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UNIVERSITY COLLEGE CORK



HIF-1 α role in oxygen-dependent radio- and chemosensitivity

Thesis presented by
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Under the supervision of
Dr Ashley Allshire

for the degree of
Doctor of Philosophy

April, 2014

DEPARTMENT OF PHARMACOLOGY & THERAPEUTICS

Head of Department: Prof. Thomas Walther

Dedicated to

my parents *Ewa* and *Miroslaw*

my sister *Anna*

my husband *Musharaf*

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Declaration

The work contained herein is being submitted to the National University of Ireland for the degree of PhD. Accordingly, I declare that this thesis is my own work and has not been submitted for another degree at University College Cork or elsewhere. This project was supervised throughout by Dr Ashley Allshire, Department of Pharmacology & Therapeutics at University College Cork.

Signed:

Kinga Gebolys

Date:

4th April 2014

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List of Abbreviations

aa	amino acid
Ah	aryl hydrocarbon
AP-1	activator protein 1
ARD	arrest-defective-1 protein (acetyltransferase)
ARNT	Ah nuclear translocator
Asn	asparagine
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BSA	albumin from bovine serum
cAMP	cyclic adenosine monophosphate
CDK	cyclin-dependent kinase
CoCl ₂	cobalt chloride
CREB	cAMP response element binding protein
DAG	diacylglycerol
DGK	diacylglycerol kinase
DMSO	dimethyl sulfoxide
EDHB	ethyl-3,4-dihydroxybenzoate
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
FBS	foetal bovine serum
FIH	factor inhibiting HIF
HIF	hypoxia inducible factor
H ₂ O ₂	hydrogen peroxide
HRE	hypoxia response element
Gy	Gray
IC ₅₀	half maximal inhibitory concentration
ID	inhibitory domain
JNK	c-Jun amino terminal kinase
Lys	lysine
MAPK	mitogen-activated protein kinase

MH	moderate hypoxia
MV	megavolt
NFκB	nuclear factor kappa B
NLS	nuclear localisation signal
NO	nitric oxide
ODDD	oxygen-dependent degradation domain
OER	oxygen enhancement ratio
PAS	Per-Arnt-Sim domain
PBS	phosphate buffered saline
PHD	prolyl hydroxylases
PI3K	phosphatidylinositol-3-kinase
P _{O₂}	oxygen partial pressure
Pro	proline
pVHL	von Hippel-Lindau tumour suppressor
RB	retinoblastoma protein
ROS	reactive oxygen species
R59949	3-[2-[4-(bis(4-fluorophenyl)methylene)-1-piperidinyl]ethyl]-2,3-dichloro-2-thioxo-4(1H)-quinazolinone
SAPK	stress activated protein kinase
Ser	serine
SH	severe hypoxia
siRNA	small interfering RNA
SOD	superoxide dismutase
SRC-1	steroid receptor coactivator
TAD	transactivation domain
TIF	transcription intermediary factor
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
Ub	ubiquitin
VEGF	vascular endothelial growth factor
5-FU	5-fluorouracil

Abstract

Poor oxygenation (hypoxia) is a common characteristic of human solid tumours, and is associated with cell survival, metastasis and resistance to radio- and chemotherapies. Hypoxia-induced stabilisation of hypoxia-inducible factor-1 α (HIF-1 α) leads to changes in expression of various genes associated with growth, vascularisation and metabolism. However whether HIF-1 α plays a causal role in promoting hypoxic resistance to antitumour therapies remains unclear. In this study we used pharmacological and genetic methods to investigate the HIF-1 α contribution to radio- and chemoresistance in four cancer cell lines derived from cervical, breast, prostate and melanoma human tumours. Under normoxia or hypoxia (<0.2% or 0.5% oxygen) the cells were exposed to either a standard irradiation dose (6.2 Gy) or chemotherapeutic drug (cisplatin), and subsequent cell proliferation (after 7 days) was measured in terms of resazurin reduction. Oxygen-dependent radio- and chemosensitivity was evident in all wild type whereas it was reduced or abolished in HIF-1 α (siRNA) knockdown cells. The effects of HIF-1 α -modulating drugs (EDHB, CoCl₂, deferoxamine to stabilise and R59949 to destabilise it) reflected both HIF-1 α -dependent and independent mechanisms. Collectively the data show that HIF-1 α played a causal role in our *in vitro* model of hypoxia-induced radioresistance whereas its contribution to oxygen-dependent sensitivity to cisplatin was less clear-cut. Although this behaviour is likely to be conditioned by further biological and physical factors operating *in vivo*, it is consistent with the hypothesis that interventions directed at HIF-1 α may improve the clinical effectiveness of tumour treatments.

Chapter 1

General Introduction

What is cancer?

Cancer is the generic term used to describe a large group of complex and progressive genetic diseases that can affect any part of the body. Carcinogenesis is a multistep process of malignant formation that involves three main stages: initiation, promotion and progression. Transformation of the normal cell into a cancer cell can occur due to sequential accumulation of random mutations in genes responsible for the regulation of cell proliferation, cell death and DNA repair. Mutagens - physical or chemical carcinogenic agents that lead to heritable DNA damage - can also initiate the process of cancer development (Essigmann & Wood, 1993). During the promotion stage of cancer development, tumours enlarge due to reduced ability of abnormal cancer cells to balance the cell division and cell death processes. This leads to cell accumulation and formation of benign tumour masses. At the last stage of cancer tumour cells gain the ability to spread into surrounding tissues by invading blood vessels and lymph nodes leading to metastasis elsewhere in the body (malignant neoplasm).

Risk factors for cancer in humans include family history, obesity, smoking or inhaling tobacco, as well as occupational and environmental exposure to carcinogens. Further contributory risk factors for cancer are infections such as hepatitis, human papillomavirus or *Helicobacter pylori* associated with liver, cervical and stomach cancers respectively.

There are three main types of cancer including gland and duct cancers (*carcinomas*), cancers of muscle, bone, artery or nerve tissue (*sarcomas*), and cancers that start in lymphatic or blood-forming tissue (*leukaemias*). Each type has a different prognosis for cure as well as mortality and survival rates. Moreover, various cancer

types respond best to different treatment regimes that can include surgery, radiotherapy, chemotherapy, photodynamic therapy or hormone therapy, either applied separately or in combination.

The most frequent types of cancer in humans include those affecting the lung and bronchus, the female breast and cervix, the prostate, the neck and head, the colon and rectum. According to the World Health Organisation cancer is the second most common noncommunicable disease leading to premature death, with lung cancer predominant among men and breast cancer among women (World Health Statistics, 2012). Figure 1.1 shows the ten most commonly diagnosed cancers in Ireland between 2008-2010 (National Cancer Registry, 2013). New cancer cases are also increasing in Ireland, with total number of cases in 2010 being 56 % higher than in the mid 1990s. The most frequently diagnosed invasive forms of cancer in Ireland are breast and prostate cancer which represent almost one third of all cancers diagnosed in women and men respectively (National Cancer Registry, 2013).

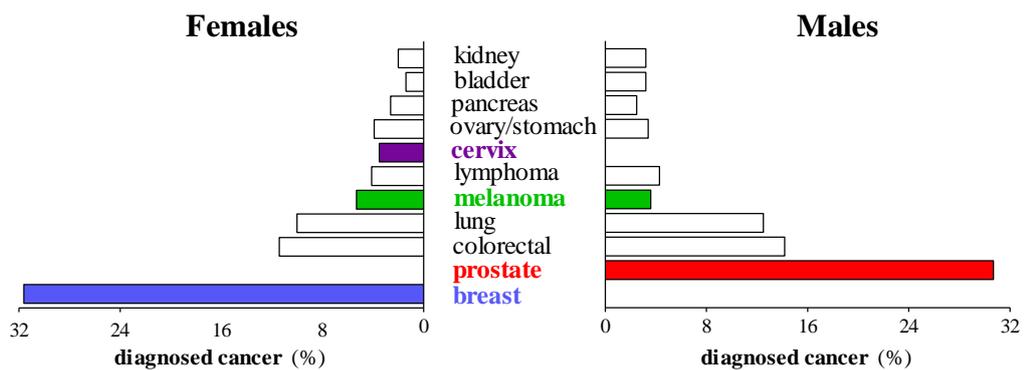


Figure 1.1. **Relative incidence of the main cancers diagnosed in Ireland between 2008-2010.** Data are taken from the *Cancer in Ireland 2013* report published by the National Cancer Registry, Ireland (p. 4). Highlighted are the cancer types from which cell lines were used in this study to test the role of hypoxia-inducible factor-1 α in oxygen-dependent radio- and chemosensitivity.

Tumour biology

Tumour vasculature

Over 90% of all human cancers are solid tumours in which excessive growth begins from a single cell that has accumulated several critical mutations. The newly developing tumour eventually reaches a size that is limited by availability of nutrients, so that further progression depends on the formation of new blood vessels (angiogenesis) to provide oxygen and nutrients, and to remove catabolites from the tumour mass. The physiology and microenvironment of solid tumours differ significantly from those of normal tissue, mainly due to dissimilar arrangement of their blood vessels. Whereas normal tissue has relatively regular, well-ordered blood vessels that are organized in a way to adequately oxygenate surrounding tissue, the blood vessels of solid tumours show structural and functional abnormalities. They are often dilated, tortuous and highly irregular and include arteriovenous shunts, temporary occlusions and blind ends (Brown & Giaccia, 1998). Consequently the tumour vasculature is structurally defective and shows chaotic and heterogeneous blood flow (Vaupel, 2004). This leads to reduced oxygen delivery to many tumour cells that in turn causes development of hypoxic or even necrotic areas within the tumour (Fig. 1.2). Reduced oxygen availability is one of the main regulatory factors that contributes to progressive growth and increased migration elsewhere in the body (metastasis), and is also associated with poor prognosis and resistance to anticancer therapies.

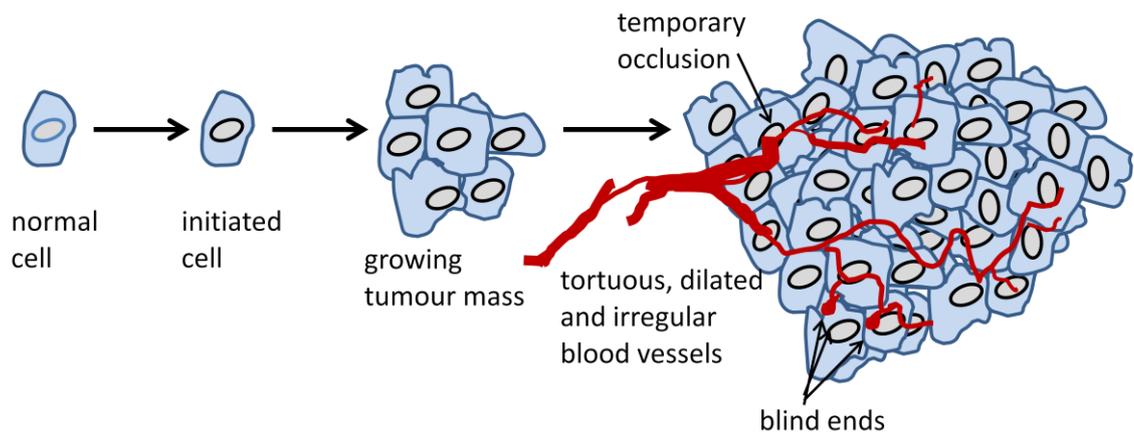


Figure 1.2. **Main stages of tumour development.** *Growth of a new solid tumour begins when a single initiated cell undergoes uncontrolled divisions. After reaching a size where further growth is limited by the availability of oxygen and nutrients the tumour mass is forced to develop its own vascular system. By contrast with normal tissues tumour blood vessels are irregular, dilated, have temporary occlusions and blind ends.*

Oxygenation status of tumours

Molecular oxygen (dioxygen) is indispensable for survival of all aerobic organisms. The latter have an ability to maintain cellular and tissue oxygen homeostasis, and respond to reduced oxygen conditions by activating signaling pathways which regulate proliferation, angiogenesis or programmed cell death. Such responses depend on oxygen-sensing mechanisms that are essential for development and normal functioning of the body organs, but may also contribute to growth and metastasis of tumours.

Inhaled air normally includes 21% O₂, which corresponds to a partial pressure of ~150 mmHg at sea level. Partial pressure of oxygen in the alveoli (~100 mmHg) falls progressively through arterial blood, capillaries and cells, and reaches a minimum (~9

mmHg) in mitochondria (Law & Bukwirwa, 1999). In normal tissues P_{O_2} ranges between 40-60 mmHg but in tumour tissue as a result of severe structural defects of tumour vessels and disturbed microcirculation, oxygen delivery is reduced. Therefore most human solid tumours larger than 1 mm^3 in volume contain a substantial fraction of cells that are hypoxic (Fig. 1.3), with a median P_{O_2} less than 10 mmHg (1.4% O_2 , Brown, 2002). These include advanced breast cancer (Vaupel *et al.*, 1991; Tomes *et al.*, 2003), cervical cancer (Hoeckel *et al.*, 1999; Vaupel *et al.*, 2001), head and neck carcinoma (Nordsmark *et al.*, 1996; Urtasun *et al.*, 1996; Brizel *et al.*, 1997), squamous carcinoma (Evans *et al.*, 2000), brain tumours (Rampling *et al.*, 1994), small-cell lung cancer (Urtasun *et al.*, 1996), prostate cancer (Movsas *et al.*, 1999) and melanoma (Lartigau *et al.*, 1997).

