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Can Gene Therapy Overcome the Problem of Hypoxia in Radiotherapy?

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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 ($pO_2 < 0.3\%$; 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[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[2,3,4]. 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)[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[5], carcinomas of the uterine cervix[6,7] and of the head and neck[3,8] 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)[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 *p53* mutations and have consequently lost their apoptotic potential[10,11]. Also, squamous cell carcinomas of the uterine cervix characterised by pronounced hypoxia[12] and low apoptotic index[13] showed a high probability for lymphatic spread and recurrence, despite adjuvant treatment with radiation or chemotherapy in addition to radical surgery[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[14], and new delivery systems like E1B gene-attenuated adenoviruses[15], lentiviruses, polylysine constructs, leukocytes and bacteria are being developed[14]. In targeting hypoxic cells, a further obstacle is represented by their reduced metabolism, proliferation rate[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[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 *Clostridium* and tumour-invasive *Salmonella* auxotrophs.

The ability of *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[18,19]. Spores of *C. beijerinckii* genetically engineered to produce the *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[18]. *In vitro* conversion of the prodrug CB 1954 (see final section for details) to a cytotoxic agent by clostridia-produced NTR demonstrated the therapeutic potential 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 *C. acetobutylicum* and *C. oncolyticum*[19].

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[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[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[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 phosphoramide mustard)[21]. A hypoxia-responsive promoter (see next section) conferred an additional level of selectivity to the system. However, the rate limiting activation (hydroxylation) of cyclophosphamide 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)[23]. 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, 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[23], 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)[24]. Although both subunits are constitutively expressed, HIF-1α is hypoxia-regulated *via* post-translational stabilisation and transactivation by several additional factors[25,26]. 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[25] and the HIF-1α subunit was found to be overexpressed in 68% of the tumour types analysed[27].

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[28]. 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 (O_2 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% O_2[29]. 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% O_2; ** = 0.02% O_2) or anoxic incubation intervals: ref 28, 30, 31: 16h; 29: 24h; 32, 33^X: 6h; 33^-: 18h. h: human, m: murine.

HRE	Basal promoter	Reporter gene	Cell line	Hypoxic/oxic induction	Anoxic/oxic induction	Ref.
3×mPGK-1	mPGK-1	CD2	HT1080	1.4	1.9	28*
3×mPGK-1	minHSV TK			2.3	2.2	
3×mPGK-1	9–27			2.2	4.1	
mPGK-1	mPGK-1	hEPO	C2C12		2.7	29
3×hENO	SV40	luciferase	T47D	120		30*
3×hENO				63		
2×mLDH-A				81		
4×mLDH-A				65		
4×hEPO				255		
3×mPGK-1				146		
3×mPGK-1 + VEGF 3' UTR				~ 300		
mPGK-1	SV40	β-gal	HT1080	15		31*
			MCF-7	16		
			Ovcar-3	12		
			HS 906(D).Mu	8.5		
			C2C12	18		
			SkMC	50		
hVEGF	hVEGF	luciferase	HT1080		8.2	32
			SCCVII		3.3	
			EMT-6/KU		4.2	
			HepG2		8.5	
hEPO	SV40		HT1080		2	
			HepG2		5	
5×hVEGF	h VEGF		HT1080		20	
5×hVEGF	hVEGF+minE1b				44	
5–10×hVEGF	SV40	luciferase	HT1080	54–57		$33^{x,**}$
5–10×hVEGF+5' VEGF UTR				23–27		$33^{x,**}$
5–10×hVEGF+5' VEGF UTR	E1b			56–60		$33^{x,**}$
5×hVEGF + 5' VEGF UTR	E1b			131		$33^{-,**}$
5×hVEGF + 5' VEGF UTR + 3' VEGF UTR				193		$33^{-,**}$

DNA construct was studied in mice with C2C12-EPO cells implanted in their dorsal flank. Serum EPO levels in animals exposed to 7% O_2 were twice as high than in controls kept at 21% O_2[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[30]. 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_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_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 β-galactosidase (β-gal) transgene expression was observed, with levels of hypoxic induction comparable to the full-length CMV[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[32,33]. In this cell line, a six-hour hypoxic (0.02% O_2) incubation induced a 40–50 fold increase in luciferase activity[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[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[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[30–33] represent a clear improvement over the constructs used in earlier studies[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 *in vivo* and *in vitro* results (summarised in table 1) demonstrated the selectivity of the system and its potential for tumour-specific targeting of therapeutic gene expression.

