an oxygen enhancement ratio (OER) of ~2, i.e. double the dose of radiation was necessary to induce 50% cell kill compared withoxic cells. When pre-incubated with 0.1 mM IAA, HRP+ cells showed a 3.6-fold increase in sensitivity to radiation (figure 6B), and the OER was reduced to 1.2. The response of control cells transfected with the marker CD4 was not significantly different whether the monolayers were pre-exposed to IAA or buffer only (figure 6A).

4. Discussion

The use of gene therapy protocols to enhance the effect of ionizing radiation holds promise for
Figure 6. Effect of HRP/IAA GDEPT on the radiosensitivity of T24 cells in anoxia. CD4+ (A) and HRP+ (B) cells were exposed to 0.1 mM IAA for 24 h prior to irradiation. Means ± SE are shown. Lines are best-fit curves. Cell survival was normalized to exposure to IAA, to account for drug toxicity. Survival data for oxic cells without IAA are shown for comparative purposes. —▽—, CD4+ T24 cells, no prodrug, air; —△—, CD4+ cells, no prodrug, anoxia; —○—, CD4+ cells + 0.1 mM IAA, anoxia; —▼—, HRP+ cells, no prodrug, air; —△—, HRP+ cells, no prodrug, anoxia; —●—, HRP+ cells + 0.1 mM IAA, anoxia.

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Figure 6. Effect of HRP/IAA GDEPT on the radiosensitivity of T24 cells in anoxia. CD4+ (A) and HRP+ (B) cells were exposed to 0.1 mM IAA for 24 h prior to irradiation. Means ± SE are shown. Lines are best-fit curves. Cell survival was normalized to exposure to IAA, to account for drug toxicity. Survival data for oxic cells without IAA are shown for comparative purposes. —▽—, CD4+ T24 cells, no prodrug, air; —△—, CD4+ cells, no prodrug, anoxia; —○—, CD4+ cells + 0.1 mM IAA, anoxia; —▼—, HRP+ cells, no prodrug, air; —△—, HRP+ cells, no prodrug, anoxia; —●—, HRP+ cells + 0.1 mM IAA, anoxia.

anticancer therapy. The results in this study show that human tumour cells engineered to express the plant enzyme horseradish peroxidase (HRP), which selectively activates the prodrug indole-3-acetic acid (IAA) into a cytotoxic agent, show an increase in radiation-induced toxicity in oxic as well as in anoxic conditions.

Statistical evaluation of combined radiation and GDEPT protocols has shown that in vitro SER values of 1.2 can significantly increase local control after radiotherapy (Lambin et al. 2000). In the present studies, values between 2.1 and 5.6 were measured in oxic conditions, depending on prodrug concentration. Moreover, the sensitization effect could be detected at doses of radiation as low as 1 Gy, indicating that multificationed treatment (usually 2 Gy/fraction, over 6–8 weeks) in a clinical setting may be effective. No major difference was detected in the radiosensitization potential of the two produgs tested (IAA and 1-Me-IAA), with IAA being slightly more potent (figures 3 and 5).

Delivery of a foreign gene alone (HRP or the marker CD4) did not itself alter cellular radiosensitivity. Similar findings were reported by other investigators who utilized retroviral (Kim et al. 1994, 1995, Khil et al. 1996) or adenoviral vectors (Hanna et al. 1997, Blackburn et al. 1999, Valerie et al. 2000) for GDEPT. More importantly, Mansur et al. (2001) observed that neither retroviral nor liposome-mediated delivery of the glutathione peroxidase (GPX) gene resulted in an effect on radiosensitivity of human T cells and Chinese hamster ovary (CHO) fibroblasts. On the other hand, an increase in sensitivity to radiation was observed in cells transduced with adenovirus containing the cytosine deaminase gene (Pederson et al. 1997, Stackhouse et al. 2000).

True radiation sensitizers have no lethal effect of their own. The doses of prodrug tested reduced the survival of HRP+ cells to ~30% at 0.1 mM and ~0.6% at 0.5 mM after 24 h (figure 2). Further selective toxicity could be achieved with the addition of radiation. These levels of prodrug were found to be less than one-half the concentration required to reduce cell survival by 50% (IC50) in HRP− T24 and FaDu cells (Greco et al., in press). Doses in this range are likely to be achievable in vivo and may represent a possible clinical scenario. In mice, 250 mg/kg IAA and 50 mg/kg 3-F-IAA i.p. resulted in tumour prodrug levels of ~0.5 mM and ~0.2 mM respectively, and plasma levels in excess of ~1 mM, with no associated toxicity (authors’ own unpublished observations). Also, transient rather than stable transfectants were utilized in this study in order to mimic a clinical gene therapy protocol.

Limited transfection efficiency (10–14% in FaDu and 20–25% in T24 cells) compared with the levels of cell kill was measured (figures 2–6), therefore a bystander effect was induced, whereby the activated prodrug induced toxicity and radiosensitization also of surrounding untransfected tumour cells. Activated IAA can cross cell membranes (Folkes et al. 1999) and has been observed to induce strong bystander killing, with no requirement for cell-to-cell contact (Greco et al. 2000b).

Significant synergy of HRP/IAA GDEPT and X-ray exposure was also detected in the extreme tumour condition of anoxia. Anoxic HRP+ cells pre-exposed to 0.1 mM IAA were 3.6-fold more sensitive to radiation than buffer-treated controls, and the OER was reduced from 2 to 1.2. Hypoxic radiosensitization may result in increased therapeutic effectiveness, and be exploited for hypoxia-targeted gene therapy (Dachs et al. 1997). To the authors’ knowledge, this is the first report demonstrating efficacy of a combined radiation and GDEPT protocol in oxic as well as anoxic conditions. HRP/IAA was also toxic in cells at intermediate oxygen concentrations (Greco et al., in press), which appear to be important for the outcome of a fractionated radiotherapy schedule (Wouters and Brown 1997).

Similar SER values were measured when prodrug incubation was performed prior to or after X-rays, suggesting that activated IAA may increase susceptibility to ionizing radiation and also interfere with post-irradiation repair processes. To date, the
activated drugs and the cellular targets involved in HRP/IAA-mediated cytotoxicity and radiosensitization have not been identified. However, of the stable oxidic products, 3-methylene-2-oxindole (MOI, figure 1) has been reported to be toxic in Escherichia coli and some plants, to react with glutathione (GSH) and to bind to sulphydryl regions of histone DNA or RNA (Folkes and Wardman 2001). Exposure of hamster fibroblast V79 cells to IAA or 5-F-IAA activated by purified HRP resulted in a decrease of cellular GSH up to ~70% of control levels (Folkes et al., in press). Also, when activated IAA was incubated with plasmid DNA in a cell-free system, DNA adducts and strand breaks were observed (Folkes et al. 1999). In the absence of oxygen, MOI cannot be formed. The observed toxicity is likely to be free-radical based due to the results in anoxia (Greco et al. 2000b). Skatolyl-type radicals readily abstract hydrogen from donor molecules and have been shown to react with biomolecules such as DNA (Folkes et al. 1999) or lipid, and could lead to cell damage by the formation of secondary radicals in key biological targets.

Depletion of intracellular thiols has been shown to have a radiosensitizing effect 

*(in vitro) (Clark et al. 1984, Varnes et al. 1984, Biaglow et al. 1986) and 

*(in vivo) (Bump et al. 1982, Stevens et al. 1995). The effect has been observed in both anoxic and anoxic conditions (Varnes et al. 1984, Debieu et al. 1985), with preferential sensitization of cells at low or intermediate oxygen levels (Bump et al. 1982, Clark et al. 1984, Debieu et al. 1985, Scott et al. 1993, Stevens et al. 1995). GSH depletion did not induce any significant alteration of the OER in normal mouse skin (Stevens et al. 1995), V79 (Mitchell et al. 1983) and A549 human lung carcinoma cells (Biaglow et al. 1983a, Mitchell et al. 1983). A decrease in OER has been observed in cells that show no oxic response to thiol depletion (Biaglow et al. 1983b).

However, GSH depletion is unlikely to be solely responsible for the effects shown in this paper. The reduction in GSH levels to 70% after treatment with activated 5-F-IAA (Folkes et al., in press) is probably insufficient to account for the SER achieved, as a reduction to >90% was necessary to achieve full radiation sensitization (Clark et al. 1984). Moreover, GSH depletion would only be expected to radiosensitize prior to irradiation, while HRP/IAA was shown to induce sensitization also after irradiation. Additional biochemical alterations, probably involving the DNA macromolecule, are likely to take place. It is clear that further studies are now warranted to shed light on the possible mechanisms associated with HRP/IAA-mediated radiosensitization. DNA damage and thiol depletion will be quantitatively analysed 

*(in vitro), in cells treated with IAA and purified HRP in combination with ionizing radiation.

With 50% of all human cancer patients treated with radiation, improvement of the efficacy of a radiotherapy schedule remains a major issue in cancer research. Numerous clinical trials have focused on combining radiation with conventional chemotherapeutic agents, such as doxorubicin, methotrexate, 5-fluorouracil and cisplatin (Stewart and Saunders 1997). However, normal tissue toxicity remains a major limiting factor with these protocols. The results presented here demonstrate that IAA and 1-Me-IAA selectively enhance radiation-induced lethality in human tumour cells expressing the enzyme HRP at doses of prodrug achievable 

*(in vivo). Selective radiosensitization was observed in oxic as well as radiosensitive anoxic cells, and testing in animal models is now warranted. In conclusion, the combination of HRP-gene therapy protocols with ionizing radiation can represent an interesting and promising approach to overcome limitations of tumour biology.

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5-Fluoroindole-3-acetic acid: a prodrug activated by a peroxidase with potential for use in targeted cancer therapy

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Abstract

Indole-3-acetic acid and some derivatives are oxidized by horseradish peroxidase, forming a radical-cation that rapidly fragments (eliminating CO\(_2\)) to form cytotoxic products. No toxicity is seen when either indole-3-acetic acid or horseradish peroxidase is incubated alone at concentrations that together form potent cytotoxins. Unexpectedly, 5-fluoroindole-3-acetic acid, which is oxidized by horseradish peroxidase compound I 10-fold more slowly than indole-3-acetic acid, is much more cytotoxic towards V79 hamster fibroblasts in the presence of peroxidase than the unsubstituted indole. The fluorinated prodrug/peroxidase combination also shows potent cytotoxic activity in human and rodent tumor cell lines. Cytotoxicity is thought to arise in part from the formation of 3-methylene-2-oxindole (or analogues) that can conjugate with thiols and probably DNA or other biological nucleophiles. Levels of the fluorinated prodrug in the murine carcinoma NT after intraperitoneal administration of 50 mg/kg were about 200 pM. Although these were 4–5-fold lower than plasma levels (which reached 1 mM), the integrated area under the concentration/time curve in tumors over 2 hr was ~20 mM min, almost double the exposure needed to achieve ~90–99% cell kill in human MCF7 breast or HT29 colon tumor cell lines and CaNT murine cells in vitro, although the human bladder T24 carcinoma cell line was more resistant. The high cytotoxicity of 5-fluoroindole-3-acetic acid after oxidative activation suggests its further evaluation as a prodrug for targeted cancer therapy involving antibody-, polymer-, or gene-directed delivery of horseradish peroxidase or similar activating enzymes. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Indole-3-acetic acid; 5-Fluoroindole-3-acetic acid; Peroxidase; Horseradish peroxidase; Prodrug; 3-Methylene-2-oxindole

1. Introduction

Approaches to cancer therapy involving prodrugs activated by tumor-targeted enzymes, such as in the ADEPT strategy [1], have utilized many different prodrugs. Examples include alkylating mustards masked either by conjugation (activated by carboxypeptidase), or deactivated nitroarene moieties (activated by nitroreductase) [2]. We have identified an alternative prodrug/enzyme combination that is worthy of exploration because of the known low toxicity of the prototype prodrug and the potential of the activating enzyme to be modified to reduce immunological reactions. Indole-3-acetic acid (IAA) (Fig. 1, 1), a plant growth hormone, is oxidized by horseradish peroxidase (HRP) to form products cytotoxic to mammalian cells [3]. The initiating hypothesis was based on the possibility that IAA/HRP would initiate lipid peroxidation, as found in liposome models [3–6]. The free radical formed on one-electron oxidation of IAA (the indolyl radical-cation, Fig. 1, 2) was known to fragment in ~40 μs, releasing CO\(_2\) to form the skatolyl radical 3. In the presence of oxygen this forms the skatole peroxyl radical 4, the presumed reactive intermediate in lipid peroxidation [7]. However, cytotoxicity was demonstrated in experiments in which IAA was oxidized either by HRP or by radiolysis and the stable products then added to cells [8], indicating the short-lived peroxyl radical was not itself the damaging species. Lipid peroxidation was not detectable in mammalian cells after cytotoxic treatments, also pointing to other mechanisms of cytotoxicity [9]. Toxicity has been measured in Chinese hamster lung fibroblast V79 cells with a range of different IAA analogues, and differences
in toxicity were observed with different substituents. Damage to liposome model systems and plasmid DNA was seen [8], but the cause of cellular toxicity remains unclear.

HRP compounds I and II (Cpds I and II, oxidation states shown in Fig. 1) are key oxidizing intermediates in the action of the peroxidase [10–12] (see Fig. 1). These oxidize IAA in the absence of hydrogen peroxide to the indolyl radical-cation 2, the precursor of the carbon-centered skatolyl radical 3 [13,14]. This radical can abstract a hydrogen atom from DNA in anoxia [8], or react with oxygen to form the skatole hydroperoxyl radical 4. This by further steps (see Fig. 1) leads to the major products: indole-3-aldehyde (5), indole-3-carbinol (6), skatole hydroperoxide (7), oxindole-3-carbinol (8) and 3-methylene-2-oxindole (MOI, 9) [11,15]. The latter is a known product of the IAA/HRP reaction [16], and is a candidate for the putative toxic species in aerobic systems due to its reactivity towards cellular nucleophiles, such as thiols or DNA, as illustrated in the formation of the GSH adduct, Fig. 1 (10) [9,17–19].