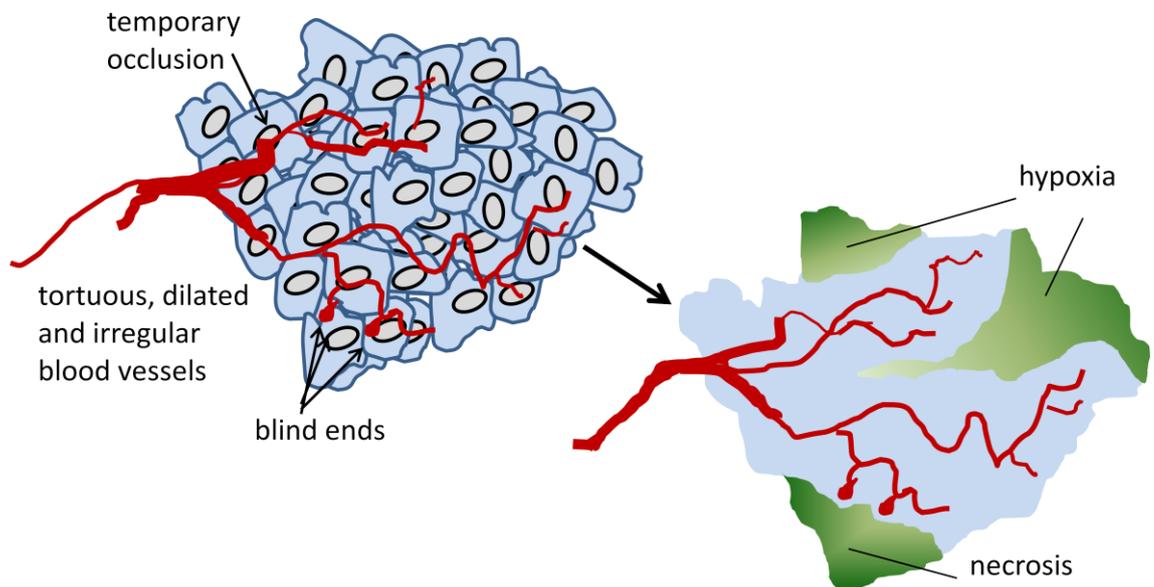


Figure 1.3. **A consequence of irregular vasculature in solid tumours.** *As rate of oxygen consumption begins to exceed delivery, relative hypoxia develops. If this hypoxia becomes extreme it leads to cell death through necrosis.*

Changes in blood flow cause the pattern of hypoxia in solid tumors to be highly dynamic but two main types of oxygen deficiency in tumours can be distinguished. Low oxygenation status of solid tumours at a micro level results from diffusion-limited oxygen delivery to cells when distance from functional blood vessels is between 70 - 150 μm (*chronic hypoxia*), first observed by Thomlinson and Gray (1955) in human lung carcinoma. In addition oxygen deficiency occurs at macro level in solid tumours due to temporarily slowed or stopped blood flow through a vessel (*transient* or *acute hypoxia*) which when reopened leads to reoxygenation of hypoxic tissue. This acute type of hypoxia was first demonstrated by Brown (1979) in mouse tumours then confirmed by others (Chaplin *et al.*, 1986; Trotter *et al.*, 1989; Hill *et al.*, 1996; Kimura *et al.*, 1996).

Measurement and modeling of tumour hypoxia

Oxygen deficiency has a profound effect on tumour physiology and biology (described later) and can be studied either at the level of the whole organism (*in vivo*) or in *in vitro* models based on cell culture. Oxygenation status in solid tumours can be measured using either invasive or noninvasive methods (Chapman, 1991; Hoeckel & Vaupel, 2001; Krohn *et al.*, 2008). The standard invasive technique by which intratumour hypoxia can be measured uses an oxygen-sensitive electrode mounted on a stiff needle which is then advanced automatically through the tissue (Schneiderman & Goldstick, 1978; Kallinowski *et al.*, 1990; Kavanagh *et al.*, 1996). However this technique is limited to accessible tumours, for example those of the cervix (Hoeckel *et al.*, 1991; Heockel *et al.*, 1993; Fyles *et al.*, 1998), neck and head (Brizel *et al.*, 1997) or breast (Okunieff *et al.*, 1994).

Tumour oxygenation status can also be detected by imaging the relative distribution of deoxygenated and oxygenated forms of haemoglobin (Blood Oxygen Level Dependent (BOLD) Magnetic Resonance Imaging (MRI; Al-Hallaq *et al.*, 1998; Jiang *et al.*, 2004)). Alternatively, dyes that emit phosphorescence quenched by oxygen can be used for imaging P_{O_2} in tumours (Wilson & Cerniglia, 1992; Hartmann *et al.*, 2006; Zhang *et al.*, 2010). Application of hypoxia-imaging agents such as misonidazole (Urtasun *et al.*, 1986; Gross *et al.*, 1995), fluoromisonidazole (Dubois *et al.*, 2004; Matsumoto *et al.*, 2007), pimonidazole (Olive *et al.*, 2000; Shin *et al.*, 2007) or hexafluorobenzene (Zhao *et al.*, 2003) which selectively bind to hypoxic cells have also been used extensively to measure tumour hypoxia. Non-invasive methods are also used to examine tumour blood flow, for example by application of fluorescent dyes (Hoechst 33342, DiOC₇). Both agents bind to cells adjacent to the blood vessels and undergo fluorescence changes that make it possible to visualise temporarily occluded blood vessels (Chaplin *et al.*, 1986; Trotter *et al.* 1989, 1990), indirectly reflecting acute tumour hypoxia.

Although chronic and transient hypoxia can be measured in tumours it may be difficult to identify and independently investigate the various effects in a living whole organism. To simplify this complexity various *in vitro* models have been developed in order to study cellular response to oxygen deficiency. Most commonly used are cell culture models in environmentally controlled chambers or whole incubators in which oxygen concentration in the gas phase is controlled, typically at constant level. In general *in vitro* models of cellular hypoxia can be categorized according to severity and duration of oxygen deprivation. These range from moderate ($\pm 1\% O_2$) to extreme ($<0.1\% O_2$) hypoxia, imposed for 1 h or less (Madesh *et al.*, 1999; Vordermark *et al.*,

2004) to several days (Yoshida *et al.*, 2009; Qiu *et al.*, 2011). Normally severe hypoxia is used for short insults or less extreme hypoxia over longer periods. A further approximation to *in vivo* conditions is to impose several short periods of hypoxia separated by intervals of reoxygenation (*cycling hypoxia*; Hsieh *et al.*, 2010).

Biological consequences of hypoxia

Oxygen deficiency occurring in solid tumours either acutely or chronically has important biological consequences. Low cellular oxygen concentration modulates signaling pathways controlling not only oxygen-dependent energy metabolism but also cell proliferation, cell cycle arrest and cell death (apoptosis or necrosis). It is also associated with promoting malignant tumour progression and metastasis. Furthermore oxygen deficiency increases the resistance of solid tumours to ionizing radiation or chemotherapy by direct or indirect mechanisms. In considering these effects it is important to distinguish the type of hypoxia involved because various durations and levels of oxygen deprivation differently regulate intracellular pathways as well as tumour development.

The fundamental biological role of oxygen is to participate in cell energy metabolism. In normal tissue or well oxygenated tumour tissue oxygen delivery is sufficient to support cell respiration and ATP production through oxidative phosphorylation in mitochondria. When supply of O₂ as terminal electron acceptor becomes limited cells switch to anaerobic metabolism, synthesising ATP through glycolysis. Because glycolysis is less efficient than oxidative phosphorylation (2 ATP *vs.* 38 ATP molecules generated respectively from 1 glucose molecule) a depletion of ATP levels and compensatory increase in glucose uptake is observed when cells become

hypoxic. As a consequence of intensive glycolysis, production of the end product lactic acid increases and acidifies the microenvironment of hypoxic cancer cells (Nathan & Singer, 1999). This acidosis in the tumour microenvironment has been associated with promoting tumour growth and metastasis (Estrella *et al.*, 2013).

The cell division cycle which consists of four distinct phases (G_1 , S, G_2 , mitosis) can also be affected by cellular oxygen deprivation. The transition between phases is regulated by activation of cyclin-dependent kinase (CDK)-cyclin complexes specific to the different points of the cell cycle (Morgan, 1995). In addition CDK can be regulated by cell cycle inhibitory proteins which bind to either CDK alone or CDK-cyclin complexes and inhibit their activity (Harper *et al.*, 1995; Sherr & Roberts, 1995). Most often observed is hypoxia-associated G_1 /S-phase arrest. Low oxygen concentration induces activation of the G_1 checkpoint in p53 wild-type cells, resulting in G_1 phase blockade (Zoelzer & Streffer, 2002). p53 is a tumour suppressor protein and transcription factor which can induce expression of various proteins that bind to CDK complexes and regulate the cell cycle (Shaw, 1996). In contrast, p53-independent retinoblastoma protein (RB) mediated cell cycle arrest in late G_1 phase is induced by less severe hypoxia (0.1-0.5% O_2) and attributed to decreased activity of CDK-cyclin complexes which control phosphorylation of RB needed for G_1 /S-phase transition (Krtolica *et al.*, 1998, 1999; Gardner *et al.*, 2001). Furthermore if under hypoxia the cells can enter S phase, inadequate ATP levels for support of DNA synthesis result in S phase arrest (Amellem *et al.*, 1994). Similarly p53 mutant cells, in which G_1 checkpoint control by p53 protein is lost, are able to enter but not complete the S phase due to energy depletion induced by hypoxia (Zoelzer & Streffer, 2002). In addition reoxygenation following prolonged extreme hypoxia (<0.0004% O_2) causes a

resumption in cell cycle progression, but only the subpopulation of cells arrested in G₁ phase during oxygen deficiency were able to complete the cell cycle whereas others blocked in S-phase were permanently arrested in G₂ after reoxygenation (Amellem & Pettersen, 1993; Koritzinsky *et al.*, 2001).

Besides the effects on energy metabolism and cell cycle progression, reduced oxygen concentration can induce a cascade of events leading to apoptotic cell death. The programmed cell death pathway (apoptosis) caused by a wide range of extra- and intracellular cellular signals is an energy-dependent signaling process which involves activation of a set of cysteine proteases (caspases) during a complex cascade of molecular events (reviewed in Rastogi *et al.*, 2009). The effect of reduced oxygen concentration on apoptosis is complex as it can either promote or inhibit the process. In general, apoptotic activity is unchanged following exposure of tumour cells to moderate hypoxia (0.3-1% O₂). However extreme hypoxia (<0.005% O₂) causes a significant increase in apoptosis (Acs *et al.*, 2004), which for less severe oxygen depletion (<0.1% O₂) may be cell-type specific (Weinmann *et al.*, 2004). Mechanisms by which oxygen deficiency can cause apoptosis involve hypoxia-induced translocation of pro-apoptotic protein Bax from the cytosol to mitochondria (Saikumar *et al.*, 1998) and decreased mitochondrial membrane potential (McClintock *et al.*, 2002) which in turn causes release of cytochrome c and caspase activation. Reactive oxygen species (ROS)-dependent caspase activation by hypoxia (0.5% O₂) was also found to induce apoptosis (Kim & Park, 2003). In addition, hypoxic stress (< 0.2% O₂) induces accumulation of p53 protein (Koumenis *et al.*, 2001) which may lead to activation of apoptosis through Apaf-1 and caspase-9 as downstream effectors (Soengas *et al.*, 1999). Conversely, oxygen deficiency may also induce cell resistance to apoptosis by promoting adaptation

to the stress conditions and thereby enhancing cell survival. Hypoxia (<0.1% O₂) was reported to reduce levels of pro-apoptotic Bax and Bad proteins in tumour cells (Erler *et al.*, 2004). In addition hypoxia (1% O₂) inhibited tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by blocking translocation of Bax from the cytosol to mitochondria and subsequent cytochrome c release (Kim *et al.*, 2004). Moreover increase of the pro-apoptotic protein Bcl-X_L protected tumour cells from hypoxia-induced apoptosis (Santore *et al.*, 2002). Hypoxia was also reported to increase apoptosis inhibitory protein-2 (IAP-2) levels which indirectly suppressed caspase activation (Dong *et al.*, 2001). In summary, inadequate oxygen delivery to hypoxic tumour cells can contribute to either cell death or cellular adaptation and survival. A balance exists between the factors inducing these opposite effects in cells, so when disturbed by reduced oxygen tension may drive either cell death or survival (Greijer & van der Wall, 2004).

Oxygen deprivation also induces activation of signaling cascades such as mitogen activated protein kinases (MAPKs) pathway. This family of protein kinases with diverse biological functions include extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2, also known as p42/p44 MAPKs) and stress-activated protein kinases (SAPKs), p38 MAPKs and c-Jun N-terminal kinases (JNKs; Karin, 1998; Garrington & Johnson, 1999). In general the Ras-Raf-MEK-ERK1/2 pathway responds to factors involved in cell proliferation, survival or differentiation, whereas the JNK/SAPK and p38 pathways regulate responses to cellular stresses or inflammatory cytokines (Roux & Blenis, 2004; Koul *et al.*, 2013). Hypoxia-induced activation of ERK1/2 has been reported in various cell lines and suggested to contribute to angiogenesis (Berra *et al.*, 2000), cell migration (Lester *et al.*, 2005), keloid formation

(Zhang *et al.*, 2006), increasing cell-cell or cell-extracellular matrix adhesion (Blaschke *et al.*, 2002) and apoptosis resistance (Liu *et al.*, 2010a). In addition to common cellular stress factors such as heat injury, ionizing radiation or osmotic shock, hypoxia has also been reported to activate JNK/SAPK and p38 stress kinases (Laderoute *et al.*, 1999). That increase was further associated with hypoxia-induced apoptosis of human melanoma cells (Kunz *et al.*, 2001), expression of interleukin 8 in human ovarian carcinoma cells (Xu *et al.*, 2004) or increased survival and invasiveness of prostate cancer cells (Khandrika *et al.*, 2009). Downstream targets for these signaling cascades include transcription factors such as activator protein 1 (AP-1), cAMP response element binding protein (CREB) and Elk-1, expression of which has also been shown to be induced by hypoxia (Bandyopadhyay *et al.*, 1995; Meyuhas *et al.*, 2008). In general AP-1 and CREB are involved in cellular processes such as transformation, apoptosis, proliferation and differentiation (Angel & Karin, 1991; Andreucci *et al.*, 2002; Sakamoto & Frank, 2009). In addition CREB was reported to play a role in immune response (Wen *et al.*, 2010). Another transcription factor induced under hypoxia is nuclear factor kappa B (NF κ B; Koong *et al.*, 1994). NF κ B proteins are mainly regulators of immune, inflammatory, stress and apoptotic responses of cells to various stimuli (Campbell & Perkins, 2006; Vallabhapurapu & Karin, 2009). However most changes in gene expression under hypoxic conditions are mediated by hypoxia-inducible factor-1 (HIF-1) which is commonly present in tumours (Zhong *et al.*, 1999). In general HIF-1 regulates expression of various target genes related to cell energy metabolism, apoptosis, survival etc. (described later). In addition, multiple transcription factors may cooperate to activate specific target genes (Michiels *et al.*, 2001; Kunz *et al.*, 2002; Brant & Fabisiak, 2013; Nakayama, 2013).