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[36,37], phagocytosis of apoptotic vesicles[38], induction of immune response against the tumour[39,40] and diffusion of soluble toxic metabolites[37,41] 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[28], 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[43,44]. 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[43]. 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[44].

Bioreductive drugs such as mitomycin C, tirapazamine (SR 4233), RSU 1069 and CB 1954 have been extensively utilised as hypoxia-selective cytotoxins (HSCs)[45,46]. 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[47,48].

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

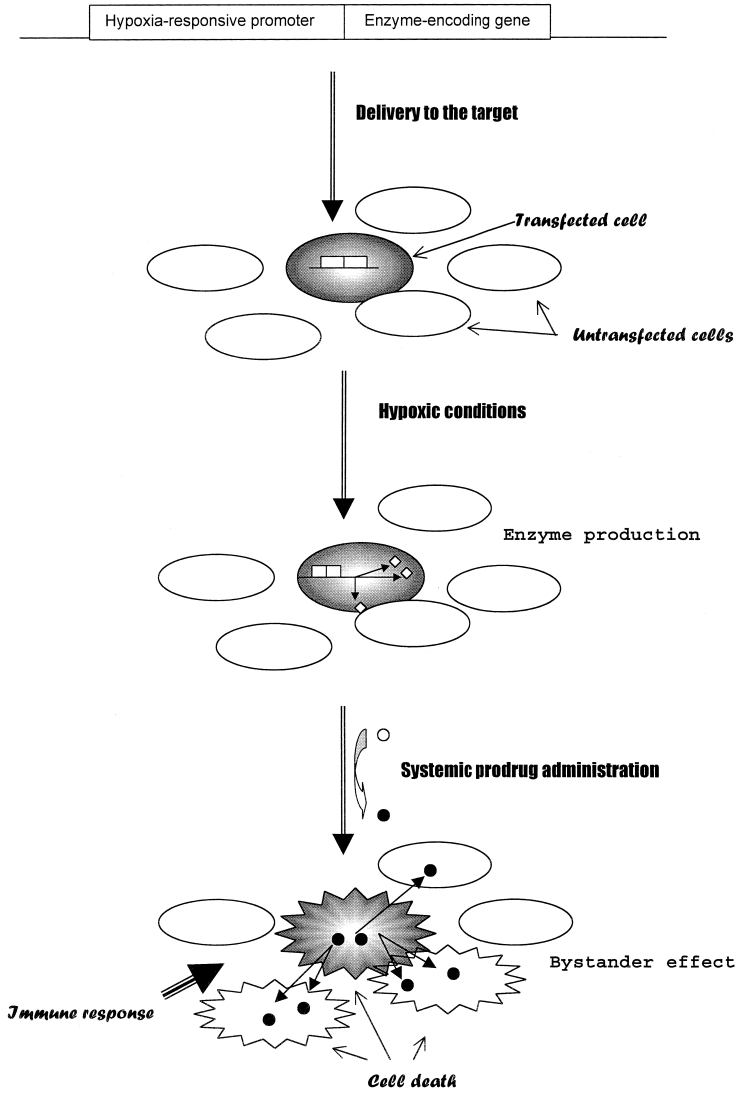

Fig. 1. Schematic diagram of hypoxia-regulated gene directed enzyme-prodrug therapy. A DNA construct containing an enzyme-encoding gene controlled by a hypoxia-responsive promoter is delivered to the tumour using viral or non-viral vectors. At best 10D–20% of the target cells will express the foreign 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[49]. 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[50].

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, EO9, 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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