The rates of oxidation of simple amines and phenols by the key peroxidase intermediates HRP Cpds I and II show a marked dependence on substituents that modify the redox properties [11,20]. This was also found to be the case with substituted indole-3-acetic acids [21,22]. Substitution by electron-withdrawing halogen moieties, such as fluorine, is expected to deactivate indole-3-acetic acids towards oxidation by HRP: redox relationships for oxidation of IAA derivatives by HRP Cpds I using either Hammett sigma substituent parameters or reduction potentials of the radicals were established [21,22]. (Oxidation of most substrates by Cpds II is usually several-fold more slowly than Cpds I, although parallel redox relationships are observed.) However, factors other than the rates of oxidation or decarboxylation by HRP may control activity. We show here that 5-fluorooindole-3-acetic acid (FIAA), is indeed oxidized an order of magnitude more slowly than IAA by HRP Cpds I, but is much more toxic when activated, indicating that the effects of substituents on the reactivity of products as well as the rate of activation has to be considered.
2. Materials and methods

2.1. Materials

HRP type IV-A, FIAA, IAA, l-buthionine sulfoximine (BSO), fetal calf serum (FCS), Eagle’s modified medium (EMEM) and spinner modified EMEM (SMEM), non-essential amino acids, penicillin, streptomycin, l-glutamine, trypsin and phenol-red free Hanks’ balanced salt solution were obtained from Sigma. Monobromobimane was obtained from Molecular Probes. Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Life Technologies. Other chemicals were from Merck, AnalaR grade.

2.2. Cells

Chinese hamster V79 lung fibroblast, HT29 human colon carcinoma, MCF7 human breast carcinoma, CaNT mouse carcinoma, and T24 human bladder carcinoma cells were obtained from the European Collection of Cell Cultures. V79 cells were maintained in EMEM supplemented with 10% FCS, or SMEM supplemented with 7.5% FCS. CaNT cells were maintained as attached monolayers in EMEM supplemented with 10% FCS, HT29 cells and MCF7 cells similarly in EMEM supplemented with 10% FCS and 1% non-essential amino acids, and T24 cells similarly in DMEM supplemented with 10% FCS. All media were supplemented with 2 mM l-glutamine, 100 unit/mL penicillin and 100 µg/mL streptomycin. All cells were subcultured by trypsin removal of the cells (1 x 0.5% porcine trypsin, 0.2% EDTA).

2.3. Measurements of cytotoxicity

Indole stock solutions were prepared daily in 1 or 10% ethanol and protected from light. The pH was adjusted to 7.4 for high final concentrations of indoles. For cytotoxicity experiments, V79 cells were allowed to attach, from spinner culture, for at least 1 hr before administration of the drug; HT29, MCF7 and T24 cells were allowed to attach for at least 4 hr, and CaNT cells overnight (16 hr) following trypsin removal. Cell survival experiments were carried out as previously described [8]. Attached cells on Petri dishes (200–20,000 cells) were treated with 2 mL indoles (50 or 100 µM) and HRP (1.2 µg/mL) in phenol red free Hanks’ balanced salt solution, then washed with 2 mL Hanks’, and left to form colonies in EMEM for 7 days. After growth, colonies were fixed with 75% methanol and stained with 1% (w/v) crystal violet. Colonies containing >50 cells were counted and surviving fractions (SF) calculated relative to untreated controls.

2.4. Effects on intracellular glutathione

Cellular glutathione (GSH) depletion was measured by plating 10^6 cells in phenol red free DMEM. The cells were left to attach for 1 hr, then 50 µM IAA or FIAA added in phenol red free Hanks’ solution, with or without 1.2 µg/mL HRP. Samples were taken from 0 to 2 hr. Cells were washed with 4 x 2 mL Hanks’ solution and scraped in 1 mL 50 mM perchloric acid/1 mM EDTA before freezing at —20°. Duplicate samples were thawed and solid matter removed by centrifugation. Supernatants were analyzed for GSH content after derivatization with monobromobimane (MBB) [23]. Samples (0.4 mL) were mixed with mercaptoethanol (25 µL, 100 µM), MBB (25 µL, 10 mM) and Tris–HCl (250 µL, 2 M containing 1 mM EDTA) for 15 min in the dark. The samples were then acidified with HCl (50 µL, 6 M) and interfering substances removed by extraction with 0.5 mL dichloromethane, retaining the sample in the aqueous phase. HPLC involved a 250 mm x 4 mm Hypersil 50DS column eluting at a flow rate of 2 mL/min with a gradient of NH4H2PO4 (40 mM)/H2PO4 (10 mM)/l-octanesulfonic acid (5 mM) and 10–40% acetonitrile/water (75% (v/v)) over 10 min. Detection was by fluorescence (Perkin-Elmer LS40 detector, excitation 398 nm, emission 476 nm).

The effect of GSH depletion in V79 cells was carried out by treating 3 x 10^5 V79 cells/mL in SMEM with 0.1 mM BSO overnight (16 hr). The cells were counted the following day and plated on Petri dishes in phenol red free DMEM for 1 hr prior to drug treatment. The cytotoxicity of 50 µM FIAA or IAA was then measured as already described and compared to non-GSH depleted cells. GSH depletion was checked by washing treated cells in Hanks’ solution, lysing in 50 mM PCA/1 mM EDTA and storing at —20° for HPLC analysis.

2.5. Analysis of products

The products of oxidation of FIAA with HRP, and reactivity of FMOI with thiols were measured by oxidizing 0.1 mM FIAA with 10 µg/mL HRP in Hanks’ for 2 hr. Excess GSH or cysteine (1 mM) was added and the loss of FMOI demonstrated by HPLC. Compounds were eluted on a reversed phase RP8 column (100 mm x 3.2 mm) with 50 mM ammonium acetate, pH 5.1 (A) and methanol (B) with a gradient of 20–60% B in 8 min at 0.9 mL/min. Detection was by a Waters photo diode array detector extracting at 250 nm. Mass spectral analysis (LCMS) was carried out using a Waters ThermaBeam mass detector, operating in scan mode over an appropriate mass range using electron impact ionization.

2.6. Kinetics of reaction with HRP compound I

The rate of reaction of FIAA with HRP compound I, formed from a 1 s premixing of equimolar HRP and hydrogen peroxide (0.47 µM), was carried out as previously described [21] using a Hi-Tech SF-61 DX2 double mixing stopped-flow spectrophotometer equipped with a xenon lamp. Formation of HRP compound II was detected
at 411 nm at 25°C in 10 mM phosphate buffer pH 7 with 50 mM potassium bromide.

2.7. Distribution of FIAA in murine tissues

FIAA (5 mg/mL) in 2% (v/v) ethanol/water was adjusted to pH 7.4 with NaOH and injected i.p. (50 mg/kg) in female CBA mice bearing the CaNT tumor. The mice were sacrificed up to 2 hr after drug administration by decapitation, and the blood (heparin-coated tubes) and tissues removed and immediately placed on ice. The whole blood was spun down and the plasma stored at −20°C. Tissue samples were weighed and homogenized in four to nine volumes of ice-cold water added. The homogenized tissue was stored at −20°C before HPLC analysis.

For HPLC analysis, plasma (50 μL) was mixed with IAA internal standard (130 μM, 25 μL) and the protein precipitated with acetonitrile (50 μL). The samples were spun down and the supernatant injected directly for HPLC analysis. For tissue levels, samples (250 μL) were mixed with IAA (130 μM, 25 μL) and precipitated with 250 μL acetonitrile for direct injection for HPLC. HPLC analysis was carried out with a Hypersil 50D S 125 mm x 4.6 mm column eluting with A: 75% acetonitrile and B: 20 mM ammonium acetate (pH 5.1) with a gradient of 15–70% A in 10 min at 2 mL/min. Detection was at 290 nm using a Waters 486 variable wavelength detector. Calibration curves were linear up to 1 mM FIAA (R = 0.9998) and recovery of added IAA was >95%.

3. Results

3.1. Differential cytotoxicity of FIAA with/without HRP

Toxicity towards V79 cells of 50 μM FIAA or IAA with 1.2 μg/mL HRP was measured after 0–2 hr exposure (Fig. 2). No surviving cells (SF < 10−5) could be detected with 100 μM FIAA + 1.2 μg/mL HRP after treatment for only 1.5 hr. Treatment with IAA, FIAA or HRP alone at these concentrations had no detectable effect on cell survival.

In other experiments, V79 cells were depleted of GSH by treatment with BSO overnight. Cellular GSH levels were measured and were <0.1% of initial values. This resulted in enhanced cytotoxicity of 50 μM IAA and 1.2 μg/mL HRP. GSH depletion also enhanced the cytotoxicity of the FIAA/HRP combination at treatment times up to 1.5 hr, although at 2 hr the effect of BSO treatment was not evident (Fig. 2).

Cytotoxicity of the FIAA/HRP combination was also analyzed in a range of human and rodent tumor cell lines: human breast carcinoma MCF7 cells, human colon carcinoma HT29 cells, human bladder carcinoma T24 cells, or mouse carcinoma NT cells. Since these were more resistant to the treatment than V79 cells, 100 μM concentrations of FIAA were used. The relationships between survival and exposure time were qualitatively similar to those shown in Fig. 2; Table 1 summarizes the cytotoxic responses, and compares them with measurements or published values of the intracellular GSH levels. No toxicity was detectable with any of the cell lines with HRP or prodrug alone at these drug concentrations and times.

The effects of varying concentrations of FIAA and HRP were investigated using V79 or CaNT cells exposed for 5 hr (Fig. 3). The concentrations of FIAA required to kill 50% of the cells (IC50) in the presence of 1.2 μg/mL HRP were estimated as 6 μM with V79 cells, or 17 μM with CaNT cells. In the absence of HRP the IC50 of FIAA was 2.5 or 5 mM with V79 or CaNT cells, respectively, after 5 hr exposure (Fig. 3(A)). A ~400-fold differential (in concentration terms for equal cytotoxic effect) was thus demonstrated between indole toxicity with or without HRP with V79 cells, and a ~300-fold differential for CaNT cells.

### Table 1

Cytotoxicity of FIAA (100 μM) with HRP (1.2 μg/mL) towards rodent and human cells after 2 hr exposure

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Surviving fraction at 2 hr</th>
<th>GSH/NPSH (μmol/g protein)</th>
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<tr>
<td>V79</td>
<td>&lt;10−4</td>
<td>20±</td>
</tr>
<tr>
<td>MCF7</td>
<td>0.0054 ± 0.0003</td>
<td>9±</td>
</tr>
<tr>
<td>HT29</td>
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<td>29±</td>
</tr>
<tr>
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<td>Not known</td>
</tr>
<tr>
<td>T24</td>
<td>0.185 ± 0.060</td>
<td>16±</td>
</tr>
</tbody>
</table>

* This work.
[24]
[25]
[26]
After 5 hr, 50% cell kill was shown with only ~1.5 ng/mL HRP and 50 μM FIAA in V79 cells (Fig. 3(B)).

3.2. Reaction of an oxidation product with GSH

The putative toxic product of oxidation, 5-fluoro-3-methylene-2-oxindole (FMOI), was expected to react with thiols (see Fig. 1). HRP oxidation of FIAA produced a product identified as FMOI by its mass spectrum after HPLC separation (Fig. 4, peak 5), which was lost after incubation in excess GSH with the formation of a more polar compound (Fig. 4, peak 2). This is believed to be a conjugate with the thiol. Similar results were also seen with cysteine. (Using electron impact ionization, mass spectral confirmation of the mass of this product was not possible; only the fragment corresponding to the oxindole residue was observed.)

In order to investigate the role that GSH depletion may have in cellular toxicity, the GSH concentrations were measured in V79 cells treated with 50 μM IAA or FIAA and 1.2 μg/mL HRP for up to 2 hr. Intracellular GSH levels decreased with time, with a maximum loss of ~65% of initial (control) levels of GSH after 2 hr (Fig. 4). No effect on GSH levels was seen with either indole or HRP alone.

3.3. Relative oxidation rates of FIAA and IAA

FIAA was found to react with HRP compound 1 with a rate constant of \((3.82 \pm 0.08) \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) at pH 7. This is an order of magnitude lower than reaction with IAA, \((3.79 \pm 0.07) \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) [22]. Despite the much lower rate of oxidation, major products of FIAA oxidation by HRP corresponded to those formed with IAA: 5-fluorindole-3-carbinol, 5-fluoro-2-oxindole-3-carbinol and 5-fluoro-3-methylene-2-oxindole were detected by UV–VIS absorbance and mass spectrometry (Fig. 4). The absorption spectra of the products were very similar to those reported for IAA oxidation [15] with corresponding shifts in the mass spectra and HPLC retention.

3.4. Distribution of FIAA in tumor-bearing mice

Fig. 5 shows levels of FIAA in several tissues after i.p. administration of 50 mg/kg FIAA. No metabolites of FIAA were seen in any of the samples. FIAA was cleared quite slowly from plasma, with a half-life of 1.5 hr. Tissue concentrations were considerably lower than plasma. The
4.1. Effect of fluorine substitution on oxidation by HRP and the mechanisms of cytotoxicity

Fluorination substitution introduces an electron-withdrawing group, and from the well-established redox dependence of the rate constant for reaction of HRP Cpd I [21,22,27], FIAA was predicted to react with HRP much more slowly than IAA itself. This was confirmed by stopped-flow spectrophotometry, HRP Cpd I reacting with FIAA ~10-fold more slowly than IAA at pH 7. These rate constants for reaction with Cpd I are mirrored by the relative rates of loss of parent indole and formation of products upon treatment with HRP (data not shown). V79 cells treated with 5-^H-IAA and HRP showed intracellular accumulation of ^H, especially in the nuclear fraction, compared to controls without HRP [8,9]. This indicates that an oxidation product is binding to cellular and nuclear components. It is unlikely that the oxidation products of FIAA are qualitatively different to those of IAA, as similar products were detected by HPLC and identified by UV–VIS spectrophotometry and MS detection following HRP oxidation. If the product that is binding to cellular components is MOI or FMOI (electrophiles susceptible to nucleophilic attack) one would expect fluorine substitution, by its electron withdrawing nature, to enhance the electrophilic properties of the methylene group. MOI has been shown to bind to DNA histones [19], and RNA [28] in plants and DNA plasmids [8].

4.2. Relative cytotoxicity of IAA and FIAA and variations between cell lines

FIAA was shown to be much more cytotoxic than IAA when activated by HRP, using 50 μM prodrug (Fig. 2) and using 100 μM prodrug (Table 1) (previously published data for IAA [3] showed a SF of 0.015 with 100 μM IAA + 1.2 μg/mL HRP after 2 hr). This is not consistent with the relative reactivities of the key oxidizing intermediate, Cpd I (also expected to be representative of Cpd II) and points to the importance of substitution on the reactivities of products, such as the methyleneoxindole (Fig. 1, 9 and analogues).