When cells adapt to hypoxic conditions they can acquire metastatic potential (Sutherland 1998; Cairns & Hill 2004). For example short exposure of prostate cancer cells to hypoxia (1% O₂) was reported to increase their invasive capacity as well as survival (Dai *et al.*, 2011). If such hypoxic cells which do not experience diminution of their invasive potential are part of the tumour then metastasis is more likely to develop. Hypoxia-induced metastasis was associated with increased expression of vascular endothelial growth factor (VEGF) in human melanoma cells (Rofstad & Danielsen, 1999). Recently HIF-induced transcriptional activation of genes encoding angiopoietin-like protein 4 (Ang-4), L1 cell adhesion molecules and lysyl oxidase family members which promote invasion and metastatic niche formation has been suggested to mediate breast cancer metastasis (Semenza, 2012).

Finally, oxygen deficiency is known to impair tumour response to radio- and chemotherapy. Therefore there is considerable interest in understanding the molecular and signaling mechanisms that contribute to this resistance, as potential targets to improve the clinical effectiveness of anticancer treatments.

Hypoxia Inducible Factor (HIF) pathway

One of many responses of mammalian cells to reduced oxygen level is stabilisation and accumulation of hypoxia-inducible factors (HIFs). These transcriptional activators mediate cell adaptation to hypoxia by binding to specific regulatory sequences of target genes and thereby controlling their expression. HIFs are heterodimers consisting of an α -subunit (HIF-1 α , HIF-2 α or HIF-3 α) the availability of which is oxygen-dependent, and a stable β -subunit (HIF-1 β) which is constitutively present.

All three HIF- α subunits have similar domain structure and mechanisms of activation, translocation into the nucleus after stabilisation, heterodimerisation with the β -subunit and binding to the HRE of target genes (reviewed in Zagorska & Dulak, 2004). HIF-1 α and HIF-2 α subunits share 48% total amino acid (aa) sequence identity (Ema *et al.*, 1997; Tian *et al.*, 1997), whereas the N-terminal bHLH-PAS domain of HIF-3 α (described later) shares 57 and 53% aa sequence identity with that of HIF-1 α and HIF-2 α respectively (Gu *et al.*, 1998). Both HIF-1 α and HIF-2 α contain two transactivation domains (TADs) but show only partly overlapping gene specificity, due to differences in one of their TAD domains (Hu *et al.*, 2007). C-terminal TAD is homologous between the subunits and promotes expression of their common target genes, whereas distinct N-terminal TAD sequences confer target gene specificity on the two isoforms (Wang *et al.*, 2005; Dayan *et al.*, 2006). HIF-1 α and HIF-2 α also have distinct expression patterns. While HIF-1 α is ubiquitously present, HIF-2 α expression is mainly limited to endothelium, kidney, heart, lung and small intestine (Gordan *et al.*, 2007). Moreover HIF-1 α expression was correlated with initial stages of tumour

development whereas HIF-2 α was associated with later more advanced stages, promoting metastasis and development of a more aggressive phenotype (Raval *et al.*, 2005; Holmquist-Mengelbier *et al.*, 2006). In some cases HIF-1 α and HIF-2 α may also play opposing regulatory roles. For example HIF-1 α antagonises c-Myc function whereas HIF-2 α enhances it (Gordan *et al.*, 2007). HIF- α isoform-specific transcriptional selectivity was also reported to cause opposite effects on tumour xenografts, with HIF-1 α slowing and HIF-2 α promoting growth (Raval *et al.*, 2005). These authors also reported that the two α -isoforms reciprocally influence one other's expression *in vitro*. Moreover although hypoxia-induced stabilisation is common to both HIF-1 α and HIF-2 α , the severity and duration of hypoxia can regulate the two subunits differently. For example in lung cancer cells acute hypoxia induced HIF-1 α and HIF-2 α protein (0.5% O₂ x 4 h) but more prolonged insults (0.5% O₂ x 12 h) preferentially stabilised HIF-2 α (Uchida *et al.*, 2004). Hypoxia-induced relative stabilisation of HIF-1 α and HIF-2 α is also cell type specific (Bracken *et al.*, 2006; Yu & Hales, 2011).

Although HIF-1 α is the most extensively studied isoform, and the role of HIF-2 α has also been investigated more recently, significantly less is known about HIF-3 α . However expression of HIF-3 α in kidney (Hara *et al.*, 2001) as well as in vascular cells (Augstein *et al.*, 2011) decreased expression of HIF-1/HIF-2 target genes, suggesting that HIF-3 α may act as a negative regulator of the HIF pathway. So far however the roles of HIF-1 α and HIF-2 α have been studied far more extensively than that of HIF-3 α in ischaemic and cancer biology or as potential targets to improve the clinical effectiveness of cancer treatments.

Discovery and structure of HIF- α

As outlined already, hypoxia-inducible factor 1 (HIF-1) is a key transcriptional factor that regulates cellular response to hypoxia. A major consequence of severe tissue hypoxia is the up-regulation of erythropoietin (EPO) gene expression (Goldberg *et al.*, 1987; Semenza *et al.*, 1990) that results in erythropoiesis and thereby increases the O₂-carrying capacity of blood. Before HIF-1 was discovered, the hypoxia response element (HRE) had been localised in the 3'-enhancer region of the EPO gene (Beck *et al.*, 1993). Subsequently it was shown that a hypoxia-inducible enhancer binding site 1 (HIE-BS1) sequence located within this 3'-enhancer region is the target for a nuclear transcriptional factor induced in response to reduced oxygen tension via *de novo* protein synthesis (Semenza & Wang, 1992). Further analysis confirmed general involvement of HIF-1 in regulation of gene transcription in response to hypoxia in various mammalian cell types, including those in which the EPO gene is not expressed (Wang & Semenza, 1993). Finally purification by DNA affinity chromatography led to isolation and identification of encoding cDNA sequences showing that HIF-1 protein is a heterodimeric complex comprised of two subunits: 120kDa HIF-1 α and 91-94kDa HIF-1 β (Wang *et al.*, 1995). The HIF-1 α subunit was found to be a 826 amino acid (aa) protein hitherto unknown, whereas constitutively expressed HIF-1 β subunit (789 aa) is the product of the aryl hydrocarbon (Ah) nuclear translocator (ARNT) gene which was previously identified as a component of the DNA binding form of the Ah receptor (Hoffman *et al.*, 1991; Reyes *et al.*, 1992). HIF-2 α (also named endothelial PAS protein, HIF-like factor, HIF-related factor), discovered shortly after HIF-1 α was found to be a 874 aa polypeptide (Flamme *et al.*, 1997), whereas HIF-3 α is 662 aa residues long (Gu *et al.*, 1998).

Structural analysis of HIF- α and HIF-1 β showed that both subunits contain a basic helix-loop-helix (bHLH) domain and two PER-ARNT-SIM (PAS) domains (A and B) in their N-terminal region (Wang *et al.*, 1995; Ema *et al.*, 1997; Gu *et al.*, 1998). These domains together mediate the protein-protein heterodimer formation that is required for HIF-1 binding to DNA (Jiang *et al.*, 1996). Further toward their C-terminus HIF-1 α and HIF-2 α contain two transactivation domains (TADs), separated by an inhibitory domain (ID) (Jiang *et al.*, 1997; Pugh *et al.*, 1997; O'Rourke *et al.*, 1999). N-terminal TAD overlaps with an oxygen-dependent degradation (ODD) domain required for HIF-1 α protein labelling for proteasomal degradation. HIF-3 α lacks the structures for transactivation found in the C-terminus of the other α -isoforms (Hara *et al.*, 2001). In addition, PEST-like motifs common to many proteins with short half-lives are common (31% of all amino acids) in the C-terminal part of the HIF-1 α molecule (Wang *et al.*, 1995). Moreover HIF-1 α contains two nuclear localisation signals, one near the C-terminus (C-NLS) of the protein and the other in its N-terminal region (N-NLS; Kallio *et al.*, 1998). By contrast, the HIF-1 β subunit contains only one TAD in its C-terminal region. Figure 1.4 shows the domain structure of HIF- α isoforms and HIF-1 β , together with specific hydroxylation, acetylation and phosphorylation sites (described later).

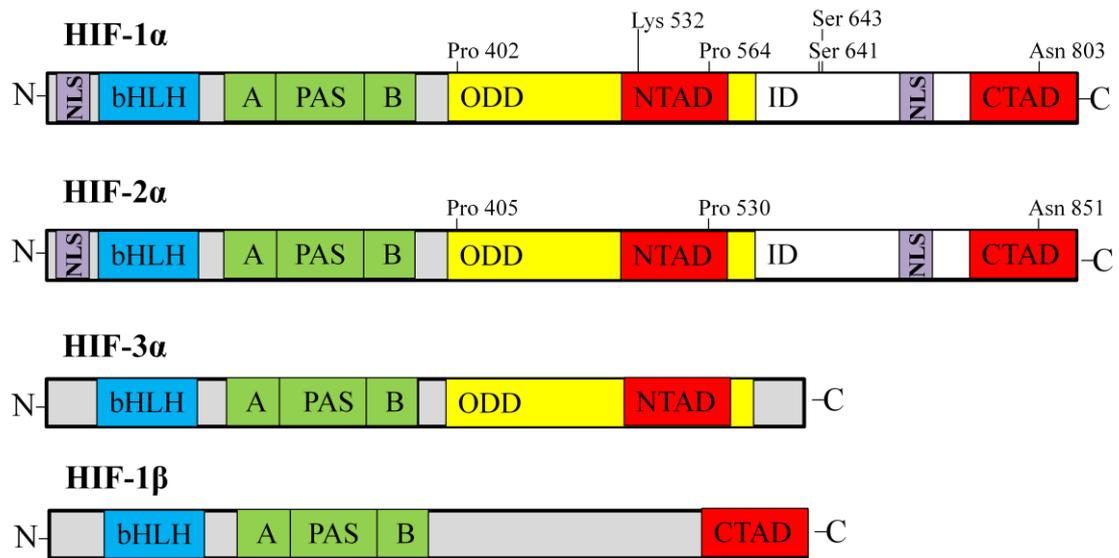


Figure 1.4. **Structures of human HIF- α isoforms and the HIF-1 β subunit.** Both α and β subunits contain basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) A and B domains. HIF- α isoforms also contain an oxygen-dependent degradation (ODD) domain, transactivation domains (NTAD and CTAD; NTAD only in HIF-3 α), nuclear localisation signal (NLS) domains and inhibitory domain (ID) whereas HIF-1 β has only one transactivation domain (CTAD). Hydroxylation, acetylation and phosphorylation sites are also indicated. Abbreviations: Pro – proline, Lys – lysine, Asn – asparagine, Ser – serine, HIF – hypoxia inducible factor.

HIF-1 α regulation through post-translational modification

Post-translational modifications including hydroxylation, acetylation and phosphorylation control HIF-1 α protein stabilisation and regulate its subsequent transcriptional activity, as outlined below.

Hydroxylation

Both HIF-1 α and HIF-1 β mRNA are constitutively present in most human tissues cell lines, independent of oxygen availability (Gradin *et al.*, 1996). However the protein levels of HIF-1 α/β are differently regulated by oxygen tension. HIF-1 β protein levels are sustained (Kallio *et al.*, 1997) under aerobic conditions whereas levels of HIF-1 α protein range from low (Huang *et al.* 1996; Kallio *et al.* 1997; Formento *et al.* 2005) to undetectable (Wang *et al.*, 1995; Ioannou *et al.*, 2010). In the presence of oxygen specific proline residues (Pro402 and Pro564 for HIF-1 α , Pro405 and Pro530 for HIF-2 α , Pro492 for HIF-3 α) located within the ODD domain are hydroxylated by HIF prolyl hydroxylase domain (PHD) enzymes (Epstein *et al.*, 2001). PHD enzymes belong to a family of dioxygenases which require molecular O₂ and 2-oxoglutarate as co-substrates, and iron (Fe²⁺) and ascorbate as cofactors (Schofield & Zhang, 1999; Bruick & McKnight, 2001; McNeill *et al.*, 2002; Masson & Ratcliffe, 2003). Once hydroxylated HIF-1 α can bind to the β -domain of von-Hippel-Lindau (VHL) tumour suppressor protein (Maxwell *et al.*, 1999; Bonicalzi *et al.*, 2001), part of the elongin C/elongin B/cullin-2 E3 ubiquitin ligase complex, leading to its degradation by the 26S proteasome (Cockman *et al.*, 2000; Kamura *et al.*, 2000; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). Since PHD enzymes require the presence of oxygen, 2-oxoglutarate, Fe²⁺ and

ascorbate for full activity, reduced availability of any of these limits HIF-1 α hydroxylation (Knowles *et al.*, 2003; Page *et al.*, 2008). Consequently HIF-1 α is stabilised under hypoxic conditions. Conversely, hypoxia-induced SUMOylation opposes this effect by promoting HIF-1 α binding to VHL protein, enhancing ubiquitination and degradation via a proline hydroxylation-independent mechanism (Cheng *et al.*, 2007).