The variation in cytotoxicity of FIAA/HRP with the various cell lines may be compared with reported differences in cellular GSH levels (Table 1). The prodrug/HRP combination was cytotoxic in the order T24 < CaNT < HT29 < MCF7 < V79 cells. Cellular GSH levels measured in different studies are not in the same ranking order, although protein levels may differ and cytoplasmic GSH
concentrations cannot be inferred directly. However, T24 bladder carcinoma cells are known to be chemoresistant [29]. Other possible interpretations of correlation, such as the compartmentation of GSH [30], have been discussed in a study of the links between cellular GSH and ADEPT involving a masked alkylating mustard [31]. Treatment with either indole together with HRP results in approximately the same extent of thiol loss (Fig. 4) in spite of FIAA being much more toxic than IAA in the presence of HRP, suggesting that other cellular damage is occurring that is associated with toxicity.

4.3. Tissue distribution of FIAA and the potential cytotoxicity of FIAA/HRP in vivo

Fluorine substitution in the 5-position may block the usual IAA hydroxylation site during metabolism, explaining the absence of detectable metabolites; it is possible that most FIAA is excreted unchanged, although this needs to be confirmed. The van der Waals radius of fluorine is only about 13% greater than that of hydrogen, and binding of FIAA to any receptor sites should be broadly similar to IAA. Hence, it is likely that the doses of IAA or FIAA tolerated by animals might be not dissimilar. The dose of FIAA used in the present study (50 mg/kg) equals 0.26 mmol/kg. IAA can be administered to mice at 2 mmol/kg, and IAA is known to be tolerated by humans in doses of 100 mg/kg (0.57 mmol/kg) [32]. The present work shows that the lower dose of 50 mg (0.26 mmol/kg) FIAA administered to mice results in tumor levels of about 200 μM FIAA. Such levels are 5- to 10-fold higher than those resulting in high levels of cytotoxicity in hamster fibroblasts or murine tumor cells after 5 h exposure in the presence of HRP (Fig. 3).

Possibly a more realistic comparison is between the area under the concentration/time curve (AUC) in the tumor and the exposure needed for cell kill in vitro. After 2 h, this is 20 mM min in the murine tumor (Fig. 5). The exposure corresponding to the treatment in vitro for the conditions described in Table 1 is almost half this level (12 mM min), after which cell kill is ~99 or ~90% in human tumor breast (MCF7) or colon (HT29) cell lines, respectively. However, the chemoresistant T24 bladder carcinoma cell line is less sensitive to FIAA/HRP.

Targeting of HRP to a tumor by antibody-, polymer-, or gene-directed methods would allow tumor specificity to be achieved, since mammalian peroxidases are ineffective in activating the prodrug. Fig. 3(B) shows that significant cytotoxicity can be achieved with HRP levels much smaller than 1.2 μg/mL used in most of the experiments. Preliminary experiments (data not shown) by R.B. Pedley and S. Cooke (University College London/Royal Free Hospital) have demonstrated that it is possible to conjugate HRP to the tumor-targeting anti-CEA antibody used in ADEPT studies. Obviously, to predict the likely magnitude and differential effects on tumors relative to normal tissues in vivo in such a strategy requires much further work. However, the effects of variations in concentrations of both prodrug and enzyme reported here provide a basis for the studies needed.

The alternative gene-directed (GDEPT) strategy is being explored in this institute. IAA has recently been shown to be selectively toxic towards T24 human bladder carcinoma cells transfected with a mammalian expression vector containing the HRP cDNA [33].

Although the details of the mechanism of cytotoxicity of the combination of FIAA and HRP remain uncertain, the formation of the electrophilic methyleneoxindole oxidation product and reactivity towards a nucleophile has been demonstrated. More importantly, the cytotoxic effectiveness of the fluorinated prodrug is significantly higher than that of the parent IAA. Further work to understand the enhanced activity conferred by fluorine substitution, by extending this work to other halogenated or alternative electron-withdrawing substituents, is merited. Direct measurements of the cytotoxicity and chemical reactivity of the putative (oxindole) cytotoxins are also needed. However, the present study has clearly demonstrated that the combination of a halogenated indole acetic acid and HRP has the properties required to justify further evaluation of its potential in targeted cancer therapy in vivo.

Acknowledgments

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[8] Folkes LK, Dennis MF, Stratford MRL, Candeias LP, Wardman P. Peroxidase-catalyzed effects of indole-3-acetic acid and analogues on...
Can Gene Therapy Overcome the Problem of Hypoxia in Radiotherapy?

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Radioresistance/Gene delivery/HRE/GDEPT/Bioreductive drugs

Studies have shown that reduced oxygen tension (hypoxia) in solid tumours adversely affects the outcome of radiotherapy. Despite being an independent prognostic marker of poor treatment outcome, hypoxia represents a physiological difference that can be utilised for selective cancer treatment. Since severe hypoxia (pO\textsubscript{2}\textless;0.3%; 2.5 mmHg) does not occur in normal tissue, it may be exploited for therapeutic gain. Accurate targeting of oxygen-deprived cells within a tumour mass may be achieved using hypoxia-targeted gene therapy. For gene therapy three separate issues need to be considered: 1) delivery of a gene to the tumour, 2) regulation of gene expression and 3) therapeutic efficacy. Each of these aspects is outlined here, with a view to gene therapy of the hypoxic tumour environment. It is proposed that by combining hypoxia-selective gene delivery with hypoxia-specific gene expression and oxygen-sensitive prodrug activation, radioresistant hypoxic tumour tissues may be effectively targeted.

HYPOXIA IN SOLID TUMOURS

Multiple factors contribute to the resistance of solid malignancies to radiotherapy, including intrinsic genetic and extrinsic physiological determinants. Properties such as blood flow, tissue oxygenation, nutrient supply, pH distribution and bioenergetic status can markedly influence therapeutic response to ionising radiation. Blood vessels within the tumour mass are highly irregular, tortuous and elongated, with arterio-venous shunts, blind ends, incomplete

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List of abbreviations: PGK: phosphoglycerate kinase; EPO: erythropoietin; LDH: lactate dehydrogenase; VEGF: vascular endothelial growth factor; iNOS: inducible nitric oxide synthase; HIF: hypoxia inducible factor; HRE: hypoxia-regulatory element; ENO: enolase; SV: simian virus; CMV: cytomegalovirus; UTR: untranslated region; GDEPT: gene-directed enzyme/prodrug therapy; HSV TK: herpes simplex virus thymidine kinase; GCV: ganciclovir; CD: cytosine deaminase; FC: fluorocytosine; HRP: horseradish peroxidase; IAA: indole-3-acetic acid; HSC: hypoxia selective cytotoxin; NTR: nitroreductase; P450R: NADPH:cytochrome P450 reductase.
endothelial linings, increased vascular permeability and irregular blood flow\textsuperscript{1,2}, giving rise to perfusion-limited \(O_2\) delivery. Additionally, the inadequate vascular geometry relative to the volume of oxygen consuming cells creates diffusion-limited \(O_2\) delivery. Therefore, compared to their normal tissues of origin, human tumours are characterised by areas of reduced oxygen tension\textsuperscript{3,3,4,9}. While in normal tissues polarographic electrode measurements of oxygen partial pressure (pO\(_2\)) are in the 24–66 mmHg (3.1–8.7% \(O_2\)) range, the pre-therapeutic oxygenation status of human malignancies presents median pO\(_2\) readings from 2 mmHg (~0.3% \(O_2\), cervical carcinomas) to 28 mmHg (3.9% \(O_2\), breast carcinoma), with fractions of measurements below 2.5 mmHg ranging from 5% (soft tissue sarcoma) up to 82% (FIGO III cervical carcinoma)\textsuperscript{2}.

A correlation between the presence of hypoxia and response to radiotherapy has been shown in a range of human tumour sites. Studies performed in patients with soft tissue sarcomas\textsuperscript{5}, carcinomas of the uterine cervix\textsuperscript{6,7} and of the head and neck\textsuperscript{8,9} confirmed that the presence of hypoxic regions adversely affects locoregional control and/or disease-free survival after primary radiotherapy. Radiation resistance induced by the classical "oxygen effect" is unlikely be the only explanation, since tumour oxygen status has been observed to be the most important prognostic factor for treatment outcome in cervical carcinoma, irrespectively of the therapeutic modality (i.e. surgery vs. radiotherapy)\textsuperscript{9}. Hypoxia-induced modifications of gene expression may contribute to this poor prognostic outlook, giving rise to more aggressive locoregional disease and enhanced invasive capacity. For example, experimental evidence suggests that hypoxia selects for tumour cells that have acquired \textit{p53} mutations and have consequently lost their apoptotic potential\textsuperscript{10,11}. Also, squamous cell carcinomas of the uterine cervix characterised by pronounced hypoxia\textsuperscript{12} and low apoptotic index\textsuperscript{13} showed a high probability for lymphatic spread and recurrence, despite adjuvant treatment with radiation or chemotherapy in addition to radical surgery\textsuperscript{13}. Thus, hypoxia not only provides an environment directly facilitating radio-resistance, but also encourages the evolution of phenotypic changes inducing permanent resistance to treatment.

However, since severe hypoxia does not occur in normal tissues, it represents an attractive target for selective cancer therapy. Hypoxia-targeted gene therapy is the latest approach that aims to exploit this unique physiological feature of solid tumours, with the major goal to eradicate radioresistant malignant populations, whilst sparing normal tissue from damage.

**HYPOXIA-TARGETED GENE DELIVERY**

The efficient delivery of DNA to tumour sites remains a formidable task, but progress has been made in recent years using both viral and non-viral methods. Vehicles such as retro- and adenoviruses, liposomes and naked DNA injection or electroporation are currently adopted in the clinical trials\textsuperscript{14}, and new delivery systems like E1B gene-attenuated adenoviruses\textsuperscript{15}, lentiviruses, polylysine constructs, leukocytes and bacteria are being developed\textsuperscript{14}. In targeting hypoxic cells, a further obstacle is represented by their reduced metabolism, proliferation rate\textsuperscript{16}, gene transcription and translation, which could affect DNA uptake and
gene expression. However, by using a non-viral method, it has recently been demonstrated
that in vitro transfection and transgene expression can be obtained even in extreme anoxic
conditions\textsuperscript{17}. Although in vivo data on specific transfer of genetic material to hypoxic cells are
still lacking, promising approaches are under investigation, utilising bacteria and macrophages
as cellular vehicles to deliver therapeutic modalities. Examples of prokaryotic vectors include
obligate anaerobic bacteria of the genus \textit{Clostridium} and tumour-invasive \textit{Salmonella}
auxotrophs.

The ability of \textit{Clostridium} to selectively germinate and replicate in necrotic and hypoxic
regions of solid tumours has been recognised since the 1950s, and makes them a promising
tumour-selective vehicle for gene therapeutics\textsuperscript{18,19}. Spores of \textit{C. beijerinckii} genetically engi­
neered to produce the \textit{Escherichia coli} enzyme nitroreductase (NTR) have been intravenously
injected in tumour-bearing mice, and NTR protein was detected in all tumours tested but not
in any normal tissue\textsuperscript{18}. \textit{In vitro} conversion of the prodrug CB 1954 (see final section for
details) to a cytotoxic agent by clostridia-produced NTR demonstrated the therapeutic poten­
tial of this approach. Tumour selective spore germination was also observed in rhabdomyo­sarcoma-bearing rats injected with five different bacterial strains, the most efficient species
being \textit{C. acetobutylicum} and \textit{C. oncolyticum}\textsuperscript{19}.

Attenuated hyperinvasive auxotrophic mutants of \textit{Salmonella typhimurium} can selectively
target tumour tissues and amplify in necrotic spaces to levels in excess of $10^9$ bacteria
per gram of tissue\textsuperscript{20}. While the ability to replicate in tumour tissue provides inherent anti­tumour activity, it is their ability to deliver therapeutic proteins to cancer cells in vivo that
may confer utility for gene therapy strategies.

The utilisation of macrophages as vehicles for hypoxia-selective gene therapy has been
recently demonstrated\textsuperscript{21}. It is known that macrophages infiltrate solid malignancies to form a
significant proportion of the tumour solid mass, predominating in areas of hypoxia and
necrosis\textsuperscript{22}. Differentiated macrophages transduced with an adenoviral vector containing the
human cytochrome P450 2B6 (CYP 2B6) gene were found to infiltrate human tumour
spheroids and induce tumour cell death when the spheroids were incubated with the prodrug
cyclophosphamide (converted by CYP 2B6 into the alkylating agent phosphoramidemustard)\textsuperscript{23}. A hypoxia-responsive promoter (see next section) conferred an additional level of
selectivity to the system. However, the rate limiting activation (hydroxylation) of cyclophos­
phamide is an oxygen-dependent reaction that is inhibited by hypoxia. The macrophages
themselves did not appear to be affected by the CYP 2B6/cyclophosphamide treatment, which
may make them a suitable vehicle for this gene therapy approach.

**HYPOXIA-TARGETED GENE EXPRESSION**

Gene expression is regulated both at transcriptional and post-transcriptional levels. To
enhance or restrict transcription, transcription factors bind to particular DNA sequences that
are located either within the promoter region or up to several kilobases up- or down-stream.
The stability of mRNA and protein can be modified to regulate the synthesis of the final
product. In order to engineer neoplasia-targeted gene expression, gene regulation specific to certain tissue-types, disease-types, conditions or stimuli (such as hypoxia or radiation) can be exploited.

The adaptive response to cellular hypoxia involves the modulation of the synthesis of multiple proteins controlling processes such as glucose homeostasis, angiogenesis, vascular permeability and inflammation. Hypoxia-inducible genes include phosphoglycerate kinase 1 (PGK-1), erythropoietin (EPO), lactate dehydrogenase A (LDH-A), glucose transporters-1 and -3, vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS)

The DNA regulatory elements controlling the expression of oxygen-responsive genes have been defined in most cases, and involve the specific binding and transactivation by various inducible, phosphorylation-dependent and/or redox sensitive transcription factors, including Hypoxia Inducible Factor-1 (HIF-1), Activator Protein-1 (AP-1), Nuclear Factor kB (NF-kB), p53 and the Heat Shock Transcription Factor. Published evidence indicates that only HIF-1 is specifically oxygen-responsive, while the other transcriptional systems appear to contribute to the response to hypoxia via related redox and metabolic changes. Affinity purification and molecular cloning of HIF-1 showed it to function as a heterodimer consisting of two basic-helix-loop-helix proteins, HIF-1α and HIF-1β (previously identified as ARNT, aryl receptor nuclear translocator, which is part of the xenobiotic response). Although both subunits are constitutively expressed, HIF-1α is hypoxia-regulated via post-translational stabilisation and transactivation by several additional factors. To modulate gene expression, HIF-1 specifically binds to hypoxia-responsive elements (HREs), enhancers containing the core sequence 5′- (A/G)CGT(G/C)G(C)-3′, localised at varying distances and orientations of the coding region of several hypoxia-regulated genes. The HRE/HIF-1 regulation system was shown to be common to all mammalian cells and human tissues tested to date and the HIF-1α subunit was found to be overexpressed in 68% of the tumour types analysed.

The high frequency of HIF-1 expression across many human tumours of diverse tissue origin represents a possible therapeutic target for HRE-directed gene therapy of the hypoxic environment. It has been demonstrated that marker gene expression regulated by the murine PGK-1 HRE could be induced in hypoxic tumour cells. Production of the marker protein CD2 in stably transfected human fibrosarcoma cells HT1080 increased with increasing length and severity of hypoxia. Compared to oxygen levels typical of normal tissues, radiobiologically relevant hypoxia (O2 concentration <0.3%) induced a three-fold increase in gene expression (table 1). Following anoxia and subsequent reoxygenation a 7–8-fold induction was observed. When the transfected tumour cells were grown as xenografts in nude mice, expression of the CD2 gene was limited to areas adjacent to necrosis. To analyse this system on a single cell basis, tumour-bearing mice were exposed to a bioreductive drug (which induces DNA cross-links only in hypoxic cells) and X-rays (preferentially generating DNA strand breaks in oxic cells). By analysing individual tumour cells with the comet assay combined with CD2 immunostaining, it could be demonstrated that increased CD2 expression was only seen in hypoxic tumour cells. Similarly, murine C2C12 myoblasts engineered to express the human EPO gene regulated by the murine PGK-1 promoter showed a 2.7-fold increase in gene expression in anoxia and a 3.2-fold induction at 1.3% O2. The in vivo response of this
Table 1. *In vitro* studies of hypoxia-regulated transgene expression, utilising different hypoxia-responsive promoters. The basic components of the DNA constructs and the cell lines used are indicated. Gene expression has been evaluated after various hypoxic (* = 0.1% O₂; ** = 0.02% O₂) or anoxic incubation intervals: ref 28, 30, 31: 16h; 29: 24h; 32, 33*: 6h; 33**: 18h. h: human, m: murine.

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<td>5 × hVEGF + S’ VEGF UTR</td>
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<td>5 × hVEGF + S’ VEGF UTR + 3’ VEGF UTR</td>
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DNA construct was studied in mice with C2C12-EPO cells implanted in their dorsal flank. Serum EPO levels in animals exposed to 7% O₂ were twice as high than in controls kept at 21% O₂.²⁹

To achieve significant gene expression in a therapeutic context, specific and robust transcriptional activation is required. In order to increase the hypoxic/oxic inducibility ratio of
hypoxia-responsive promoters, a series of DNA constructs containing fragments of the murine PGK, murine LDH, human EPO and human enolase (ENO) genes were inserted into the context of the basal simian virus (SV) 40 promoter\textsuperscript{10}. In transiently transfected human mammary tumour T47D cells, the EPO-chimeric promoter exhibited the most stringent regulation in hypoxia (255-fold induction at 0.1% O\textsubscript{2}), while the PGK HREs showed the highest absolute levels of expression (more than the strong cytomegalovirus (CMV) promoter), at the expense of selective regulation (146-fold at 0.1% O\textsubscript{2}; see table 1). The hypoxia response was augmented by two-fold by inserting at the C-terminus of the reporter gene luciferase a 150 bp oligonucleotide spanning the 3' untranslated region (UTR) of VEGF, which is involved in hypoxia-induced mRNA stability. The PGK HRE promoter was inserted in an adenoviral vector and in a panel of transduced cell lines a low basal level of $\beta$-galactosidase ($\beta$-gal) transgene expression was observed, with levels of hypoxic induction comparable to the full-length CMV\textsuperscript{31}.

The hypoxic response system appears to be specifically effected by the cellular background, since cell lines of diverse origin respond differently (table 1). In two studies on fibrosarcoma HT1080 cells transiently transfected with constructs containing fragments of the human EPO and VEGF genes, the best differential response to hypoxia was obtained by combining five copies of the 35 bp VEGF HREs with the adenoviral E1b minimal promoter\textsuperscript{32,33}. In this cell line, a six-hour hypoxic (0.02% O\textsubscript{2}) incubation induced a 40–50 fold increase in luciferase activity\textsuperscript{32}. An even higher hypoxic/aerobic ratio (~500) was obtained when the five VEGF HREs were linked to the minimal CMV, with a marker protein production similar to the full-length CMV\textsuperscript{33}. Interestingly, in this study, the inclusion of the 3' VEGF UTR decreased hypoxic gene expression. However it has been demonstrated that the VEGF mRNA not only contains destabilising elements in its 3' UTR, but also in its 5' UTR and coding region\textsuperscript{34}, and stabilisation of the mRNA in response to hypoxia is completely dependent on the cooperation of elements in each of these three regions.

It is currently not clear whether the large fold-inductions by HRE-controlled genes in response to hypoxia in later reports\textsuperscript{30-33} represent a clear improvement over the constructs used in earlier studies\textsuperscript{28}, since transient rather than stable transfection methods were employed. Also, the end point in early studies was the immunological detection of a cell surface protein, whereas luciferase assays measure the conversion by the enzyme of many substrate molecules to light units. The luciferase assay therefore further amplifies any increase in transcription, making a direct comparison to other marker assays difficult.

These \textit{in vivo} and \textit{in vitro} results (summarised in table 1) demonstrated the selectivity of the system and its potential for tumour-specific targeting of therapeutic gene expression.

\textbf{HYPOXIA-TARGETED GENE-DIRECTED ENZYME/PRODRUG THERAPY}

Genetic immunopotentation, mutation compensation and molecular chemotherapy are the three major approaches in the design of therapeutic genes for cancer gene therapy. In the first case, the tumour immunogenicity is enhanced by the insertion of genes that encode cytokines
or costimulatory molecules; mutation compensation aims to inactivate oncogenes or induce tumour suppressor gene expression, while in molecular chemotherapy a "suicide" gene is delivered to the tumour cells (reviewed in 35). In this latter approach, the enzyme expressed by the therapeutic gene is not toxic per se, but is able to convert a non-toxic compound (prodrug) into a potent cytotoxin (gene-directed enzyme/prodrug therapy, GDEPT, figure 1). Although GDEPT is a complex two-stage system, it is characterised by two basic advantages: the amplification effect, due to the ability of each enzyme to activate many prodrug molecules, and the bystander effect. The bystander phenomenon can be defined as an extension of the killing effects of the active drug to untransfected cells, which do not express the foreign enzyme. Transfer of toxic metabolic products through gap junctions, phagocytosis of apoptotic vesicles, induction of immune response against the tumour and diffusion of soluble toxic metabolites have been shown to be involved in the killing of neighbouring untransfected cells. Thus, even if, as with current protocols, systemic delivery results in (at best) 10% of the tumour cells expressing the therapeutic gene, tumour eradication may still be achieved.

The most well known examples of enzyme/prodrug combinations in cancer GDEPT are the herpes simplex virus thymidine kinase (HSV TK)/ganciclovir (GCV) and the E. coli cytosine deaminase (CD)/5-fluorocytosine (5-FC) systems (reviewed in 42). Since they interfere with DNA synthesis, both the HSV TK/GCV and the CD/5-FC combinations need cell proliferation for their action and are generally not suitable to target slowly dividing hypoxic cells. Although tumour cells transfected with a hypoxia-induced CD-encoding gene could be sensitised to 5-FC during subsequent drug exposure in air, no cell kill could be detected when CD-expressing cells were treated in anoxia (own observation). Analogously, cells transfected with the HSV TK gene could not be sensitised to GCV when exposed to the prodrug in anoxic conditions (unpublished results). These observations suggest that cell-cycle independent cytotoxins will be essential to successfully eradicate radioresistant hypoxic tumour cells.

A novel enzyme/prodrug system for GDEPT, consisting of the plant enzyme horseradish peroxidase (HRP) and the non toxic plant hormone indole-3-acetic acid (IAA), is currently being developed. The efficacy of the HRP/IAA system was evaluated in vitro by exposing human bladder carcinoma T24 cells transfected with HRP-encoding genes to the prodrug IAA. Significant cytotoxicity could be evoked after two-hour exposure only, and it was further increased after 24 h incubation. A substantial bystander effect due to the transfer of soluble toxic metabolites was also observed (unpublished data). Anoxic incubation did not affect the efficacy of the system, indicating that the HRP/IAA combination has the potential to kill the hypoxic subpopulation in solid tumours.

Bioreductive drugs such as mitomycin C, tirapazamine (SR 4233), RSU 1069 and CB 1954 have been extensively utilised as hypoxia-selective cytotoxins (HSCs). These classes of prodrug are particularly suitable to target hypoxic tumour cells since endogenous activation in normal tissue is restricted by the presence of oxygen. They are currently adopted in the clinic or in clinical trails, generally in combination with radiotherapy.

The mustard prodrug CB 1954, which originally resulted in the single agent cure of Walker rat tumours, is efficiently activated by the rodent enzyme DT diaphorase into a DNA
Fig. 1. Schematic diagram of hypoxia-regulated gene directed enzyme-prodrug therapy. A DNA construct containing an enzyme-encoding gene controlled by a hypoxia-responsive promoter is delivered to the tumour using viral or non-viral vectors. At best 10D-20% of the target cells will express the foreign gene. Therapeutic gene expression is activated selectively in hypoxic conditions and the enzyme is synthesised in the transfected cells. After systemic injection of the prodrug, cytotoxic activation takes place at the target only, and the bystander effect allows the eradication of neighbouring untransfected cells.

- ○: transfected cell
- □: untransfected cell
- ◊: enzyme
- ○: inactive prodrug
- ●: activated drug
- ●○: dead cells
cross-linking agent, but showed little toxicity against human tumour cells. However, since the *E. coli* nitroreductase (NTR) is very active towards this prodrug, infection of colorectal and pancreatic tumour cells with a NTR-encoding retrovirus resulted in 50 and 500-fold increase, respectively, in sensitivity to CB 1954, compared to the parental lines\(^{40}\). A significant bystander effect was also demonstrated, which was not dependent on cell-cell contact\(^{41}\). Transgenic mice studies of NTR expression under the control of a T cell-specific promoter showed selective CB 1954 toxicity in the thymus and the spleen of systemically treated animals\(^{40}\).

The redox-sensitive flavoprotein, NADPH:cytochrome P450 reductase (P450R), is an important endogenous bioactivator of many nitroaromatic, aromatic N-oxide and quinone “triggered” HSC\(^{51}\). P450R over-expression in human fibrosarcoma (HT1080) or breast cancer (MDA231) cells transfected with the human P450R cDNA conferred increased sensitivity to tirapazamine, RSU 1069, mitomycin C and porfiromycin\(^{51-53}\). Selective hypoxic targeting could be further refined by incorporating an optimised PGK-1 HRE/SV40 chimeric promoter to regulate the expression of P450R\(^{52}\). In transfected HT1080 cells anoxic incubation produced a 3.4-fold increase in enzyme activity\(^{52}\) and a 30-fold enhancement of *in vitro* cytotoxicity of the 2-nitroimidazole bioreductive prodrug RSU 1069\(^{53}\). HT1080 tumour xenografts were established and treated with a combination of 10 Gy X-rays and the precursor of RSU 1069, RB 6145\(^{53}\). Compared to radiation alone, a significant increase in specific growth delay was observed in the transfected tumours, but not in the untransfected xenografts.

Hypoxia-targeted gene therapy represents an interesting and promising tumour-selective approach, with potential to significantly improve the outcome of radiation therapy. By combining selective delivery of therapeutic modalities to hypoxic tumour areas with hypoxia-dependent transcriptional control and oxygen-sensitive prodrug metabolism, hypoxic radioresistant tumour cells may be selectively targeted *in vitro* and *in vivo*. It is now warranted to consider the potential of gene therapy as an adjuvant for radiotherapy.

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INTRODUCTION

One of the major goals in antitumour therapies is to target toxic agents to tumour cells selectively and specifically, whilst sparing normal tissue from damage. This may be achieved by gene therapy that can combine highly specific gene delivery with highly specific gene expression. The first clinical gene therapy protocol was approved in 1989 (Rosenberg et al., 1990), and since then more than 400 clinical trials have started world-wide, with over 50% related to cancer.

For gene therapy three separate issues need to be considered: (1) delivery of a gene to the tumour, (2) regulation of gene expression and (3) therapeutic efficacy. This review will focus on the choice of therapeutic genes for cancer gene therapy, in particular on molecular chemotherapy or “suicide” gene therapy. In this approach, the gene delivered to the target encodes an enzyme which is not toxic per se, but is able to convert a non-toxic compound (prodrug) into a potent cytotoxin (gene-directed enzyme/prodrug therapy, GDEPT; Fig. 1).

The purpose of this article is to review the progress made in the design and application of GDEPT strategies. The most widely used enzyme/prodrug combinations already in clinical trials (e.g., herpes simplex 1 virus thymidine kinase/ganciclovir and cytosine deaminase/5-fluorocytosine), as well as novel approaches (carboxypeptidase G2/CMDA, horseradish peroxidase/indole-3-acetic acid) are described, with a particular attention to translational research and early clinical results.

ENZYME/PRODRUG COMBINATIONS FOR CANCER GDEPT

Prodrugs are chemicals that are inert even at relatively high doses, but can be converted to toxic species at the target. Ideally, specific activation of a prodrug is the result of the metabolism by an enzyme that is either unique to the tissue or at higher concentrations at the tumour site. Even though prodrug treatment of animal tumours with high levels of endogenous activating enzymes has been successful (Connors and Whisson, 1966; Khan and Ross, 1967; Cobb et al., 1969), clinical results were disappointing, since human cancers that contained satisfactory levels of activating enzymes were rare and not associated with any particular type of tumour (Connors, 1995). To overcome this problem and achieve high levels of enzyme at the target, two
different approaches are being investigated, antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT).

In the choice of the appropriate enzyme/prodrug combination, priority should be given to the enzyme. From past experience, it is likely that suitable prodrugs can be designed for almost any enzyme substrate specificity (Connors, 1995). For ease of handling and possible protein modification, the enzyme should be monomeric, of low molecular weight and with no requirement for glycosylation. It should have high catalytic activity under physiological conditions, fast and efficient prodrug activation even at low concentrations of the substrate (high $K_{cat}$ and low $K_{m}$), without dependence on further catalysis by other enzymes. Expression of the enzyme itself should not lead to cytotoxic effects; the bystander effect required (see the Bystander Effect section) would not be achieved if the cells were killed by the action of the enzyme alone. The reaction pathway should also be different from any endogenous enzyme, in order to avoid cytotoxic activation of the prodrug in normal tissues. This is the main drawback of utilising proteins of human origin, which, on the other hand, have the potential advantage of avoiding complications of acquired immunity, in particular after prolonged administration or prolonged protein expression.