HIF- α is also regulated by oxygen-dependent hydroxylation of an asparagine residue (Asn803 for HIF-1 α , Asn851 for HIF-2 α) within the CTAD domain (Ruas *et al.*, 2002). This reaction is catalysed by factor inhibiting HIF-1 (FIH-1) enzyme, which like PHDs belongs to the family of 2-oxoglutarate and iron dependent dioxygenases which use oxygen as a co-substrate (Hewitson *et al.*, 2002). In the presence of oxygen Asn hydroxylation prevents HIF- α from associating with p300/Creb-binding protein (CBP) coactivator (Kallio *et al.*, 1998; Ema *et al.*, 1999; Sang *et al.*, 2002). As a consequence, recruitment of accessory transcriptional coactivators including steroid receptor coactivator (SRC-1) and transcription intermediary factor 2 (TIF-2) by transactivation domains is blocked (Carrero *et al.*, 2000), thereby inhibiting HIF-1 α transcriptional activity and regulation of its target genes (Lando *et al.*, 2002). Under hypoxia hydroxylation by FIH-1 is suppressed, allowing recruitment of co-activators and formation of the transcriptional complex (Lando *et al.*, 2002). Collectively, decreased oxygen availability limits the catalytic functions of both PHD and FIH-1 enzymes, leading to HIF- α stabilization, accumulation and finally translocation from the cytoplasm into the nucleus where the protein dimerizes with HIF-1 β (Chilov *et al.*, 1999). In this way active HIF-1 complexes can bind to HREs of target genes and modulate their transcription. HIF-1 binding to DNA is through a mandatory core

sequence 5'-(A/G)CGTG-3' (Semenza *et al.*, 1996) located in either the enhancer (5'- or 3'-flanking) or promoter regions of HREs of target genes (reviewed in Maxwell *et al.*, 2001; Zagorska & Dulak, 2004). Although HIF-1 α stabilization is hypoxia-dependent its nuclear translocation is not, as the latter occurs even when HIF-1 α is stabilised under normoxic conditions (Hofer *et al.*, 2001).

Acetylation

HIF-1 α protein stability and transcriptional activity are also regulated to a lesser extent by other post-translational modifications influenced by oxygen availability. These include acetylation of a lysine residue (Lys532) within the ODD domain by an arrest-defective-1 (ARD1) acetyltransferase, which promotes destabilisation of HIF-1 α by enhancing its interaction with pVHL (Jeong *et al.*, 2002). ARD1 may act on HIF-1 α only in the cell cytosol as the enzyme appears to be absent from the nucleus (Jeong *et al.*, 2002). Lys532 substitution with arginine causes stabilisation of HIF-1 α under normoxia, confirming the critical role of this residue in HIF-1 α regulation (Tanimoto *et al.*, 2000). However hypoxia reduces both ARD1 mRNA and protein levels, contributing to HIF-1 α stabilisation (Jeong *et al.*, 2002). Acetylation of HIF-2 α by sirtuin 1, a redox-sensing deacetylase, at lysines 385, 685, 741 has been correlated with increased transcriptional activity during hypoxia (Dioum *et al.*, 2009). Figure 1.5 shows a summary of oxygen-dependent HIF-1 α regulation.

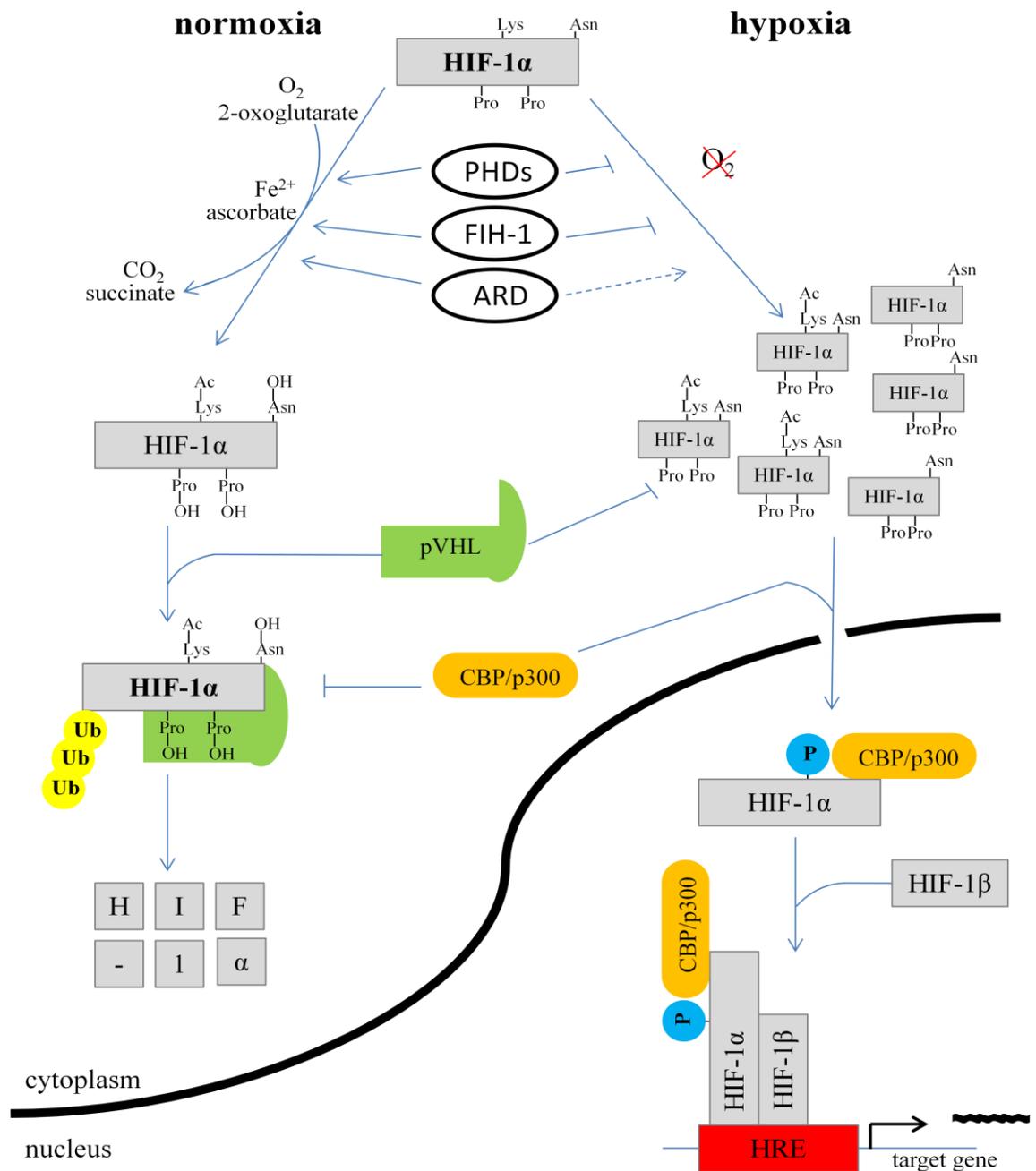


Figure 1.5. **Oxygen-dependent regulation of HIF-1 α .** Under normoxia HIF-1 α undergoes hydroxylation which targets it for proteasomal degradation. Under hypoxia PHD and FIH-1 activity are blocked so stabilised HIF-1 α protein translocates into the nucleus where it dimerizes with HIF-1 β , then binds CBP/p300 coactivator before interacting with HRE motifs to upregulate target genes. Acetylation of HIF-1 α by ARD is less oxygen-dependent and enhances the protein destabilisation.

Phosphorylation

A third post-translational modification that contributes to HIF-1 α regulation is its phosphorylation by MAPK (Richard *et al.*, 1999; Sodhi *et al.*, 2001; Kwon *et al.*, 2005). In contrast to hydroxylation and acetylation which both destabilise HIF-1 α , phosphorylation by p42/p44 MAPK has no effect on HIF-1 α stability or DNA binding activity (Hofer *et al.*, 2001; Hur *et al.*, 2001) yet enhances its transcriptional activity (Richard *et al.*, 1999). More detailed studies showed that MAPKs phosphorylate HIF-1 α on serine residues within the ID domain (Ser641 and Ser643; Mylonis *et al.*, 2006), retaining HIF-1 α in the nucleus (Mylonis *et al.*, 2008; Triantafyllou *et al.*, 2008). HIF-1 α can also be phosphorylated on serine 247 by casein kinase 1 which does not affect either stability or nuclear accumulation but impairs dimerisation with HIF-1 β (Kaloussi *et al.*, 2010).

The phosphatidylinositol-3-kinase (PI3K)/protein-serine-threonine-kinase (AKT) signalling pathway has also been implicated in regulating HIF-1 α protein level since pharmacological inhibition of PI3K activity attenuates HIF-1 α stabilisation by hypoxia (Mazure *et al.*, 1997; Zhong *et al.*, 2000; Gort *et al.*, 2006; Yang *et al.*, 2009). Non-hypoxic activation of HIF-1 α by Angiotensin II in vascular endothelial cells (Page *et al.*, 2002) and by mitochondrial respiratory complex III (Chandel *et al.*, 2000) both involve ROS activation of the PI3K/Akt pathway which in turn lead to HIF-1 α stabilisation. PI3K/Akt might also contribute to HIF-1 α stabilisation by promoting the expression of heat shock proteins (Hsp) which protect HIF-1 α from pVHL-independent protein degradation (Zhou *et al.*, 2004). However others reported that hypoxia-induced

stabilisation of HIF-1 α or expression of its target genes is independent of the PI3K/Akt pathway (Arsham *et al.*, 2002).

Other mechanisms of HIF-1 α regulation

Several other extra- or intracellular stimuli can regulate HIF-1 α stabilisation. These include free radicals of oxygen. ROS-dependent control is complex and often indirect, depending on the oxygenation status of cells as well as origin, type and concentration of ROS involved. For example H₂O₂ was shown to increase HIF-1 α levels in normoxic pulmonary artery smooth muscle cells (BelAiba *et al.*, 2004). The authors found that increased levels of superoxide dismutase (SOD) which promotes the dismutation of superoxide to H₂O₂ led to stabilisation of HIF-1 α , whereas this response was inhibited when H₂O₂ levels were reduced by catalase and glutathione peroxidase. In addition increased ROS were found to stabilise HIF-1 α under hypoxic conditions in some cancer cell lines (Chandel *et al.*, 2000; Guzy *et al.*, 2005). Conversely in other cell lines hypoxia-induced HIF-1 α stabilisation was found to be suppressed by increased ROS generation under similar conditions (Wartenberg *et al.*, 2003; Sibenaller *et al.*, 2014). Reactive nitrogen species also affect HIF-1 α regulation. At low concentrations NO inhibits HIF-1 α stabilization under hypoxic conditions (Huang *et al.*, 1999 ; Agani *et al.*, 2002 ; Hagen *et al.*, 2003) through inhibition of mitochondrial respiration (Mateo *et al.*, 2003), whereas at high concentration NO stabilizes HIF-1 α independently of oxygen concentration through mitochondria-independent pathways (Mateo *et al.*, 2003). On the basis that HIF-1 α accumulation was attenuated by antioxidants (N-acetyl-L-cysteine or ascorbic acid) NO-dependent HIF-1 α stabilization was also suggested to involve formation of free radicals (Quintero *et al.*, 2006). In addition post-translational

HIF-1 α S-nitrosylation has also been reported, via cysteine 800. This enhances recruitment of p300/CBP co-activator to the CTAD domain of HIF-1 α , causing an increase in its transcriptional activity (Yasinska & Sumbayev, 2003).

HIF-1 α function is also regulated by various growth factors and inflammatory cytokines. These include insulin (Zelzer *et al.*, 1998; Stiehl *et al.*, 2002; Biswas *et al.*, 2007), insulin-like growth factor (Zelzer *et al.*, 1998; Feldser *et al.*, 1999), epidermal growth factor (Zhong *et al.*, 2000), human epidermal growth factor receptor 2 (Laughner *et al.*, 2001), interleukin-1 β (Haddad, 2002; Stiehl *et al.*, 2002; Qian *et al.*, 2004) and tumour necrosis factor α (Haddad & Land, 2001). The mechanism involves activation of either PI3K/Akt or MAPK, or generation of ROS. Angiotensin II, thrombin and platelet-derived growth factor were also shown to increase HIF-1 α expression and HIF-1 transcription complex activation through intracellular ROS production (Richard *et al.*, 2000; Gorlach *et al.*, 2001; Page *et al.* 2002).