The selected prodrug should be freely diffusible throughout the tumour (possibly a neutral species), chemically stable under physiological conditions and have suitable pharmacological and pharmacokinetic properties. For significant therapeutic gain, the released drug should be at least a 100-fold more toxic than the prodrug. The toxic agent should also have a half-life that allows diffusion to the surrounding untransfected cells (bystander effect), but ensures that any drug escaping into the circulation will be inactive. Moreover, the induced cytotoxicity should be cell cycle phase- or proliferation-independent, to kill a wide range of tumour cell populations.

Several combinations have been proposed for GDEPT. Most of them do not fulfil all the above requirements, including those currently adopted in the clinical trials. In some cases, active DNA replication (S phase) is an essential requirement for cytotoxicity (e.g., herpes virus 1 thymidine kinase (HSV TK)/ganciclovir (GCV)); in other systems, the prodrug requires further metabolism by endogenous enzymes (cytosine deaminase (CD)/5-fluorocytosine (5-FC); nitroreductase (NTR)/CB1954).

Some activated drugs are characterised by a long half-
life (CD/5-FC), others are not membrane-permeable and depend on cell-to-cell contact to diffuse into neighbouring cells (HSV TK/GCV). Nevertheless, some promising preclinical and clinical data have been reported, and they will be discussed in the following sections.

THE Bystander EFFECT

The bystander phenomenon, initially described by Mooltien (1986), can be defined as an extension of the killing effects of the active drug to untransfected neighbouring cells (Fig. 1). This implies that even if only 5–10% of the target cells are genetically modified and express the therapeutic gene, tumour eradication may still be achieved. After treatment with 5-FC, for example, tumour regression could be observed when only 4% of the tumour population expressed CD (Huber et al., 1994). The bystander effect is crucial for a successful GDEPT strategy, since with the protocols currently adopted in the clinical trials the transfection efficiency is unlikely to be greater than 10%. It is clear that a prerequisite for a substantial bystander effect is the lack of cytotoxicity of the enzyme itself, even when produced at a high rate.

Two major categories of bystander effect have been identified: local or immune-mediated. In the first case, the killing of neighbouring cells is due to the transfer of toxic metabolites via gap junctions (Elshami et al., 1996; Mesnil et al., 1996; Dilber et al., 1997; Touraine et al., 1998), via apoptotic vesicles (Freeman et al., 1993; Colombo et al., 1996), or through the diffusion of soluble toxic metabolites (Huber et al., 1994; Wei et al., 1995; Bridgewater et al., 1997; Lawrence et al., 1998; Greco et al., 2000; Stibrbling et al., 2000).

Relying only on gap junctions could be restricting, since cell-to-cell contact is required, and a number of tumour tissues have been shown to down-regulate intercellular gap junction communication (Holder et al., 1993; Mesnil et al., 1996; Touraine et al., 1998). This is the major limitation of purine nucleosides, which cannot passively diffuse across cell membranes when phosphorylated (see next section for details). In the case of freely diffusing species a key role to obtain a considerable but limited bystander effect is played by the drug half-life, which, assuming diffusion ranges in tumours of 100 to 200 µm, should be of about 1 min (Patterson and Harris, 1999). The local bystander effect can also be induced by the contact with dead or dying cells (“Kiss of death”), due to the transfer of apoptotic factors (Freeman et al., 1993; Frank et al., 1998).

Evidence in animal models (in vivo) suggests that a systemic immune response may play an important role in inducing bystander killing (Freeman et al., 1997). The presence of an intense inflammatory infiltrate has been described in the regressing tumours of immunocompetent animals treated with GDEPT systems (HSV TK/GCV, Caruso et al., 1993; Vile et al., 1997; Yamamoto et al., 1997; CD/5-FC, Consalvo et al., 1995; Kuriyama et al., 1999a). Moreover, it has been noticed that the bystander effect was significantly reduced in immunodeficient athymic mice (Vile et al., 1994; Gagandeep et al., 1996; Pavlovic et al., 1996; Ramesh et al., 1996; Bi et al., 1997; Kuriyama et al., 1999a) and after sublethal irradiation (Ramesh et al., 1996). The immune stimulation does not only enhance local tumour killing, but it can also induce the regression of distant tumour deposits (“distant bystander effect”; Dilber and Smith et al., 1997), with major implications in the prognosis of patients with microscopic metastases.

The impact of the bystander effect on the success of gene therapy strategies is so important that a number of studies are currently focused on enhancing the efficacy of untransfected surrounding cells. Gap junctional communication, for instance, can be enhanced by regulating the production of connexins (Cx), membrane proteins considered to be the building blocks of gap junctions and the major factors responsible for the gap junction-mediated bystander phenomenon (Mesnil and Yamazaki, 2000). The transfer of Cx-encoding genes (Cx43, Cx32, Cx40) or chemically induced Cx-overexpression has been shown in tissue culture (in vitro) (Elshami et al., 1996; Ghoumari et al., 1998; Kunishige et al., 1998; Carpystinos, et al., 1999; Andrade-Roentel et al., 2000) and in vivo (Dilber et al., 1997; Park et al., 1997; Duflot-Dancer et al., 1998; Touraine et al., 1998) to increase intercellular communication and the transfer of toxic agents. It is important to notice that in some human tumour cells not only expression but also correct surface localisation of Cx43 are necessary components of the bystander effect (McMasters et al., 1998), and that Cx-cotransfection appears not to be applicable to all tumour systems (Cirenei et al., 1998).

Another attractive tool to enhance the bystander effect is the HSV 1 virion protein VP22. Once synthesised in infected cells, VP22 can spread very efficiently via a Golgi-independent pathway to surrounding uninfected cells, where it specifically accumulates in the nucleus (Elliott and O’Hare, 1997). Due to these peculiar trafficking properties, delivery of DNA constructs containing the VP22 gene fused to the gene encoding the marker green fluorescent protein (GFP) resulted in a significant spread of the VP22-GFP protein to the nucleus of untransfected cell monolayers (Duflot et al., 1998). The VP22-GFP spread appeared to be a general phenomenon, common to all cell types tested to date (Wybranietz et al., 1999). Therapeutic advantage of this “biologically active” bystander effect for GDEPT was demonstrated by coupling the VP22 gene to the HSV TK gene, which produced significant bystander killing in vitro and tumour regression in vivo, regardless of cellular gap junctional activity (Dilber et al., 1999). Other cancer gene therapy approaches may also benefit from VP22 transgene fusions, since a VP22-p53 chimeric protein retained its ability to “colonise” recipient nuclei and induce apoptosis in p53-negative human osteosarcoma cells (Phelan et al., 1998).

To improve the in vivo bystander effect and enhance the immunological response to the tumour, strategies aim to combine tumour immunisation with GDEPT systems. Cotransfection of cytokine- and suicide gene-based vectors followed by prodrug treatment has met with varying results (Chen et al., 1995; Castleden et al., 1997; Coll et al., 1997; Moriuchi et al., 1998; Nanni et al., 1998; Pizzato et al., 1998; Cao et al., 2000). Combined adenoviral delivery of CD/5-FC and murine interleukin-2 (IL-2) to mice inoculated with erythroleukemia cells induced more potent tumour growth inhibition and longer survival than the separate treatments (Ju et al., 1998). Safety and some clinical benefit was
demonstrated in four patients with glioblastoma multi-
forme, who underwent standard surgery and radio-
therapy followed by HSV TK/GCV combined with IL-2
gene delivery (Palù et al., 1999).

**HERPES SIMPLEX VIRUS THYMIDINE**

**KINASE/GANCICLOVIR**

To date, the most well-studied enzyme/prodrug stra-
ty in cancer GDEPT is undoubtedly HSV TK with the
nucleoside analogue GCV.

GCV and related agents, widely used in the treatment
of HSV infection in humans, are poor substrates for the
mammalian nucleoside monophosphate kinase, but can be
converted (1000-fold more) efficiently to the mono-
phosphate by TK from HSV 1 (Fig. 2). Subsequent reac-
tions catalysed by cellular enzymes lead to a number
of toxic metabolites, the most active ones being the
triphosphates (Fig. 2). GCV-triphosphate competes with
deoxyguanosine triphosphate for incorporation into
elongating DNA during cell division, causing inhibition
of the DNA polymerase and single strand breaks (Elion,
1983; Mar et al., 1985). These characteristics make the
HSV TK/GCV combination particularly suitable for the
eradication of rapidly dividing tumour cells invad-
ing non-proliferating tissue. On the other hand, since
activated GCV is an S-phase specific cytotoxic, it is
necessary that the target cells are actively dividing at
the time of the exposure, or that the prodrug is con-
tinuously administered to allow them to start replicat-
ing the DNA.

In the last 15 years, more than 400 papers have
discussed the potentiality of HSV TK/GCV for cancer
GDEPT. Preclinical studies using adeno- and retroviral
vectors were performed in many different animal models
and successful results were reported for established
rodent liver metastases (Caruso et al., 1993), murine
hepatocellular carcinomas (Kuriyama et al., 1999 b),
rodent glioblastomas (Short et al., 1990; Culver et al.,
1992), human head and neck carcinomas (O'Malley,
1995), human mesotheliomas (Smythe et al., 1995) and
several other tumour types. Nevertheless, the HSV TK/
GCV-induced mechanisms of cell killing have not been
completely elucidated, and both apoptotic (Freeman
et al., 1993; Wei et al., 1999; Beltinger et al., 2000; Thust
et al., 2000) and non-apoptotic (Kaneko and Tsukamoto,
1995, Vile et al., 1997, Melcher et al., 1998) pathways
have been reported.

The relationship between p53 gene status, p53-me-
diated apoptosis and the sensitivity of human tum-
ours to HSV TK/GCV is controversial. p53 Played a
significant role in the in vivo response of oesophageal
cancer, as demonstrated in two human lines, T. Tn and
TE2 cells, with truncated and wild type p53 respectively,
implanted in immunocompromised nude mice (Matsu-
bara et al., 1999). Exposure to HSV TK/GCV induced p53
accumulation and translocation of the receptor CD95 to
the cell surface, with subsequent recruitment of Pas-
associated death domain and trigger of the apoptotic
caspase cascade, in human SH-EP neuroblastoma cells
(Beltinger et al., 1999), and in murine tumours, includ-
ing B16F10 melanoma, NG4TL4 sarcoma, H6 hepatoma
and 1ME7R.1 hepatoma (Wei et al., 1999). Mitochon-
dria appear to have a major involvement in HSV TK/
GCV-induced apoptosis, by regulating both the initia-
tion (through p53 accumulation) and the effector phase
of apoptosis (through the release of cytochrome c into
the cytosol and caspase activation; Beltinger et al., 2000).
On the other hand, endogenous p53 status did not
correlate with sensitivity to HSV TK/GCV in human
SKOV-3 ovarian and Hep3B hepatocellular carcinoma
cells (Xie et al., 1999) and in some breast cancer cell lines
(Li et al., 1999).

One of the main drawbacks of the HSV TK/GCV
system is that the highly charged triphosphate is
insoluble in lipid membranes. This impairs the diffusion
of the drug and makes cell-to-cell contact necessary for
bystander killing. Nevertheless, preclinical studies
showed that tumour regression could be achieved when
only 10% of the tumour cells expressed HSV TK (Caruso
et al., 1993; Freeman et al., 1993). This phenomenon has
been proposed to result from transfer of activated GCV
through gap junctions (Elshami et al., 1996; Dilber et al.,
1997; Touraine et al., 1998; Mesnil and Yamazaki, 2000)
or exchange of apoptotic vesicles (Freeman et al., 1993;
Colombo et al., 1995). Interestingly, transfer of toxicity
in some murine cell lines occurred before there was
evidence of apoptotic degeneration, indicating that apo-
posis could be the result, not the cause, of the bystander
effect (Denning and Pitts, 1997). It is likely that a major
part in the in vivo bystander killing is played by the host
immune system. HSV TK/GCV treatment resulted in
infiltration of CD4+ and CD8+ T cells and macrophages,
as well as increased expression of IL-2, IL-12, interferon-
γ , tumour necrosis factor-α and granulocyte/macro-
phage colony-stimulating factor, suggesting that the
induced cell killing creates a cytokine-rich immunostim-
ulatory environment (Gagandeep et al., 1996; Dilber et al.,
1997). The generation of immunostimulatory
signals in vivo appeared to be predominant in non-
apoptotic tumours, and associated with the induction
of genes of the inducible heat shock protein family
(Melcher et al., 1998).

An immune-related antitumour response could also
account for the "distant bystander effect". GCV treat-
ment of head and neck squamous cell carcinoma xeno-
grafts in nude mice resulted not only in the eradication
of genes of the inducible heat shock protein family
(Melcher et al., 1998).
of TK⁺ tumours, but also in the delayed regression of untransduced tumours in the contra-lateral flank (Wilson et al., 1996), which was abrogated in mice with severe combined immunodeficiency (SCID) (Bi et al., 1997). Interestingly, in a plasmacytoma model, local and some distant bystander effects could be observed also in SCID mice (Dilber et al., 1996). This suggests that T and B lymphocytes, absent in SCID mice, may not be involved in HSV TK/GCV-mediated distant bystander killing, and that the effector mechanism may depend on the treated tumour.

Another aspect of distant bystander killing is the "vaccination effect". After eradication of primary transplanted tumours, experimental animals challenged with untransduced tumour cells showed rejection or growth delay of the new malignancies (Yamamoto et al., 1997; Kruse et al., 2000), suggesting that HSV TK/GCV treatment may be beneficial in the prevention of recurrence.

On the basis of these animal studies, the first gene therapy trial using HSV TK/GCV to treat ovarian cancer was approved in 1991 (Freeman et al., 1995), and since then several other clinical studies have been undertaken. These include gene therapy of brain tumours, particularly glioblastoma multiforme (Oldfield et al., 1993; Eck et al., 1996; Izquierdo et al., 1996; Ram et al., 1997; Stockhammer et al., 1997; Klatzmann et al., 1998a; Shand et al., 1999), of metastatic melanoma (Klatzmann et al., 1998b) and prostate carcinoma (Herman et al., 1997; Shalev et al., 2000). Gene delivery has been performed by injecting HSV TK-containing replication-deficient adenoviruses (Eck et al., 1996; Herman et al., 1999; Shalev et al., 2000) or retroviral vector-producing cells (Oldfield et al., 1993; Izquierdo et al., 1996; Ram et al., 1997; Klatzmann et al., 1998a; b; Shand et al., 1999).

In the phase I clinical trials only moderate toxic events were reported, which were mostly resolved at the termination of the therapy course. Moderate therapeutic response was observed in some of the patients. In a phase I/II study using HSV TK-glutathione S-transferase (GCV resulted in the absence of recurrence in four of 12 patients at 4 months, and in one patient at 2.8 years after treatment (Klatzmann et al., 1998a). In another clinical trial for recurrent glioblastoma multiforme four of 48 patients had some regression of postoperative residual enhancement, but the therapeutic benefit was transitory and not associated with any regrowth delay (Shand et al., 1999).

Relatively poor responses could be due to insufficient gene transfer and limited distribution within the tumour mass, which only allowed the treatment of small tumours with a high density of foreign DNA (Ram et al., 1997). The growth rate of the tumour cells might also play an important role in the response to HSV TK/GCV treatment and explain the higher sensitivity of experimental tumours compared to spontaneous human malignancies. Furthermore, because of GCV toxicity, particularly to the bone marrow (Caruso, 1996), the maximum dose used in humans (10 mg/kg/day) was much lower than the doses used in most animal experiments (up to 300 mg/kg/day).

There are numerous possibilities for ameliorating treatment efficacy, notably through the improvement of gene delivery and a better understanding of the molecular mechanisms of the bystander effect. Significant benefits could also arise from the introduction of new nucleoside analogues with a higher affinity for HSV TK and fewer side effects than GCV (Balzarini et al., 1994; Balzarini et al., 1998; Dievrev et al., 1999; Thust et al., 2000), and of HSV TK mutants engineered to increase specificity and activity towards the prodrug (Black et al., 1996; Kokoris et al., 1999).

**CYTOSINE DEAMINASE/5-FLUOROCYTOSINE**

The system consisting of CD and 5-FC is similarly based on the production of a toxic nucleotide analogue. The enzyme CD, found in certain bacteria and fungi but not in mammalian cells, catalyses the hydrolytic deamination of cytosine to uracil. It can therefore convert the non-toxic prodrug 5-FC to 5-fluorouracil (5-FU), which is then transformed by cellular enzymes to potent pyrimidine antimetabolites (5-FdUMP, 5-FdUTP, 5-FUTP; Fig. 3). Three pathways are involved in the induced cell death: thymidylic synthase inhibition, formation of (5-FU) RNA and of (5-FU) DNA complexes (Springer and Niculescu-Duvaz, 1996). 5-FU is widely used in cancer chemotherapy and is the drug of choice in the treatment of colorectal carcinoma. It is characterised by...
an array of side effects and high dose levels are required for tumour response. Although not cell cycle phase-specific, 5-FU has both proliferation-dependent and proliferation-independent actions. In vitro studies showed loss of cytotoxicity of 5-FU and CD/5-FC in hypoxic tumour cells (our unpublished data). This might result in a reduction of therapeutic efficacy, since hypoxia is common to solid tumours and presents an adverse prognostic indicator.

The CD gene used for GDEPT has been cloned from *Escherichia coli* (*E. coli*; Austin and Huber, 1993) and has been shown in a number of in vitro studies to enhance mammalian cell sensitivity to 5-FC by up to 2000-fold (Ge et al., 1997). In vivo anti-tumour activity of the CD/5-FC combination has been demonstrated in several animal models, including fibrosarcomas (Mullen et al., 1994), carcinomas (Huber et al., 1993, 1994; Ohwada et al., 1996; Kanai et al., 1997; Bentires-Alj et al., 2000), gliomas (Ge et al., 1997; Ichikawa et al., 2000) and metastatic formations of different origin (Consalvo et al., 1995; Topf et al., 1998).

One of the main advantages of the CD/5-FC system is a strong bystander effect that does not require cell-to-cell contact, since 5-FU can diffuse into and out of cells by non-facilitated diffusion (Domin et al., 1993). Experiments conducted in vitro by exposing mixed transfected and untransfected populations to 5-FC showed that 1–30% of cells expressing CD could generate sufficient 5-FU to inhibit the growth of the neighbouring ones not expressing the enzyme (Huber et al., 1994; Bentires-Alj et al., 2000; Ichikawa et al., 2000), even when the cells were sparsely seeded (Huber et al., 1994; Ichikawa et al., 2000). Significant amounts of 5-FU were found in the culture medium of treated CD-positive cells (Huber et al., 1994), and the transfer of the supernatant from transfected to untransfected cells resulted in their death (Lawrence et al., 1998). Bystander killing was also observed in cells negative for gap junctions (Lawrence et al., 1998). However, uptake studies with tritiated 5-FC showed that moderate and unsatisfactory amounts of the drug accumulated intra-cellularly (Haberkorn et al., 1996), indicating that 5-FC uptake might be a bottleneck in this treatment strategy. The in vitro bystander effect was not detected in the early reports of CD/5-FC (Mullen et al., 1992) and it appears to be reduced in some cell lines (Bentires-Alj et al., 2000).

Significant bystander killing has been observed in preclinical studies. 5-FC treatment of xenografts grown from mixtures of CD-positive and negative human WiDr colorectal carcinoma cells in nude mice caused tumour regression even when only 4% of the inoculated cells expressed the enzyme (Huber et al., 1994; Trinh et al., 1995). A comparable anti-tumour effect (60% cure rate) was induced by GCV in tumours composed of 50% HSV TK-negative and positive cells (Trinh et al., 1995). Higher bystander effect in vitro and cure rate in vivo compared to HSV TK/GCV or HSV TK/acyclovir suggested that CD/5-FC is the combination of choice for Epstein–Barr virus-associated lymphomas (Rogers et al., 1996), renal cell carcinomas (Shirakawa, 1999), and thyroid carcinomas (Nishihara et al., 1998). On the other hand, anti-tumour effects and immunity to parental tumours induced by the HSV TK/GCV system were superior to those induced by CD/5-FC in a hepatocellular carcinoma model (Kuriyama et al., 1999b).

Similar to the HSV TK/GCV, the CD/5-FC combination induces an immune-mediated distant bystander effect, associated with an infiltration of natural killer cells within the tumours (Pieperfi-Carle et al., 1999). Stackhouse et al. mice pre-treated with CD/5-FC GDEPT exhibited significant resistance when rechallenged with wild type tumours (Mullen et al., 1994; Consalvo et al., 1995; Kuriyama et al., 1999a), and the “vaccination effect” appeared to be dependent on the rechallenging tumour (e.g., the eradication of CD-expressing adenocarcinomas conferred no protection against fibrosarcomas; Mullen et al., 1994). It is important to note that the CD protein itself is immunogenic (Mullen et al., 1994).

Despite the encouraging preclinical results, it becomes clear from the clinical reports that the treatment with a single GDEPT strategy may at best lead to partial response. Therefore, combinations of several genes or treatment modalities were investigated in order to increase the efficiency of such gene therapy strategies. Based on the extensive clinical experience with 5-FU as both a chemotherapeutic agent and a radiosensitisier (reviewed by McGinn and Kinsella, 1993), the combination of CD/5-FC GDEPT with ionising radiation was tested. CD-expressing SK-Ch-A-1 cholangiocarcinoma cells (Pederson et al., 1997, 1998), B16F melanoma cells (Szary et al., 1997), and WiDr cells (Khil et al., 1996; Stackhouse et al., 2000) exposed to 5-FC were selectively sensitised to radiation in a drug concentration-dependent fashion, with maximum killing enhancement after 72-h-incubation pre-irradiation (Khil et al., 1996) or, in contrast, at 3-h post-irradiation (Szary et al., 1997). Analysis of the linear quadratic or single-hit-multi-target parameters of the survival curves indicated a significant reduction in cell survival at both low and high doses of radiation (Pederson et al., 1997; Stackhouse et al., 2000). This combined strategy was tested in vivo in xenografts of squamous cell carcinoma of the head and neck (Hanna, 1997; Hamstra et al., 1999b). Daily administration of 5-FC (800 mg/kg) concomitant with radiation therapy (50 Gy, 5 Gy/day) induced significant volumetric regression and cure in three of seven tumours, while radiation or GDEPT alone produced no significant tumour control (Hanna et al., 1997). A robust bystander effect was induced, as a significant increase in survival was observed when only 25% of the tumour population expressed CD (Hamstra et al., 1999b). The combined GDEPT and radiotherapy strategy produced a measurable anti-tumour effect when clinically relevant dose regimens of radiation (2 or 5 Gy per fraction over one week) where analysed (Stackhouse et al., 2000).

Specific gene expression and prodrug activation offer the possibility of combining GDEPT systems to enhance the antitumour activity of the single treatments, without increasing systemic toxicity. Delivery of CD-HSV TK fusion genes followed by GCV and 5-FC treatment conferred upon gliosarcoma, mammary carcinoma and prostate tumour cells prodrug sensitivity (Rogulski et al., 1997a; Uckert et al., 1998; Blackburn et al., 1998, 1999) and radiosensitisation (Rogulski et al., 1997a; Blackburn et al., 1999), equivalent to or better than that observed for each system independently. The
mechanism underlying the synergistic cytotoxicity of concomitant CD/5-FC and HSV TK/GCV therapies is unclear, and it appears to be related to the enhancement of GCV phosphorylation by HSV TK after 5-FU treatment (Agbi et al., 1998). In vivo analysis showed high efficacy of the combined systems, further enhanced by radiotherapy (Rogulski et al., 1997b; Kim et al., 1998; Uckert et al., 1998).

Improved anti-tumour activity of CD/5-FC has been recently achieved by using the catalytically superior Saccharomyces cerevisiae CD (Hamstra et al., 1999a; Kievit et al., 1999) or by cotransfecting cells with the genes for CD and uracil phosphoribosyltransferase, able to directly convert 5-FU to 5-FUMP at the first step of its activating pathway (Adachi et al., 2000; Erbs et al., 2000; Koyama et al., 2000).

5-FC has been extensively used in the past as an antifungal agent, therefore its toxicological properties in humans are well established. A phase I clinical trial involving local injection of a plasmid containing the CD gene, regulated by the tumour-selective erbB-2 promoter, and systemic 5-FC (200 mg/kg/day) demonstrated safety of this GDEPT approach for the treatment of breast cancer (Pandha et al., 1999). CD-immunohistochemistry and mRNA in situ hybridisation showed tumour-selective gene expression in 11 of 12 patients. In four patients there was evidence of tumour regression, even though two of them did not receive the prodrug, which may be due to the immunogenicity of CD. In an ongoing phase I clinical study, a CD-containing adenovirus is intratumourally administered to 18 patients diagnosed with metastatic liver disease associated with colorectal carcinoma, followed by oral administration of 5-FC (Crystal et al., 1997).

NITROREDUCTASE/CB1954

The mustard prodrug CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] is a weak monofunctional alkylator, but it can be efficiently activated by the rodent enzyme DT diaphorase into a potent DNA cross-linking agent (Knox et al., 1998b). CB1954 resulted in the single agent toxicity in the thymus and the spleen of CB1954, compared to the parental lines (Bridge water et al., 1995; Bailey et al., 1996; Green et al., 1997; Friedlos et al., 1998; McNeish et al., 1998; Weedon et al., 2000). Transgenic mice studies of NTR expression under the control of a T cell-specific promoter showed selective CB1954 toxicity in the thymus and the spleen of systemically treated animals, whereas non-lymphoid tissues were unaffected (Drabek et al., 1997). Selective tissue ablation was also demonstrated in transgenic mice engineered to specifically express the NTR gene in the luminal epithelial cells of the mammary gland (Clark et al., 1997). Even if transgenics provide a useful model for studying the in vivo efficacy of gene therapy strategies, they may not be predictive for clinical scenarios, where levels of gene transfer are unlikely to be as high. However, retro- or adenoviral-mediated NTR transfer followed by CB1954 treatment (80 mg/kg, single or double intra-peritoneal (ip) injection) has been shown to cause regression and significantly prolonged median survival of nude mice bearing human tumour xenografts of pancreatic (McNeish et al., 1998; Weedon et al., 2000), hepatocellular and squamous cell carcinomas (Djeha et al., 2000).

Apoptosis was induced in thymocytes and mammary epithelial cells of NTR/CB1954-treated animals (Clark et al., 1997; Drabek et al., 1997; Cui et al., 1999), which appears to be a consequence of DNA cross-linking and strand breakage (Clark et al., 1997; Friedlos et al., 1998). In murine L cells, morphological changes were observed, such as the generation of enlarged multinucleated cells, which have been proposed to be due to a block in DNA synthesis with continued RNA and protein synthesis (Drabek et al., 1997). Although NTR-mediated cell death by CB1954 increased the expression of wild-type p53, functional p53 was not required to induce apoptosis (Cui et al., 1999).

The NTR/CB1954 system appears to act more rapidly than most combinations, as significant cytotoxic effects have been noted after relatively short exposure times, such as 4 h in vitro (Bridge water et al., 1995) and 24 h in vivo (Cui et al., 1999). This may be due to the fact that the activated drug is a DNA cross-linking agent able to kill
both proliferating and non-proliferating cells, which do not need to enter the S-phase for cytotoxicity to take place (Bridgewater et al., 1995; Clark et al., 1997; Green et al., 1997; Weedon et al., 2000).

An efficient bystander effect was demonstrated in a number of cell lines and in animal models, regardless of cell-to-cell contact and gap junctional status (Bridgewater et al., 1997). The primary NTR-metabolites of CB1954, 2- and 4-hydroxylamines, are membrane-permeable and, after further metabolism, are able to induce single strand breaks and interstrand cross-links, respectively, in neighbouring parental cells (Friedlos et al., 1998). Mixed populations with 30% NTR-positive pancreatic cancer cells were 1000-fold more sensitive to CB1954 than untransfected cells (Green et al., 1997), and the bystander killing was measurable when only 5% of the exposed population expressed NTR (McNeish et al., 1998). On the other hand, in cell culture experiments with 5–10% NTR-positive murine L cells (Drabek et al., 1997) and mammary glands of transgenic RED20 and RED40 mice (Clark et al., 1997) no bystander effect could be observed. Also, SCID mice implanted with tumours containing either 30 or 100% of NTR-expressing Burkitt lymphoma cells treated with ip CB1954 (20 mg/kg/day) were growth inhibited but not cured (Westphal et al., 2000).

The versatility that NTR offers by metabolising a range of nitro-prodrug substrates may have clinical relevance in view of the cross-resistance observed among alkylating agents (Frei et al., 1998). Structural variations of CB1954 included N-dihydroxpropyl and (N-dimethylamino)ethyl carboxamide side chains, the use of chloro, bromo, mesyl and iodo leaving groups on the oxazaphosphorine prodrug cyclophosphamide (CP) activation. The phosphoramid mustard is able to induce DNA alkylation. Acrolein, produced in equimolar amounts, may be responsible for protein alkylation.