HIF regulation of gene expression

Many genes have been shown to be regulated by HIF-1, affecting various cell processes. For example expression of at least 2% of all genes in human pulmonary artery endothelial cells are controlled by HIF-1 (Manalo *et al.*, 2005). Since HIF-1 α is a key transcriptional regulator of cell adaptation to hypoxia, its target genes include those affecting (i) development and functioning of the vascular system (e.g vascular endothelial growth factor, VEGF and its receptors), (ii) erythropoiesis, (iii) energy metabolism (e.g. glucose transporters 1 and 3) and (iv) apoptosis. Since angiogenesis is essential for development of tissue and for organ regeneration, HIF-1 α plays an

essential role in normal vascular development during embryogenesis (reviewed in Weidemann & Johnson, 2008). However, angiogenesis is also involved in pathological processes including formation of tumour blood vessels necessary for tumour expansion. Several studies have confirmed the key role of HIF-1 α in tumour angiogenesis and progression (Maxwell *et al.*, 1997; Bos *et al.*, 2001; Jensen *et al.*, 2006; Said *et al.*, 2007). Furthermore to offset reduced oxygen transport under hypoxia, HIF-1 α induces expression of genes involved in erythropoiesis and iron-metabolism including erythropoietin (Semenza *et al.*, 1991) and transferrin (Rolfs *et al.*, 1997). Lower oxygen tension also causes cells to switch to glycolytic metabolism to meet their requirements for ATP. Thus HIF-1 α increases the expression of glycolytic enzymes and glucose transporters (Chen *et al.*, 2001; Marin-Hernandez *et al.*, 2009). Finally, HIF-1 α has been found to play both pro-apoptotic and anti-apoptotic roles. HIF-1 α expression can amplify hypoxia-induced apoptosis either by stabilizing the product of tumour suppressor gene p53 or by increasing the expression of Bcl-2 binding proteins BNIP3 and NIX and thereby inhibiting the anti-apoptotic effect of Bcl-2 (reviewed in Greijer & van der Wall, 2004). However, HIF-1 α may also play a role in prevention of apoptosis induced by hypoxia or glucose deprivation in pancreatic cancer cells (Akakura *et al.*, 2001), and apoptosis caused by *tert*-butyl hydroperoxide-induced oxidative stress and serum deprivation in HepG2 cells (Piret *et al.*, 2004).

Apart from those genes involved in cancer cell survival and adaptation to hypoxia, HIF-1 α -regulated genes also participate in cell differentiation. Hypoxia promotes arrest of tumour cells in an undifferentiated state and thereby maintains their aggressive tumorigenic potential (reviewed in Kim *et al.*, 2009). Nuclear accumulation of HIF-1 α has been correlated with poorly differentiated pancreatic carcinomas

(Couvelard *et al.*, 2005). Furthermore hypoxia induced HIF-1 expression contributed to development of osteolytic bone metastases in breast cancer by suppressing osteoblast differentiation (Hiraga *et al.*, 2007). HIF-1 has also been found to play a role in inhibiting adipogenic differentiation (Yun *et al.*, 2002; Lin *et al.*, 2006). By contrast HIF-1 α deletion impaired neuronal stem cell proliferation, differentiation and maturation (Mazumdar *et al.*, 2010).

Other genes up-regulated by HIF-1 α include participants in immune reactions (Hellwig-Burgel *et al.*, 2005), neuroprotection (Lopez-Hernandez *et al.*, 2012), drug resistance (Comerford *et al.*, 2002), genetic instability (Koshiji *et al.*, 2004) and pH regulation (Wykoff *et al.*, 2000). Although relatively less is known about down-regulation of genes by HIF-1 α , hypoxia-induced HIF-1 α was reported to suppress SOD expression directly and thereby contribute to ROS accumulation (Gao *et al.*, 2013). Proteomic analysis of hypoxic cells also showed down-regulation of several genes by HIF-1 α (Greijer *et al.*, 2005).

Pharmacological inhibition of HIF-1 α

Most of the regulatory mechanisms outlined above either directly or indirectly cause stabilisation of HIF-1 α . Given that increased HIF-1 α level leads to activation of survival pathways and subsequent tumour progression, inhibition of its expression or/and transcriptional activity has been suggested as a potential way to improve the outcome of cancer therapies (Onnis *et al.*, 2009). HIF-1 α inhibitors can be grouped according to their mechanism of action. These include agents affecting HIF-1 α mRNA expression, HIF-1 α protein translation, HIF-1 α protein degradation, HIF-1 DNA binding and transcriptional activity. Agents such as EZN-2698 (Greenberger *et al.*,

2008) and aminoflavone (Terzuoli *et al.*, 2010) have been reported to decrease HIF-1 α mRNA level both in tumour cells and xenografts. Drugs that reduce HIF-1 α protein synthesis include inhibitors of topoisomerase I and II (e.g. topotecan (Rapisarda *et al.*, 2004); tricyclic carboxamide (Creighton-Gutteridge *et al.*, 2007)), the mTOR pathway (Majumder *et al.*, 2004; Bufalo *et al.*, 2006) or receptor tyrosine kinase (Luwor *et al.*, 2005; Nilsson *et al.*, 2010). Inhibition of HIF-1 α protein translation by cardiac glycosides has also been reported (Zhang *et al.*, 2008) whereas PX-478 acts at several levels by inhibiting mRNA expression and translation as well as promoting protein ubiquitination (Koh *et al.*, 2008). Another mechanism of HIF-1 α inhibition involves the regulation of protein degradation. For example berberine-induced acetylation of HIF-1 α was reported to trigger its proteasomal proteolysis (Lin *et al.*, 2004). Curcumin-induced proteasomal degradation of HIF-1 β was reported to contribute to inactivation of HIF-1 (Choi *et al.*, 2006). Hsp90 inhibitors such as geldanamycin also cause HIF-1 α to undergo VHL-independent proteasome-mediated degradation (Issacs *et al.*, 2002). Since HIF-1 α stabilisation is followed by its nuclear translocation, dimerisation with HIF-1 β and DNA binding to HRE elements of target genes, small molecules able to interfere with any of these steps may inhibit the subsequent HIF-1 α transcriptional activity. For example chetomin prevents the interaction of HIF-1 with the transcriptional co-activator p300 (Staab *et al.*, 2007), while radicicol alters the conformation of the HIF-1 heterodimer so that its ability to interact with HRE is reduced (Hur *et al.*, 2002). Finally echinomycin (Kong *et al.*, 2005) and anthracyclines (Lee *et al.*, 2009) inhibit HIF-1 DNA binding and transcriptional regulation respectively.

Although most of these inhibitors were reported to affect HIF-1 α function *in vivo* as well as *in vitro*, most did not reach clinical trials due to toxicity or are still under investigation.

In the present study we aimed to test the hypothesis that HIF-1 α influences tumour cell response to ionizing radiation and chemotherapeutic drugs. By contributing to our understanding of the underlying biology this may in turn offer opportunities to increase the effectiveness of cancer therapy.

Using a panel of 4 cell lines from different human solid tumours, specific goals were to:

- establish *in vitro* models of oxygen-dependent radio- and chemosensitivity with relevance to *in vivo* settings
- test whether HIF-1 α contributes to hypoxia-induced radio- and chemoresistance, by transient knockdown using siRNA
- test whether pharmacological manipulation of HIF-1 α level affects oxygen-dependent radio- and chemosensitivity
- distinguish HIF-1 α -dependent from independent actions of these drugs by comparing their functional effects in wild-type and HIF-1 α knockdown cells
- analyse the functional role of HIF-1 α in oxygen-dependent radio- and chemosensitivity

Subconfluent cultures of human cervical (HeLa), breast (MCF7), melanoma (MeWo) and prostate (LNCaP) tumour cell lines under normoxia and defined hypoxia conditions (controlled atmosphere chambers) were exposed to either a standard

irradiation dose (6.2 Gy) or to chemotherapeutic agents. Subsequent cell proliferation was measured in terms of resazurin reduction, and HIF-1 α levels in cell lysates were determined by ELISA. Where a causal role for HIF-1 α could be established, specific pharmacological interventions were then evaluated as potential adjuvants to improve the clinical effectiveness of radio- and chemotherapy in cancer treatment.

Chapter 2

General Materials and Methods

Chemicals and reagents

Minimum Essential Medium Eagle (Hepes modification), RPMI 1640 medium, Hepes buffer, trypan blue, sodium pyruvate, sodium bicarbonate (7.5% solution, w/v), gentamycin, trypsin-EDTA, 7-hydroxy-3H-phenoxazin-3-one 10-oxide (resazurin), phosphate buffered saline (tablets), albumin from bovine serum (BSA), foetal bovine serum (FBS), polyoxyethylene-sorbitan monolaurate (Tween 20), Protease Inhibitor Cocktail, phenylmethylsulphonyl fluoride (PMSF) and 3,3',5,5'-tetramethylbenzidine (TMB) Liquid Substrate System for ELISA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS, pH 7.4) and L-glutamine were from Invitrogen (Grand Island, N.Y., USA). Stop Solution for terminating colour development in the horseradish peroxidase/TMB reaction was from R&D Systems (Minneapolis, USA). Cell Extraction Buffer for preparation of whole cell lysates was from Biosource (Camarillo, CA, USA). RNase-free water and 5X RNase-free buffer used for cell transfection were from Dharmacon (Thermo Fisher Scientific, USA). All other reagents were of the highest purity commercially available.

Cell Lines and Culture Conditions

HeLa (human cervical adenocarcinoma cell line), **LNCaP** (human prostatic carcinoma cell line), **MCF7** (human breast adenocarcinoma cell line) and **MeWo** (human malignant melanoma cell line) were purchased from American Type Culture Collection through LGC Promochem (Middlesex, UK). HeLa, MCF7 and MeWo cells were maintained in Minimum Essential Medium (MEM) containing Eagle's salts (Hepes modification) supplemented with 10% FBS, gentamycin (20 µg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM) and sodium bicarbonate (1.5 g/L). LNCaP cells were maintained in RPMI 1640 Medium supplemented with 10% FBS and gentamycin (20 µg/ml).

All cultures were manipulated in a BHG 2003-S laminar airflow cabinet (FASTER, Cornaredo, Italy) and incubated under standard conditions (5% CO₂, 37°C, ≥ 95% humidity) in a Forma Scientific Infrared CO₂ Incubator (Biosciences, Dublin, Ireland). HeLa, MCF7 and MeWo cells were cultured in 75 cm² polystyrene tissue culture flasks (Sarstedt, USA). LNCaP cells were grown in 75 cm² Corning CellBIND Surface flasks with a modified plastic surface to improve cell attachment (Corning Incorporated, Corning, NY, USA) (Pardo *et al.*, 2004, Pardo *et al.*, 2005). All four cell lines are adherent, and were maintained as monolayer cultures and passaged every 2-4 days (depending on cell line) when 80-90% confluent. The MycoAlert® Kit (Lonza Rockland, Inc., ME, USA) was used to test periodically that cultures remained free of mycoplasma contamination.

For experiments, cells from passages 10 to 30 were seeded at densities depending upon growth rate of cells and requirements of the end point assay then left overnight

(HeLa, MCF7 and MeWo) or for 24 hours (LNCaP) to attach. Depending on the experiment, cells were cultured in 6-well (Costar®, Corning, NY, USA), 24-well (NUNC™, Thermo Scientific, Roskilde, Denmark) or 96-well plates (Cellstar®, Greiner Bio-One, Germany), or on 55 mm or 70 mm diameter Pyrex® glass Petri dishes. Since adhesion of LNCaP cells to untreated polystyrene plastic surface is poor (seeding efficacy ~ 30%; Horoszewicz *et al.*, 1983), for this cell line we used 96-well plates with surface treatment equivalent to poly-D-lysine coating (cellGrade™ premium, BRANDplates®, Wertheim, Germany). By contrast, LNCaP cells readily attach to borosilicate glass like the other cell lines. Glass Petri dishes were used because the plastic dishes are permeable for oxygen and could act as a reservoir for oxygen, which could be slowly released into medium disturbing hypoxia conditions.

Cell Counting and Seeding

Prior to harvesting cell cultures in plastic 75 cm² flasks, medium was removed by suction and cells were washed twice with phosphate buffered saline (PBS) then detached by adding 3 ml trypsin-EDTA (25% w/v) solution. After 3-5 minutes incubation the flask was rinsed with 7 ml of fresh culture medium and the cell suspension was transferred to a 50 ml tube (Sarstedt, USA) before centrifugation at 500 x g for 5 minutes. The supernatant was aspirated and the pellet was resuspended in 10 ml of fresh medium. A sample of this cell suspension diluted 1:1 in trypan blue (0.4% w/v in PBS) was transferred to the etched glass chamber of a haemocytometer (Bright-Lane, Hausser Scientific, Horsham, USA) covered with a glass coverslip (22 x 40 mm; Menzel-Glaser, Thermo Fisher Scientific, USA), then viable cells (ranging from $1.2 \cdot 10^5$ – $1.7 \cdot 10^6$ cells per ml) were counted at 100x magnification on an inverted light

microscope (Nikon Badhoevedorp, Netherlands). Cell number per ml of suspension was calculated as:

$$\text{cells/ml} = (\text{average count/square}) \times (\text{dilution factor}) \times 10^4 (\text{chamber conversion factor})$$

Cell suspensions were then diluted with culture medium in order to seed 6, 24, 96 well plates, glass Petri dishes or 75 cm² flasks at the required densities.

Plating efficiency and cell doubling time

Cell culture growth after seeding depends both on the ability of the seeded cells to adhere to the substrate (here plastic) and on their subsequent doubling time. Therefore we estimated the plating efficiency of HeLa, LNCaP and MCF7 cells under the culture conditions used. Cells were seeded into 96-well plates (HeLa at 7,500, 15,000 and 30,000 cells/well; LNCaP and MCF7 at 15,000, 30,000 and 60,000 cells/well), then after 24 hours incubation cells were harvested and counted. Plating efficiency was calculated as cells counted at 24 h as a % of seeded cells, and found to be 219, 83 and 101% for HeLa, LNCaP and MCF7 cells respectively (Table 2.1).

HeLa			LNCaP			MCF7		
<i>seeded</i>	<i>24 h later</i>	<i>% (of) seeded</i>	<i>seeded</i>	<i>24 h later</i>	<i>% (of) seeded</i>	<i>seeded</i>	<i>24 h later</i>	<i>% (of) seeded</i>
7,500	16,400	219	15,000	10,400	69	15,000	13,600	91
	17,600	235		12,000	80		16,000	107
15,000	31,200	208	30,000	24,000	80	30,000	33,200	111
	32,800	219		26,000	87		28,000	93
30,000	65,000	217	60,000	52,000	87	60,000	66,400	111
	64,000	213		56,800	95		56,800	95

Table 2.1. **Plating efficiency of HeLa, LNCaP and MCF7 cell lines under standard culture conditions estimated from cell counts 24 h after seeding (n=2).**

Cell response to irradiation or exposure to chemotherapeutic drug was measured 7 days later in order to detect long-term changes in proliferative capacity rather than acute effects. Therefore it was necessary to use initial seeding densities that would give about 70-80% confluence at the end of the 7-day period. To do this we measured the doubling time for each cell line under the culture conditions used. Growth curves obtained (Fig. 2.1) were fitted to exponential equations (HeLa, $y = 0.1e^{0.0322x}$; MCF7, $y = 0.243e^{0.0192x}$; LNCaP, $y = 0.244e^{0.0223x}$; MeWo, $y = 0.0629e^{0.0237x}$) from which doubling times were calculated. The values obtained for each cell line were generally in good agreement with previous reports in the literature (Table 2.2).

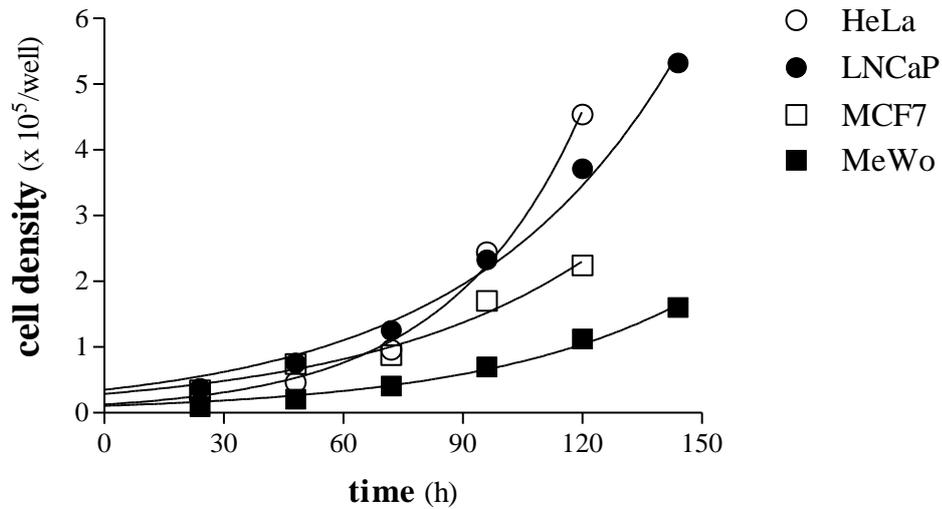


Figure 2.1. **Relative growth rates of HeLa, LNCaP, MCF7 and MeWo cell lines under standard conditions.** Cells were seeded in 24-well plates ($1.9 \text{ cm}^2/\text{well}$) at 1×10^4 (HeLa, MeWo), 3×10^4 (MCF7) or 4×10^4 (LNCaP) cells per well, then counted at 24 h intervals over 5-6 days. Data shown are from a pilot experiment.

Cell line	Doubling time (h)		Reference
	<i>Measured</i>	<i>Previously reported</i>	
HeLa	23.2	25	Benedetti <i>et al.</i> , 1991
LNCaP	36.4	30.32 ± 2.1	Liu <i>et al.</i> , 2003
		46.5 ± 5.8	Butterworth <i>et al.</i> , 2008
		60	Horoszewicz <i>et al.</i> , 1983
MCF7	40.4	36.9 ± 2.3	Smith <i>et al.</i> , 1999
MeWo	36.2	23.8	Lev <i>et al.</i> , 2004

Table 2.2. **Doubling time of HeLa, LNCaP, MCF7 and MeWo cultures (n=1) in comparison with values reported by others.**

Measurement of Resazurin Reduction

Many assays have been used to measure viability and sensitivity of mammalian cells to radiation or chemotherapeutic agents *in vitro*. Among them is the classic clonogenic cell survival method considered to be the ‘gold standard’ proliferation assay in radiobiology (Weisenthal & Lippman, 1985). The assay measures directly the ability of a single tumour cell to proliferate indefinitely and form large colonies or clones. Although various improvements including imaging and software for colony-counting (Niyazi *et al.*, 2007) have been introduced the clonogenic assay still has some drawbacks. First, its use is restricted to those cell types that can form colonies and depends on their plating efficiency and doubling times. Second, like the BrdU incorporation assay, the clonogenic assay is destructive to the cells so that they are not available for further analysis. Third, it is strongly dependent on experience in distinguishing colonies from cell clumps and in evaluating whether a cluster contains at least 50 cells, making it somewhat subjective as well as labour intensive (Hoffman 1991). Alternative assays for measuring cell growth or viability require use of radioactive biochemicals (e.g. [³H] thymidine, ⁵¹Cr release; Schlager & Adams, 1983),

reagents toxic to the cells (e.g. MTT assay; Mossmann 1983) or specific fluorescent DNA-binding dyes (Hoechst 33258; Begg & Mooken 1989). In general these methods are also time consuming and involve a series of steps.

To avoid these limitations we chose to use an enzyme-dependent resazurin reduction assay for this study because it shows a high degree of correlation with results of other cell proliferation assays (Nociari *et al.*, 1998, Cemazar *et al.*, 1999; Kawada *et al.*, 2002; Anoopkumar-Dukie *et al.*, 2005). This method has been described as a simple, non-toxic and non-destructive, one-step, sensitive, economical and reproducible method for measuring cell metabolic activity *in vitro* (Nakayama *et al.*, 1997, O'Brien *et al.*, 2000; Al-Nasiry *et al.*, 2007; Kendig & Tarloff, 2007). Resazurin is an oxidation-reduction indicator. The assay measures reduction of blue, non-fluorescent resazurin substrate to pink, highly fluorescent resorufin product (Fig. 2.2), catalysed by mitochondrial, microsomal and cytosolic enzymes (Gonzales and Tarloff, 2001). The reaction can be followed either spectrofluorometrically using an excitation wavelength in the range 530-560 nm and an emission wavelength in the range 590-610 nm, or spectrophotometrically at 570 and 600 nm.

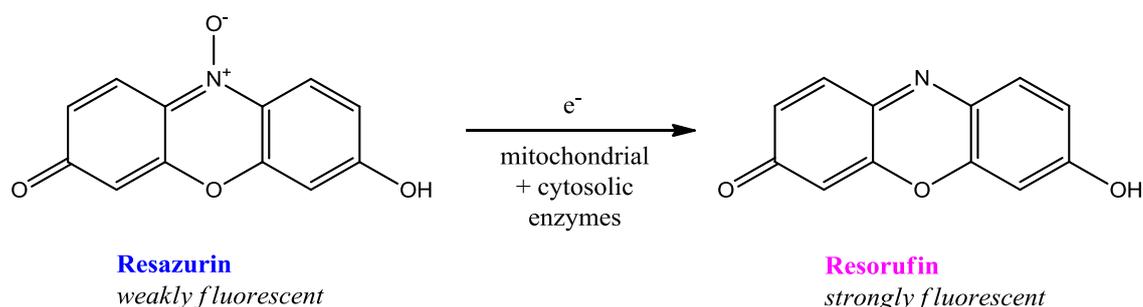


Figure 2.2. **Reduction of non-fluorescent resazurin to fluorescent resorufin by viable cells.**

For assays of metabolic capacity in cell cultures a resazurin stock solution (440 μM) was prepared in sterile PBS and filtered through a 0.45 μm syringe filter (Acrodisc[®], Life Science, NY, USA) then kept at 4°C until use. Under these conditions the dye remained stable for at least 6 months. To measure dye reduction, culture medium above cells growing in 96-well plates was aspirated, the cells were washed once with 200 μl /well of pre-warmed PBS, and then resazurin dye solution was added at a final concentration of 44 μM (O'Brien *et al.*, 2000) in culture medium (200 μl /well). In the case of LNCaP cells the washing step was omitted due to less robust cell adherence, and instead 100 μl of culture medium was removed and replaced with 100 μl of fresh medium supplemented with 88 μM resazurin to give the required final concentration. In each experiment cell-free wells containing culture medium without (background) and with 44 μM resazurin (control) were included. After incubation at 37°C for 60-120 minutes resorufin formation was measured as absorbance at 570 and 600 nm (Tecan Sunrise plate reader and X-read software, Tecan, Austria). Values were corrected for background and absorbance then used to calculate resazurin reduction as follows:

$$\% \text{ resazurin reduction} = \frac{(\epsilon_{\text{ox}}\lambda_2)(A\lambda_1) - (\epsilon_{\text{ox}}\lambda_1)(A\lambda_2)}{(\epsilon_{\text{red}}\lambda_1)(A'\lambda_2) - (\epsilon_{\text{red}}\lambda_2)(A'\lambda_1)} * 100\%$$

where:

$$\lambda_1 = 570 \text{ nm}$$

$$\lambda_2 = 600 \text{ nm}$$

$$(\epsilon_{\text{red}}\lambda_1) 155.67 \text{ M}^{-1}\text{cm}^{-1} \text{ (molar extinction coefficient of resazurin at 570nm)}$$

$$(\epsilon_{\text{red}}\lambda_2) 14.65 \text{ M}^{-1}\text{cm}^{-1} \text{ (molar extinction coefficient of resazurin at 600nm)}$$

$(\epsilon_{ox}\lambda_1)$ 80.58 M⁻¹cm⁻¹ (molar extinction coefficient of resorufin at 570nm)

$(\epsilon_{ox}\lambda_2)$ 117.22 M⁻¹cm⁻¹ (molar extinction coefficient of resorufin at 600nm)

$(A\lambda_1)$ Absorbance of test wells at 570 nm

$(A\lambda_2)$ Absorbance of test wells at 600 nm

$(A'\lambda_1)$ Absorbance of cell-free controls (resazurin + culture medium) at 570 nm

$(A'\lambda_2)$ Absorbance of cell-free controls (resazurin + culture medium) at 600 nm

Cell proliferation as measured using the Resazurin Assay

Various studies have shown that resazurin dye reduction is directly proportional to viable cell number (Nakayama *et al.*, 1997, Nociari *et al.*, 1998, Al-Nasiry *et al.*, 2007, Gould *et al.*, 2008) but becomes progressively non-linear beyond 30-50% due to substrate depletion and product accumulation. However, since cell lines differ in their metabolic capacity it is necessary to optimize the assay in terms of incubation time and cell density. Therefore cells were seeded based on their doubling times and plating efficiencies then incubated for 24 hours in order to give expected densities of 15,000, 30,000 or 60,000 cells/well at the time of resazurin addition, after which the time course of dye reduction was measured (Fig. 2.3). HeLa and MeWo cultures showed similar rates of dye reduction, **6.1** and **5.1**%/hour/ $1*10^4$ cells respectively), LNCaP cells reduced dye more slowly (**3.5**%/hour/ $1*10^4$ cells), whereas MCF7 cells were the slowest with **1.1**%/hour/ $1*10^4$ cells. Based on these data, a final (subconfluent) density of 60,000 cells/well was chosen, and a standard incubation time of 60 minutes for HeLa, 90 minutes for LNCaP and MeWo, and 120 minutes for MCF7 cells was adopted. Reproducibility measured as coefficient of variation for resazurin reduction in 8 replicate wells was within the range 4-12%.

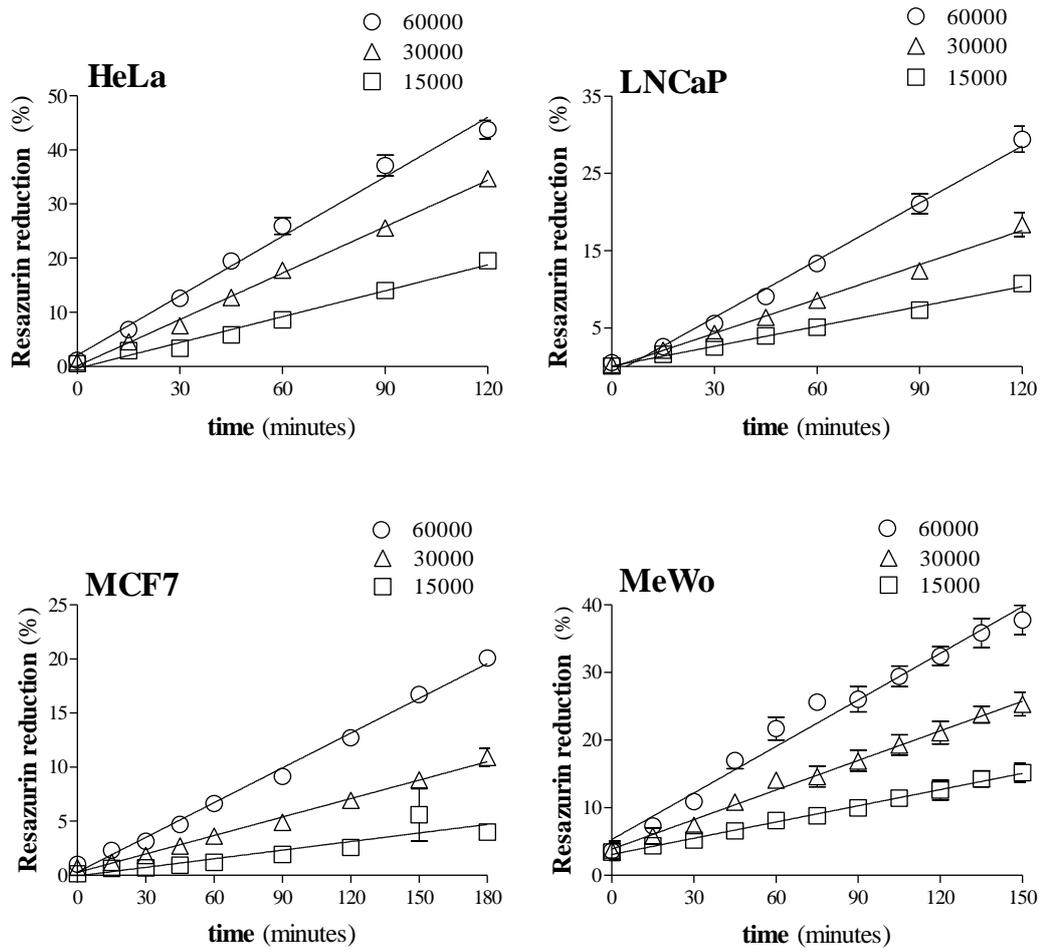


Figure 2.3. **Time course of resazurin reduction by cell lines seeded at different densities.** *HeLa* cells were seeded into a 96-well plate at 7,500, 15,000 and 30,000 cells/well while the other two cell lines were seeded at 15,000, 30,000 and 60,000 cells/well. After incubation for 24 hours at 37°C to allow cell attachment, medium over the cells was replaced with fresh medium containing resazurin dye (44 μ M). Then resazurin reduction was measured spectrophotometrically at intervals. Data are means \pm standard error from 8 (*HeLa*), 5 (*LNCaP*) and 3 (*MCF7* and *MeWo*) separate experiments. Note differences in scale of axes.