CYPs are present in the liver and in some human cancers, including colon, breast, lung, liver, kidney and prostate, which are known to express isoforms of the 3A and 1A subfamilies (Patterson et al., 1999). To reduce CP and IP side effects towards critical host tissues, such as bone marrow, kidney and heart, it was hypothesised that overexpression of CYP enzymes in genetically engineered tumour cells could lead to selective sensitisation to oxazaphosphorines. Transfected human B-lymphoblastoid cells expressed catalytically competent CYP2A6, 2B6, 2C8, 2C9 and 3A4, and were significantly growth-inhibited by CP and IP (Chang et al., 1993). CYP2B1 gene expression sensitised rat glioma and human breast carcinoma cells to CP both in vitro and in vivo (Wei et al., 1994, 1995; Chen and Waxman, 1995; Chen et al., 1996; Manome et al., 1996). In co-culture experiments, 75% decrease in proliferation was induced when only 10% of the CP-exposed population expressed CYP2B1, independently of cell-to-cell contact (Chen and Waxman, 1995; Wei et al., 1995; Chen et al., 1996).

Because the CP metabolite phosphoramid mustard does not diffuse efficiently across cell membranes, it is likely that this bystander effect is due to the diffusible precursors, such as 4-hydroxy-CP and its tautomter aldophosphamide (Wei et al., 1995; Fig. 5).

It appears that local conversion of CP is superior to its activation in the liver, probably because of the short half-life (5.2 and 3.3 min in rat and human plasma, respectively) of 4-hydroxy-CP, which would cause significant decomposition of this primary metabolite before it reaches the tumour (Chen and Waxman, 1995). Although minimal liver toxicity was detected in preclinical studies, to further protect normal tissues selective inhibitors of endogenous liver enzymes could be administered in combination with the GDEPT system, such as 21,21-dichloro-progesterone or 3,5-dimethoxy-2,6-dimethyl-1-ethyl-1,4-dihydropyridine, which are specific for liver isoforms (Halpern et al., 1989; Correia et al., 1992).
The utilisation of the CYP/CP system in combination with macrophages for hypoxia-selective gene therapy (Dachs et al., 1997) has recently been demonstrated (Griffiths et al., 2000). Differentiated macrophages transduced with an adenoviral vector containing the human CYP2B6 gene were found to infiltrate human tumour spheroids (3D in vitro tumour models) and induce cell death when the spheroids were incubated with CP. A hypoxia-responsive promoter conferred an additional level of selectivity to the system. The macrophages themselves did not appear to be affected by the CYP2B6/CP treatment, which may make them a suitable vehicle for this gene therapy approach. However, CYP/CP may not be the ideal combination for hypoxia-regulated gene therapy, since the rate limiting activation of cyclophosphamide is an oxygen-dependent reaction (Fig. 5).

**CARBOXYPEPTIDASE G2/CMDA**

In the enzyme/prodrug systems described so far the prodrug is converted to an intermediate metabolite, which requires further catalysis by cellular enzymes to form the active drug. The lack or decreased expression of these enzymes in the target cells would lead to tumour resistance. The bacterial enzyme carboxypeptidase G2 (CPG2), which has no human analogue, is able to cleave the glutamic acid moiety from the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoic acid (CMDA), releasing the DNA-cross-linking mustard drug 4-(2-chloroethyl)(2-mesyloxyethyl)aminobenzoyl-L-glutamic acid without further catalytic requirements (Springer et al., 1990; Fig. 6). The application of the CPG2/CMDA combination in ADEPT strategies has been successfully demonstrated in a number of tumour models, and phase I clinical trials showed no conversion to the active species by human enzymes and no CMDA-related toxicity (Springer et al., 1994; Bagshawe et al., 1995; Martin et al., 1997). The feasibility of CPG2-mediated GDEPT was investigated in vitro in COS cells (monkey kidney) and four human tumour epithelial lines (Marais et al., 1996). Transfection with the CPG2 gene led to the production of an active enzyme, able to convert CMDA to a cytotoxic agent within 19-h incubation. Compared to mock-transfected controls, CPG2-transfectants were characterised by an increase in sensitivity to the prodrug spanning from 11- to 95-fold (Marais et al., 1996). However, the breast tumour cell line MDA MB361 showed only modest sensitivity to CMDA activated by intra-cellular expression of CPG2, mainly because the prodrug could not enter the cells (Marais et al., 1997). To overcome this problem, CPG2 was expressed tethered to the outer surface of mammalian cells, resulting in effective cell kill by CMDA in vitro and in vivo (Marais et al., 1997; Stribbling et al., 2000).

The CPG2/CMDA system has been shown to induce a robust bystander effect. Exposure to the prodrug induced 50% and 100% WI-D cell kill in monolayers containing 1-2 and 12% CPG2+ cells, respectively (Marais et al., 1996). In nude mice, regression and cure of breast carcinoma xenografts could be obtained when only 10% of the cells expressed the tethered CPG2 (Stribbling et al., 2000). Evidence of the in vivo bystander effect was obtained measuring the proportion of apoptotic cells in the treated tumours, which was higher than the percentage of cells expressing CPG2. The effect of the surface tethered CPG2 on host immunity has not yet been reported.

To improve the efficacy of the system, a number of self-immolative prodrugs have been developed in combination with CPG2, some of them resulting in a significant increase in cytotoxicity (Niculescu-Duvaz et al., 1998 and 1999).

**HORSERADISH PEROXIDASE/INDOLE-3-ACETIC ACID**

The reaction between the plant enzyme horseradish peroxidase (HRP) and the non-toxic plant hormone indole-3-acetic acid (IAA) has been analysed in depth, but not yet completely elucidated. At neutral pH, IAA is oxidised by HRP-compound I to a radical cation, which undergoes scission of the exocyclic carbon-carbon bond to yield the carbon-centred skatolyl radical (Fig. 7). In the presence of oxygen, the skatolyl radical rapidly forms a peroxyl radical, which then decays to a number of products, the major ones being indole-3-carbinol, oxindole-3-carbinol and 3-methylene-2-oxindole (MOI; Dunford, 1999). In anoxic solution, decarboxylation of the radical cation can still take place and the carbon-centred radical preferentially reacts with hydrogen donors (Augusto, 1993; Candeias et al., 1994; Fig. 7).

When activated by purified HRP, IAA was shown to inhibit colony formation in mammalian cells, while neither enzyme nor prodrug alone were cytotoxic at the concentration or times analysed (Polkes et al., 1998, 1999). To date, the activated drug has not yet been identified. Of the stable products, indole-3-carbinol is non-toxic to V79 cells at experimentally produced concentrations, with or without HRP (Polkes and Wardman, 2000). MOI has been reported to be toxic in E. coli and some plants, to react with glutathione and to bind to sulphhydryl regions of histone DNA or RNA (Polkes and Wardman, 2000).
Non-specific activation of IAA in normal tissues is unlikely to take place, since endogenous peroxidases in human tumour cells (Folkes et al., 1998) and rat leukocytes and phagocytes (Pires de Melo et al., 1997) were significantly less efficient than HRP in converting IAA into a cytotoxin. Moreover, no toxicity was reported in patients after oral administration of 100 mg/kg IAA (Mirskey and Diengott, 1956).

The efficacy of the HRP/IAA system for GDEPT was evaluated in vitro by exposing human T24 bladder carcinoma cells transfected with an expression vector containing the HRP cDNA to the prodrug IAA (Greco et al., 2000). Prodrug activation was fast and efficient, since cytotoxicity could be evoked within a 2-h exposure, and it was further increased after 24-h incubation. A strong bystander effect was induced, since 2–3 log cell kill was observed when only 20–30% of the exposed population expressed HRP. Moreover, cell contact was not required for bystander killing, as the cells were sparsely seeded at the time of prodrug incubation and the transfer of IAA-containing medium preconditioned by HRP+ cells to HRP cells resulted in their death. Significant cytotoxicity was also detected in the extreme tumour conditions of anoxia, although different mechanisms appear to be involved in the induced cell kill.

When compared to HSV TK/GCV, the HRP/IAA combination showed increased toxicity in vitro, both in oxic and anoxic conditions (Greco et al., 2000). However, to further improve therapeutic efficacy, 10 IAA analogues were tested in combination with HRP in four different cell lines of human origin (T24; FaDu, nasopharyngeal squamous carcinoma; MCF-7, mammary carcinoma; HMEC-1, dermal microvascular endothelium; own unpublished data). In several cases increased toxicity was detected compared to the model compound IAA.

Delivery of the HRP gene to human tumours followed by treatment with IAA and analogues may provide a novel cancer GDEPT approach, with potential to target hypoxic cells, which are resistant to radio- and chemotherapy. In vivo analysis of this novel GDEPT system is currently underway.

CONCLUSIONS

Gene therapy is a promising approach, still at the early stages of development. Some major problems remain to be solved before these new strategies become routinely adopted in the clinic, one of the main challenges remaining the improvement of gene delivery, and therefore therapeutic efficacy. Nevertheless, the results described in this review and their clinical application are encouraging, and illustrate both feasibility and future promise for GDEPT as a cancer treatment. Clinical trials have already addressed the issues of safety and toxicity. As vector technology fulfils the requirement of efficient delivery, it can be anticipated that the results observed in the preclinical studies will more quickly translate into the clinic.

LITERATURE CITED


GENE DIRECTED ENZYME/PRODRUG THERAPY


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Molecular approaches to chemo-radiotherapy

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Abstract

Although radiotherapy is used to treat many solid tumours, normal tissue tolerance and inherent tumour radioresistance can hinder successful outcome. Cancer gene therapy is one approach being developed to address this problem. However, the potential of many strategies are not realised owing to poor gene delivery and a lack of tumour specificity. The use of treatment-, condition- or tumour-specific promoters to control gene-directed enzyme prodrug therapy (GDEPT) is one such method for targeting gene expression to the tumour. Here, we describe two systems that make use of GDEPT, regulated by radiation or hypoxic-responsive promoters. To ensure that the radiation-responsive promoter is be activated by clinically relevant doses of radiation, we have designed synthetic promoters based on radiation responsive CarG elements derived from the Early Growth Response 1 (Egr1) gene. Use of these promoters in several tumour cell lines resulted in a 2–3-fold activation after a single dose of 3 Gy. Furthermore, use of these CarG promoters to control the expression of the herpes simplex virus (HSV) thymidine kinase (tk) gene in combination with the prodrug ganciclovir (GCV) resulted in substantially more cytotoxicity than seen with radiation or GCV treatment alone. Effectiveness was further improved by incorporating the GDEPT strategy into a novel molecular switch system using the Cre/loxP recombinase system of bacteriophage P1. The level of GDEPT bystander cell killing was notably increased by the use of a fusion protein of the HSVtk enzyme and the HSV intercellular transport protein Vp22. Since hypoxia is also a common feature of many tumours, promoters containing hypoxic-responsive elements (HREs) for use with GDEPT are described. The development of such strategies that achieve tumour targeted expression of genes via selective promoters will enable improved specificity and targeting thereby addressing one of the major limitations of cancer gene therapy. © 2002 Published by Elsevier Science Ltd.

Keywords: Radiation; Gene therapy; Hypoxia; Promoters; CarG elements; GDEPT

1. Combination gene therapy and radiotherapy

Radiotherapy is the treatment modality of choice for most solid malignancies. However, the tolerance of surrounding normal tissues to treatment-induced injury often restricts the dose that can be delivered to the tumour to achieve cure. This problem can be partly overcome using physical techniques such as conformal or intensity modulated radiotherapy that deliver the dose to a more precisely defined tumour volume. An alternative approach is to combine radiotherapy with a pharmacological- or gene-based component, which improves the effectiveness of the radiation dose delivered to the tumour by increasing the therapeutic ratio between normal and malignant cells. Pharmacological approaches to modify tumour radiosensitivity are well established, and include for example the use of chemical radiosensitisers and inhibitors of cellular repair processes and tumour cell proliferation [1]. In contrast, the combination of radiotherapy and gene therapy technologies is an evolving discipline. Gene delivery, insufficient specificity and tumour targeting often hamper the efficacy of gene therapy approaches, but new concepts are evolving to overcome these [2,3]. This review will focus on one of these problems, describing two different strategies to selectively target the tumour, one mediated by radiation and the second by hypoxia. Both exploit selective gene promoters to activate gene-directed enzyme prodrug therapy (GDEPT) systems for use as adjuvant or concomitant with radiotherapy. GDEPT involves the delivery to the target cells of a foreign gene encoding a non-toxic enzyme which activates specific prodrugs to toxic agents at the site of conversion [4].
2. Radiation-mediated gene therapy (RMGT)

Radiation-mediated gene therapy exploits the fact that in the majority of patients receiving radiotherapy the radiation is directed to the tumour volume, providing some degree of tumour localisation for controlling the expression of therapeutic genes. Temporal and spatial control of gene expression can therefore be achieved for any genes delivered to the tumour and for any tumour types treated with radiotherapy [5–7].

2.1. Radiation-induced gene expression

A number of cell cycle control and DNA repair genes (e.g. p21WAF1/CIP1 and Early Growth Response 1 (Egr1) [8–10]) are upregulated following exposure to ionising radiation [11–15]. Some of these genes, such as WAF1 [10] and GADD45 [16] operate within p53-dependent pathways, and thus may not function efficiently in the many tumours exhibiting mutant p53 phenotypes. However, since activation of Egr1 is p53-independent, its promoter has been adopted for the regulation of therapeutic genes in the majority of the experimental models of RMGT. A 425 nucleotide region (denoted E425 here) [17] of the murine Egr1 gene was inserted upstream of the eDNA of the tumoricidal cytokine tumour necrosis factor-α (TNF-α) and delivered to human epidermoid carcinoma xenografts (SQ-20B) using a cell carrier [8], liposomes [5] or an adenoviral vector (Ad5 [18,19]). The combination of a fractionated radiation dose schedule of 40 Gy (5 Gy fractions twice a day for 4 days) and intratumorally delivered Ad5.Egr.TNF resulted in increased tumour regression by up to 90% of control values compared with maxima of 50% for vector or radiation alone [7,19]. Similarly, Joki and colleagues [20] and Takahashi and colleagues [21] used a plasmid construct containing the E425 promoter to regulate the expression of the herpes simplex virus thymidine kinase (HSVtk) suicide gene. Glialoma cells containing this vector were sensitised to the anti-herpes viral agent ganciclovir (GCV), a substrate for HSVtk, after exposure to a single radiation dose of 20 Gy.