Preparation of cell extracts

Whole cell lysates

HeLa, LNCaP or MCF7 cells were seeded in glass Petri dishes (70 mm diameter) at 1×10^6 cells per dish and left overnight to attach. The next day cultures were exposed to drug or vehicle as appropriate, immediately after which Petri dishes were placed on ice and medium over the cells was replaced with 3 ml ice-cold PBS, so that the interval between the end of cell treatment and chilling was less than 1 minute. Three minutes after placing the Petri dishes on ice cells were detached using a cell scraper (25 cm, Sarstedt, Numbrecht, Germany), transferred to ice-cold 15 ml tubes (Sarstedt) and centrifuged at $500 \times g$ and 4°C for 6 minutes. The supernatant was discarded and the cell pellet was resuspended in 0.5 ml ice-cold PBS, transferred to an 1.5 ml Eppendorf tube and centrifuged at $500 \times g$ and 0°C for 7 minutes. Again the supernatant was aspirated and the washed cells were resuspended in 100 μl ice-cold lysis buffer (comprising 3 μl of 0.3 mM PMSF and 25 μl of protease inhibitor cocktail per 500 μl of cell extraction buffer (Bioscience) prepared immediately before use). The Eppendorf tubes were incubated on ice for thirty minutes with vortexing for one minute every ten minutes, and then centrifuged at 12,000 rpm and 4°C for 10 minutes. The clear lysate was collected, a 5 μl aliquot was used for determining protein concentration while the remainder was transferred to an Eppendorf tube and stored at -80°C for later analysis of HIF-1 α level.

Nuclear extracts

Nuclear extracts for measurement of HIF-1 α DNA-binding activity were prepared using the NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific, Rockford, USA). Cells were seeded into 75 cm² tissue culture flasks at a final density of 5*10⁶ (HeLa, MCF7 and MeWo) or 6*10⁶ (LNCaP) cells per flask. Twenty-four hours later they were exposed to drug or vehicle, then immediately harvested with 3 ml trypsin-EDTA and centrifuged at 500 x g for 5 minutes at 4°C. After aspirating the supernatant, the cell pellet was washed by resuspending it in 1 ml ice-cold PBS, transferred to an 1.5 ml Eppendorf tube and centrifuged at 500 x g and 0°C for 3 minutes. The supernatant was again removed and cells were now resuspended in 500 μ l ice-cold Cytoplasmic Extraction Reagent 1 (CER I) supplemented immediately before use with 25 μ l of protease inhibitor cocktail and vortexed for 15 seconds. After 10 minutes incubation on ice, 27.5 μ l of Cytoplasmic Extraction Reagent II (CER II) was added. The tube was vortexed for 5 seconds, incubated for 1 minute, vortexed again for 5 seconds then centrifuged at 16,000 x g and 4°C for 5 minutes. The cytoplasmic extract was removed and the insoluble pellet fraction was suspended in 250 μ l of Nuclear Extraction Reagent (NER) supplemented immediately before use with 12.5 μ l of protease inhibitor cocktail. Next the sample was vortexed for 15 seconds every 10 minutes, for a total of 60 minutes then centrifuged at 16,000 x g and 4°C for 10 minutes. The clear nuclear extract was collected, a 5 μ l fraction was used for determination of the protein concentration and the remainder was transferred to an ultrafiltration tube (Amicon ultra 0.5 centrifugal filter, 30 kDa cut off; Millipore Corporation, Billerica, MA) then centrifuged at 14,000 x g and 4°C for 30 minutes. Next the ultra filter insert was inverted into a clean micro tube and spun at 1,000 x g and 4°C

for 3 minutes to recover the sample now concentrated 4-5 fold. Finally, a 1 μ l aliquot of this sample was removed for protein determination and the remainder was stored at -80°C for later analysis.

Protein concentration

This was measured using the Bio-Rad Protein Assay (Munich, Germany) which is based on binding acidic Coomassie-dye reagent (Bradford 1976). Colour development depends on the presence of basic and aromatic amino acids in the protein. Protein concentration is determined by spectrophotometric comparison with a series of bovine serum albumin (BSA) standards and measured absorbance is linear over the range 0.05-0.5 mg protein/ml.

For measurement of protein concentration in total cell or nuclear extracts, dye reagent concentrate was diluted (1:4) in distilled, deionized (DDI) water. Five concentrations of a protein standard were prepared by dilution from fresh stock solution of BSA containing 6-11 mg/ml in PBS, then 10 μ l of each standard or sample diluted 20-fold (total cell lysate), 10-fold (nuclear extract) or 40-fold (concentrated nuclear extract) in DDI water was added per well in triplicate to a 96-well plate, followed by 200 μ l of dye reagent solution. After incubation for 5 minutes at room temperature absorbance was measured at 595 nm (Tecan Sunrise plate reader and X-read software, Tecan, Austria). Figure 2.4. shows the standard curve obtained.

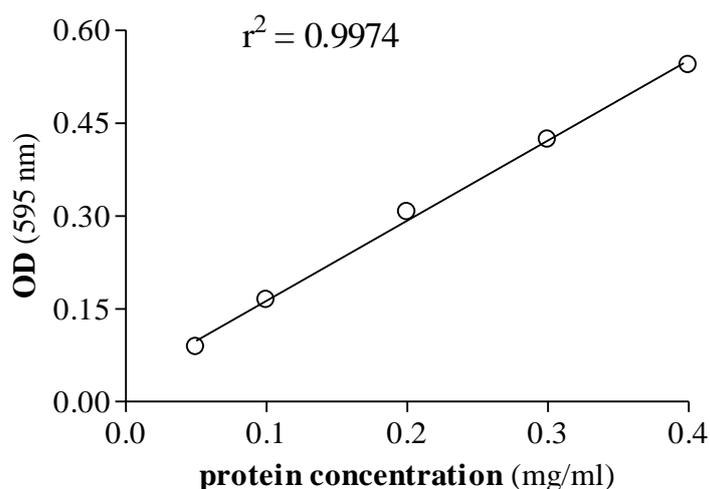


Figure 2.4. **Standard curve for Bio-Rad Protein Assay.** Results shown are means \pm SE for 114 separate experiments. Standard error bars lie within the data point circles.

Measurement of HIF-1 α level in whole cell lysates

HIF-1 α protein level can be measured by Western Blotting or Enzyme Linked Immunosorbent Assay (ELISA). Western Blotting provides an estimate of protein size based on band motility in the gel relative to molecular mass standards, while band intensity can be used for semi-quantitative estimation of the amount of protein present. Its main advantage is that more than one protein can be studied in the same sample. By contrast, ELISA provides no information about protein size and normally allows measurement of one protein only, as well as requiring use of antibodies directed at 2 separate epitopes on the protein of interest. However its advantages are that it is highly reproducible, fast and with all steps carried out in a single well of a 96-well plate.

Here we measured HIF-1 α concentration in cell lysates using a Human/Mouse Total HIF-1 α Sandwich Elisa Kit (DouSet[®] IC, R&D Systems, Minneapolis, USA). Wells of a 96-well ELISA plate (NUNC[™] Thermo Scientific, Roskilde, Denmark) were

coated with 100 μ l mouse anti-human HIF-1 α antibody (working concentration 4 μ g/ml, diluted in PBS), sealed and incubated overnight at room temperature. Next day, wells were aspirated and rinsed three times with Wash Buffer (0.05% Tween[®] 20 in PBS; 400 μ l/well/wash). After the last washing any remaining Wash Buffer was removed by inverting the plate and blotting against a clean paper towel. Then the plate was blocked by adding 300 μ l/well of Reagent Diluent (5% BSA in Wash Buffer) and incubated at room temperature for 1-2 hours. The aspiration/wash steps were repeated and finally 100 μ l of total HIF-1 α standard (using 2-fold serial dilutions and Reagent Diluent as zero standard) or cell extract (30 μ g of total cell protein diluted in Reagent Diluent per well) was added to duplicate wells. The plate was sealed and left at room temperature for 2 hours. Then unbound material was removed by aspirating and washing as before, after which 100 μ l/well of biotinylated goat anti-human HIF-1 α antibody (working concentration 400 ng/ml diluted in Reagent Diluent) was added to each well. The plate was covered with a new plate sealer and incubated at room temperature for 2 hours, washed as before, then 100 μ l/well of Streptavidin-horseradish peroxidase (HRP) (diluted 1:200 in Reagent Diluent immediately before use) was added and the plate was incubated for 20 minutes in the dark. After a further three aspirating/washing steps, 3,3',5,5'-tetramethylbenzidine (TMB) Liquid Substrate was added at 100 μ l/well, and the plate was incubated again for 20 minutes in the dark. The enzymatic reaction was stopped by adding 50 μ l/well of Stop Solution and the absorbance was measured immediately at 450 nm with the correction wavelength set at 540 nm. Figure 2.5 shows the standard curve obtained.

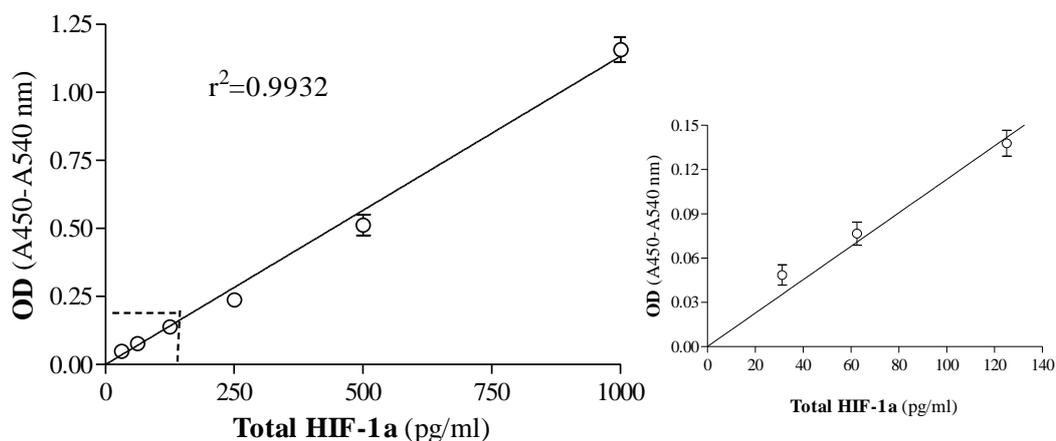


Figure 2.5 **HIF-1 α ELISA standard curve.** A six point standard curve was prepared using 2-fold dilutions of recombinant human HIF-1 α standard. Data shown are means \pm SE ($n=27$). The lower end of the concentration range is expanded on the right.

Measurement of HIF-1 DNA Binding

HIF-1 α transcriptional activity can either be inferred in terms of binding to the hypoxia response element (HRE) sequence or measured directly in terms of expression of a reporter gene such as green fluorescent protein (GFP) gene that is under HRE control. Both methods have been widely used in the literature. In this study we chose a direct DNA-binding assay because, in addition to being highly reproducible and sensitive, it is also fast and requires less extensive optimisation. The antibody used in the assay is specific for HIF-1 α and detects HIF-1 α only when bound to the oligonucleotide/HIF-1 α complex in the nuclear extract. It is assumed that at the time of DNA binding HIF-1 α has already dimerised with HIF-1 β and that all other necessary partner proteins and co-activators are also present in the nuclear extract.

DNA binding activity was determined using the Human/Mouse Active HIF-1 α Activity Assay (DuoSet[®] IC, R&D Systems, Minneapolis, USA) and following the manufacturer's instructions. Briefly, wells of a 96-well ELISA plate were coated and washed as described in the previous section before samples were added. Samples of nuclear extracts containing 50 μ g of protein were pre-incubated in Eppendorf tubes with biotinylated double-stranded (ds) oligonucleotide containing a consensus HIF-1 α binding site for 30 minutes at room temperature. Non-specific binding was measured in a parallel set of samples by further addition of unlabelled ds oligonucleotide to out-compete biotinylated oligonucleotide for HIF-1 α binding. In addition a blank containing biotinylated ds oligonucleotide without nuclear extract was included. Next, the final volume in each Eppendorf tube was adjusted to 230 μ l with Reagent Diluent and mixed gently before transfer of 100 μ l to each HIF-1 α antibody-coated well. The plate was covered with plate sealer and incubated at room temperature for 2 hours, washed (3x) as before, then 100 μ l/well of Streptavidin-HRP (diluted 1:200 in Reagent Diluent immediately before use) was added and the plate was incubated for 20 minutes in the dark. The TMB-based colorimetric reaction was performed as described above, then product formation was determined immediately by measuring absorbance at 450 and 540 nm. Each sample was analysed in duplicate wells. Figure 2.6 shows specific and non-specific DNA-binding by HIF-1 α in nuclear extracts from the 4 cell lines.