The radiation-responsive regions of the Egr1 promoter have been identified as 10 nucleotide motifs of the consensus sequence CC(A/T)G-GG, also known as CArG elements [22]. However, only the CArG elements grouped in the 5’ distal ‘enhancer’ region, appear to contribute to the radiation responsiveness of Egr1. Radiation-produced reactive oxygen intermediates (ROIs) are believed to be critical in activating the Egr1 promoter via these CArG motifs [23]. In addition, CArG elements are also known to be involved in the regulation of a number of immediate-early genes (e.g. Egr1, c-fos, β-actin) following mitogenic stimulation, and are thus often referred to as serum-response elements. This growth factor-induced response is governed by the binding of serum-response factor. Whether this transcription factor is also involved in the activation of the Egr1 gene by ionising radiation has not as yet been determined. The human and murine Egr1 promoters also contain putative binding sites for the Spl transcription factor, the Fos-Jun heterodimer API, as well as for cyclic adenosine monophosphate (AMP) and Egr1 itself [24–27], all of which may influence radiation-mediated promoter induction.

2.2. Synthetic radiation-responsive gene promoters

In order to produce a radiation-responsive promoter without such potentially antagonistic binding sites, we have developed synthetic alternatives based on isolated CArG elements [28]. These promoters consist of an enhancer region, containing the CArG elements themselves, adjacent to a basal promoter (i.e. from the cyto-megalovirus (CMV) immediate early gene) containing the essential transcriptional initiation apparatus, such as the CCAAT/TATA boxes (see Fig. 1 [28]). The enhancer is produced by cloning complementary, single-stranded, oligo-deoxyribonucleotides (ODNs) containing the CArG sequences. The synthetic promoter is positioned directly upstream of the coding sequence of the enhanced green fluorescent protein (EGFP) reporter gene [29,30] in a plasmid vector. Target cells are transfected with the plasmid construct, irradiated and subsequent EGFP production measured by flow cytometry. A synthetic promoter containing four CArG elements was shown to be responsive to radiation doses as low as 1 Gy and was more radiation-responsive (3.1-fold ± 0.2) than the wild-type Egr1 counterpart (1.86-fold ± 0.2) at an optimal 3 Gy dose (Fig. 2 [28,31]). Multiple doses of 3 Gy resulted in greater levels of induced expression than seen after a single dose. Promoters containing different numbers, sequences and arrangements of CArG elements were also evaluated. Increasing the number of CArG elements in the promoter improved the specific radio-responsiveness and reduced basal expression (data not shown). We have also demonstrated that alteration of CArG element core sequences can substantially affect the response, either positively or negatively. Although the transcription factors involved in the induction have yet to be identified, the binding of such proteins or protein complexes to the CArG elements are likely be influenced by their spatial arrangement. This is currently being investigated in our laboratory.

A GDEPT approach was then used to demonstrate the potential of CArG-activated promoters. This consisted of the HSVtk/GCV suicide gene system and a tumour cell growth inhibition assay. The HSVtk/GCV system, currently adopted in clinical trials [32], is the most well known example of enzyme/prodrug combination in cancer GDEPT. The killing effect of the HSVtk/
GCV is only seen in proliferating cells in which HSVtk can monophosphorylate GCV, which is then further phosphorylated by cellular kinases, to cause termination of DNA synthesis and cell death [33]. The HSVtk/GCV system is therefore particularly suitable for the eradication of rapidly dividing tumour cells invading non-proliferating tissue. However, HSVtk/GCV would not be the combination of choice to target the slowly dividing hypoxic population in solid tumours, which have been shown to contribute to the resistance of human tumours to chemo- and radiotherapy. Alternative enzyme/prodrug combinations able to function in non-proliferating cells would be for example carboxypeptidase 2 (CPG2) and CMDA [34,35] or horseradish peroxidase (HRP) and indole-3-acetic acid (IAA) [4,36]. The selective enhancement of radiation-mediated toxicity using GDEPT has been demonstrated both in vitro and in vivo, with some degree of synergy between radiation and gene therapy noted [37–42]. Non-transduced cells adjacent to HSVtk-containing cells can also be killed via a

![Diagram](https://example.com/diagram.png)

**Fig. 1.** (a) Strategies adopted to improve the radiation responsiveness of the synthetic CArG enhancer; increasing the number of CArG elements from the four used in the prototype (28); altering the CArG element core' sequences; introducing spacers between CArG elements to aid binding of any transcription factors involved in the radiation response. (b) Schematic representation of the radiation-responsive plasmid vector (based on PCINeo: Promega) showing chimeric enhancer/promoter and reporter (EGFP) or suicide (HSVtk) coding sequences (CDS). Restriction sites used for cloning components are italicised. The intron is present to increase expression of the CDS immediately downstream. The SV40 polyadenylation signal allows efficient processing of RNA transcripts. Amp and Neo genes are used as selectable markers for establishing clones in bacterial and mammalian cell cultures respectively. CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein.
bystander mechanism [43]. This ‘bystander effect’ is thought to be partly attributable to the diffusion of toxin molecules through gap junctions [44-46]. In vivo, the host immune system is believed to enhance this effect [47,48]. Bystander killing will be vital for prodrug-mediated gene therapy since the most efficient delivery systems will be unable to target all the cells in a tumour. Wu and colleagues [49] noted that complete tumour cell killing was achieved when only 10% of cultured melanoma cells were expressing HSVtk in the presence of GCV. Furthermore, it has been shown that significant tumour regression could be achieved in animal models when only 10% of tumour cells were producing HSVtk [50].

In our own studies, when tumour cell cultures were transected with CARG-based vectors (~20% of cells) and grown in the presence of GCV after a single 2 Gy trigger dose, cell survival was reduced to ~70%, compared with 90% for mock-transfected cultures treated similarly [28]. If this finding were to be translated into a therapeutic dose schedule in a typical 70 Gy treatment (35 fractions of 2 Gy), the resultant equivalent dose directed to the tumour would be almost 100 Gy.

The potential of using the synthetic radiation-responsive promoters was also evaluated in a preliminary study in vivo in a MCF-7 breast adenocarcinoma xenograft model. Modified cell lines were established from plasmid-transfected MCF-7 cells by G418 selection and cloning. One of these stable cell lines transfected with a synthetic promoter containing four CARG elements regulating the expression of HSVtk was used to establish xenografts in nude mice. 50 μM GCV was administered interperitoneally (i.p.) 2 days prior, during and for three days after three daily 3-Gy fractions of X-rays. The effect of each treatment was compared by assessing the time required for tumours to reach to 500 mm^3 (Table 1). An increase in tumour growth delay was evident for those tumours treated with GCV combined with irradiation, compared with GCV or radiation treatment alone. Despite the small size of this study, it clearly demonstrated that the synthetic radiation-responsive promoters are functional in vivo, an observation that supported our earlier studies in whole tumours using EGFP as a reporter system.

We have also investigated the use of an intercellular transport protein (the HSV protein vp22) to enhance the bystander effect. As reported by Elliot and O’Hare [51], vp22 is an efficient intercellular transporter, able to transfer into neighbouring cells and translocate to the nucleus of the target cell. The fusion protein product of the chimeric vp22/EGFP gene was efficiently exported into as many as 200 nuclei of cells surrounding the original transfectant. Chimeric vp22 proteins are known to function in a wide range of cell lines [52] and deliver proteins that modify the cell response to radiation damage [53]. This active transport has been achieved using vp22HSVtk fusion proteins that retain enzyme...
Table 1

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Radiation Treatment</th>
<th>GCV Dosing (IP)</th>
<th>Days to 500 mm³</th>
</tr>
</thead>
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<tr>
<td>MCF-7</td>
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<td>8.9 ± 2</td>
</tr>
<tr>
<td>MCF-7</td>
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<td>8 x 50 µM</td>
<td>11.2 ± 5</td>
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</tr>
<tr>
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<td>11.3 ± 6</td>
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<tr>
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<td>8 x 50 µM</td>
<td>24.9 ± 5</td>
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<td>3 x 3 Gy</td>
<td>8 x 50 µM</td>
<td>33.1 ± 3</td>
</tr>
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</table>

* Treatment commenced when tumours were of the same size. MCF-7/pE4TK cells were established by transfecting MCF-7 cells with plasmids containing HSVrA under the control of the radiation-responsive four CArG promoter, and then enriched by neomycin selection so that all cells contained the HSVrA gene. Tumours were divided into groups and either sham-irradiated, irradiated with three doses of 3 Gy (24 h interval), given intraperitoneal (i.p.) injections of ganciclovir (GCV, 8 daily doses of 50 µM) or vehicle controls, or combinations of the treatments. The largest tumour growth delay, indicating the highest toxicity, was seen in irradiated tumours that were combined with GCV treatment. These data indicate that the synthetic promoter is functional in vivo.

In order to ensure long-term, high-level expression of therapeutic genes following the modest radiation-induction of the CArG promoters, we have devised a novel ‘molecular switch’ system based on the Cre/LoxP recombinase of bacteriophage P1 (Fig. 3 [31,56]). Using this strategy, the radiation-responsive promoter drives the expression of the Cre recombinase gene, which in turn activates a transcriptionally-silenced tumour sensitising gene by loxP site-mediated recombination. Therefore, a single activating dose of radiation could lead to HSVrA gene expression via the strong constitutive CMV promoter, thereby producing a substantial amplification of the activation signal. Indeed, in dual-plasmid transfection experiments using the EGFP reporter system, we demonstrated an 8.2-fold (± 1.2) increase in numbers of brightly fluorescent cells after a radiation trigger, compared with unirradiated control cultures (Fig. 2 and [31]). In addition, these cells were on average nearly 15 times brighter than those produced when the CArG promoter directly (without the Cre/
loxP switch) regulated EGFP expression. This translates to an approximately 40-fold induction of EGFP production via the switch, compared with 3.1 (±0.25)-fold when the synthetic promoter regulated EGFP expression directly. This system has now been engineered to function in a single vector, thus avoiding the necessity of having to target two vectors to each tumour cell. The use of a constitutive secondary promoter (e.g. CMV) should also ensure sustained expression of the therapeutic gene within the tumour volume after the radiation stimulus has been withdrawn. However, since this system incorporates two controlling gene promoters, the CMV promoter could be replaced by tumour-, tissue-, condition or micro-environment specific promoters [3,57,58] providing the potential for even greater specificity, selectivity and indeed safety.

3. Hypoxia-mediated gene therapy

The presence of hypoxia is a negative prognostic indicator for outcome following radiotherapy in a range of human tumour sites [59-63]. The absence of a cell killing component that can be attributed to radiation-mediated oxygen radicals is unlikely to be the only reason for the resistance of hypoxic tissue to the lethal effects of radiotherapy [64]. Resistance may also arise from modifications to gene expression induced as a direct consequence of the hypoxic environment, such as a more aggressive locoregional disease and enhanced invasive capacity [65-67]. Rather than devise therapeutic strategies of overcoming hypoxic resistance, a number of groups have chosen to exploit the hypoxic nature of tumours to gain a therapeutic advantage [4,57,68-72]. The adaptive response to cellular hypoxia involves the modulation of the synthesis of multiple proteins controlling processes such as glucose homeostasis, angiogenesis, vascular permeability and inflammation. These include, for example, phosphoglycerate kinase 1 (PGK1), erythropoietin (EPO), lactate dehydrogenase A (LDHA), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) [73]. The DNA regulatory elements controlling the expression of oxygen-responsive genes have been defined in many cases, and involve the specific binding and trans-activation by various inducible, phosphorylation-dependent and/or redox sensitive transcription factors, including HIF1 Inducible Factor 1 (HIF1), Activator Protein 1 (AP1), Nuclear Factor κB (NF κB), p53 and the Heat Shock Transcription Factor. Published evidence indicates that only HIF1 is specifically oxygen-responsive [73], while the other transcriptional systems appear to contribute to the response to hypoxia via related redox and metabolic changes.

Affinity purification and molecular cloning of HIF1 showed it to function as a heterodimer consisting of two basic-helix-loop-helix proteins, HIF1α and HIF1β (previously identified as ARNT, aryl receptor nuclear translocator, which is part of the xenobiotic response) [74]. Although both subunits are constitutively expressed, HIF1α is the hypoxia-regulated component via post-translational stabilisation and transactivation by several additional factors [75,76]. To modulate gene expression, HIF1 specifically binds to hypoxia-responsive elements (HREs), enhancers containing the core sequence 5′-(A/G)CGT(G/C)(G/C)-3′, localised at varying distances and orientations of the coding region of several hypoxia-regulated genes. The HRE/HIF1 regulation system was shown to be common to all mammalian cells and human tissues tested to date [76] and the HIF1α subunit was found to be overexpressed in 68% of the tumour types analysed [77]. The high frequency of HIF1 expression across many human tumours of diverse tissue origin represents a possible therapeutic target for HRE-directed gene therapy of the hypoxic environment. It has been shown that marker gene expression regulated by the murine PGK1 HRE could be induced in hypoxic tumour cells [68]. Following the demonstration of the successful use of radiation [28] or hypoxic-responsive gene promoters [68,78] alone, we have assessed the function of chimeric promoters containing radiation-responsive CArG elements in combination with HREs (data not shown). These dual enhancer constructs functioned in response to radiation or hypoxia alone and also in the presence of both stimuli.

4. Other gene therapy approaches

A number of radiotherapy and gene therapy strategies have been developed. High therapeutic potential was recently demonstrated for radiotherapy in combination with a trimodal approach consisting of a replication-competent oncolytic adenovirus containing a cytosine deaminaseHSVtk fusion gene [42]. This combination of radiation therapy, lytic viral therapy and double suicide gene therapy produced significant tumour regression in an experimental C33A tumour xenograft model. Manipulating the expression of the sodium and iodide symporter (NIS) gene increased the uptake of radio-labelled iodide in an in vitro spheroid model [79-81]. The overexpression of such membrane transporter proteins increases the selectivity of [131]I-metaiodobenzylguanidine therapy for neuroblastoma and ionic [131]I-administration for differentiated thyroid tumours. In contrast, gene therapy has also been exploited to decrease the side-effects of radiation-induced damage in normal tissue. For example, intratracheal injection of a vector carrying the human superoxide dismutase (SOD2) transgene prior to irradiation prevented radiation-
induced damage of transduced tissues, resulting in decreased radiation-induced oesophageal stricture [82] and decreased late pulmonary fibrosis [83]. These examples of combining radiotherapy and gene therapy illustrate the breadth of ideas being investigated. Moreover, they highlight the benefit of adopting a combinational approach in that treatment can be tailored to address individual situations.

Ultimately, the success of gene therapy will depend on the efficient delivery of transgenes to the tumour site. However, the development of strategies or vectors that can offer, precise tumour targeting will undoubtedly play a significant role in the outcome of future gene therapies for cancer.

Acknowledgements

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References