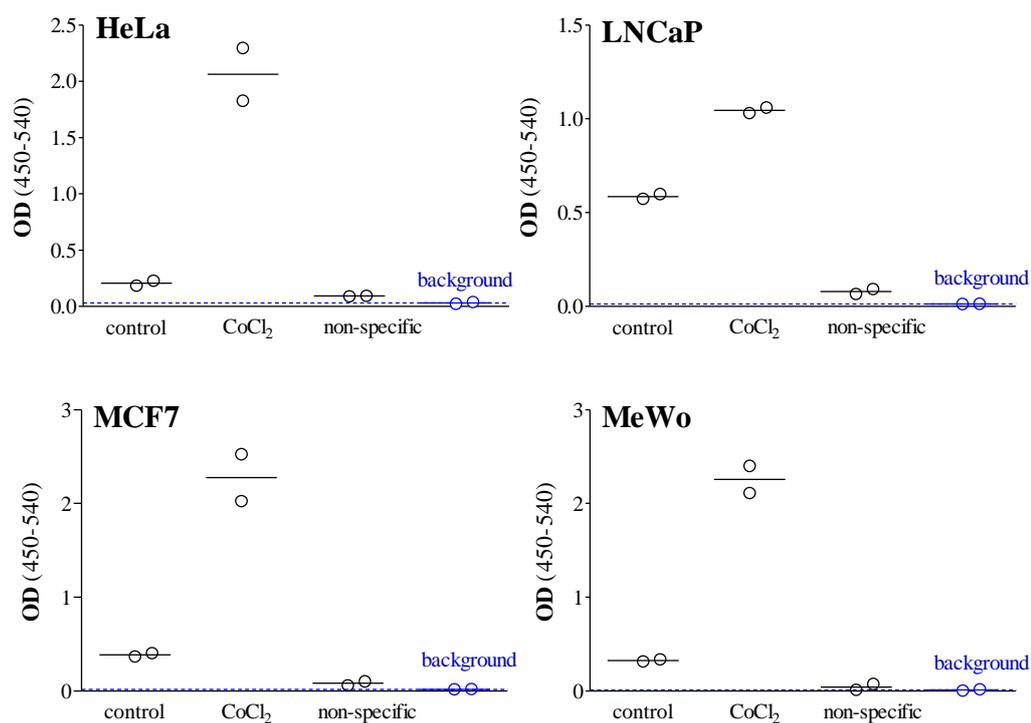


Figure 2.6. **DNA binding activity of HIF-1 α in nuclear extracts.** *Positive controls were generated from cells treated with CoCl₂ at 0.3 mM for 2 h followed by 0.1 mM for 24 h. Non-specific binding was measured in the presence of excess unlabelled ds oligonucleotide and background signal absorbance in the absence of nuclear extract averaged 0.02 \pm 0.01. Data shown are individual values from 2 independent experiments. Note difference in scale of vertical axes.*

HIF-1 α knockdown

HIF-1 α expression in cells was ablated using siGENOME SMARTpool Human HIF-1 α from Dharmacon (Thermo Fisher Scientific, USA). SMARTpool combines four siRNAs that target different regions of the same mRNA and are provided as a single reagent. Figure 2.7 shows the sequence for the gene encoding the human alpha subunit of transcription factor HIF-1, and the target positions of the siRNA sequences used to silence the gene.

siRNA resuspension

The tube containing the dried siRNA was briefly centrifuged to collect the reagent at the bottom before it was resuspended in 500 μ l of 1X RNase-free buffer (5X concentrate diluted in RNase-free water). Next, the solution was mixed for 30 minutes on an orbital shaker at room temperature after which the tube was again briefly centrifuged. Final siRNA concentration was 20 μ M, based on absorbance at 260 nm and applying a molar extinction coefficient of 376,604 L/mol*cm (manufacturer's data). Non-targeting siRNA stock was prepared in the same way, resulting in a concentration of 21 μ M. Both siRNA stock solutions were then divided into 10 μ l aliquots for storage at -20°C until further use.

1 GCGCGCGCCGGCCTGGGCAGGCGAGCGGGCGCGCTCCCGCCCCCTCTCCCTCCCCGCGCGCCCGAGCGC
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 1400 CAAAAGAGGTGGATATGTCTGGGTTGAAACTCAAGCAACTGTCATATATAACCCAAGAATTTCTCAACCA
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 ATTCCTTTTTGCTCTTGTGGTTGGATCTAACACTACTGTATTGTTTTGTTACATCAAATAAACATCTTC
 TGTGGACCAGGCAAAAAAAAAAAAAAAAAAAAAA

Figure 2.7. Sequence encoding the human HIF-1 α gene (NCBI gene accession number: NM_181054.2). *Highlighted are: the start (green) + stop (red) codons as well as the 4 sequences targeted by siRNA (blue).*

Transfection protocol

Cells were transfected by following the manufacturer's instructions. Briefly, cells were seeded in 6-well plates at 1.6×10^5 (HeLa), 3.3×10^5 (LNCaP and MCF7) or 2.0×10^5 (MeWo) cells/well in antibiotic-free medium. The next day working solutions of $4 \mu\text{M}$ siRNA mixture (50 μl) and 1:10 diluted DharmaconFECT Transfection Reagent (50 μl) were prepared in RNase-free buffer and serum/antibiotic-free medium respectively. Next these two solutions were mixed, supplemented with a further 150 μl of serum/antibiotic-free medium and incubated for 20 minutes at room temperature to form transfection reagent/siRNA complexes. Then a further 1.75 ml of serum-containing but antibiotic-free medium was added to the tube before cells were transfected with a final siRNA concentration of 100 nM. siRNA-containing culture medium was replaced 16-20 hours following transfection with fresh culture medium containing both antibiotic and serum.

Optimisation for maximal HIF-1 α knockdown

Cell lines may differ in their transfection requirements and transfection efficiency so conditions must be optimised in each case. Pilot experiments indicated that maximal HIF-1 α protein knockdown occurred at least 72 h after transfection (data not shown). This was confirmed in all 4 cell lines except MCF7, which showed greatest knockdown after 96 h (Fig. 2.8). At these times HIF-1 α protein level in normoxic HeLa, LNCaP, MCF7 and MeWo cells was reduced to 13, 28, 18 and 10% of control levels respectively.

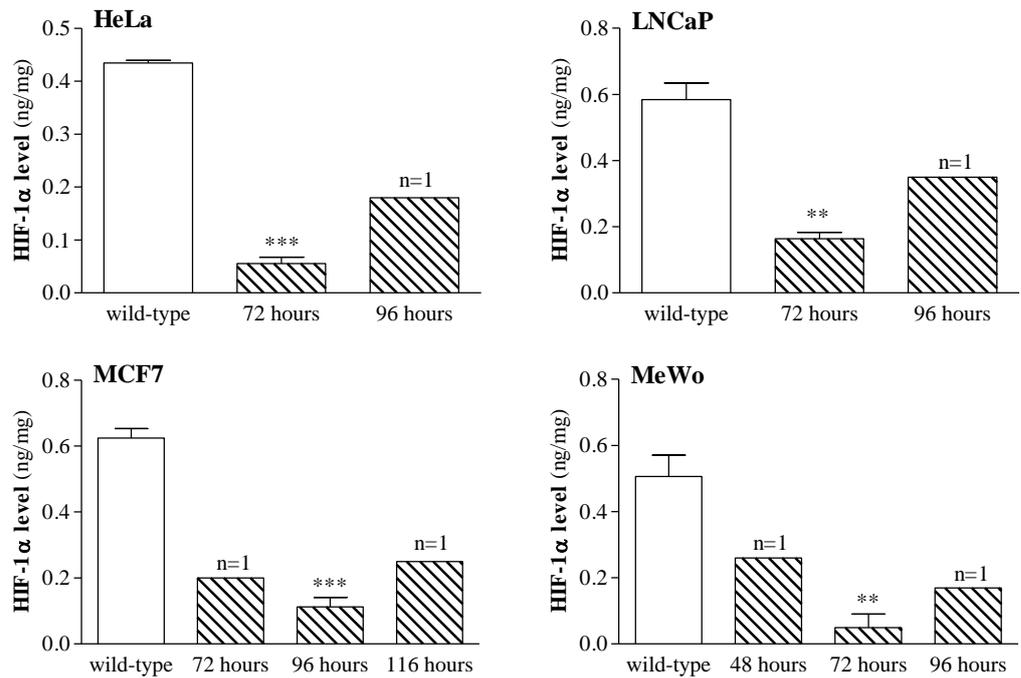


Figure 2.8. **Time course of HIF-1 α knockdown in four cell lines.** *HeLa* (1.6×10^5 cells/well), *LNCaP* and *MCF7* (3.3×10^5 cells/well) and *MeWo* (2×10^5 cells/well) cells were cultured in antibiotic-free medium and transfected with 100 nM siRNA in DharmaconFECT Transfection Reagent. At indicated time points after transfection cells were rapidly cooled to 4°C, lysed and analysed for HIF-1 α level by ELISA. Data shown are the means \pm SE from either 4 (*HeLa*, *MCF7*) or 3 (*LNCaP*, *MeWo*) experiments except where indicated otherwise. Comparisons are transfected vs. wild-type control. Significance levels are $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Note variation in scale of the vertical axes.

By contrast, exposure to either vehicle alone (DharmaconFECT Transfection Reagent) or vehicle and non-targeting siRNA did not affect HIF-1 α protein level significantly in any of the cell lines (Fig 2.9).

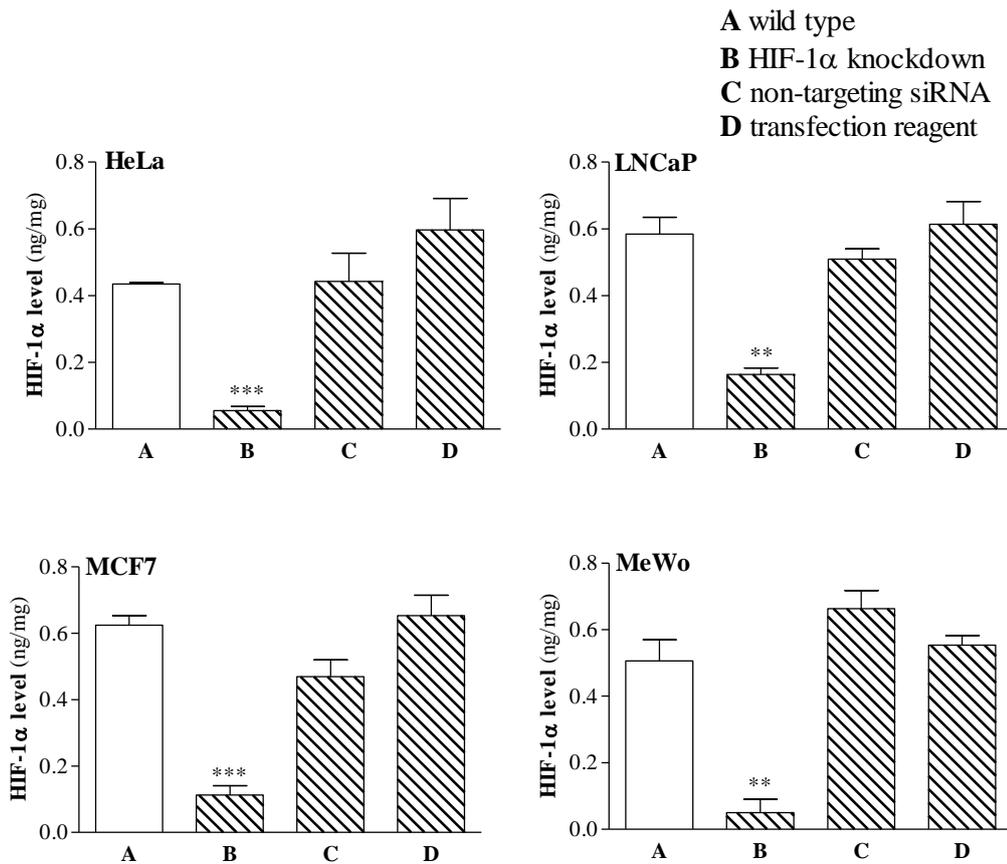


Figure 2.9. **Effects of transfection procedure on HIF-1 α level in HeLa, LNCaP, MCF7 and MeWo cells.** Cells were transfected with HIF-1 α targeting siRNA (B), non-targeting siRNA (C) or vehicle (D) either 72 h (HeLa, LNCaP, MeWo) or 96 h (MCF7) before cooling them to 4°C and then lysing for measurement of HIF-1 α level by ELISA. Data are means \pm SE from 3 experiments. Comparisons are transfected cells vs. wild type controls. Significance levels are $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

Statistical analysis

All experiments were repeated at least three times unless indicated otherwise. Data are presented as means \pm SE, and the differences between means were analysed either with the Student *t*-test for 2-way comparison or one-way ANOVA for multiple comparison, with a Tukey-Kramer post test, using Graphpad Prism Software (San Diego, CA). Significance levels were defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)).

Chapter 3

HIF-1 α role in oxygen-dependent radiosensitivity

Introduction

Radiation therapy

Ionizing radiation is one of the main treatments used in cancer therapy. It can be applied alone or as an adjuvant to surgery or chemotherapy. Radiation therapy involves either external or internal (brachytherapy) beam treatment, use of which depends on the location, size and type of the tumour. The dose of ionizing radiation delivered to the tumour is measured in Gray (Gy) and the clinical range varies depending on the form of therapy (curative or palliative) to be used. Radiation dose used also depends on the tumour type and its volume together with the area of surrounding normal tissue to be irradiated, combination with other therapies and more general factors such as patient age and health condition. For curative radiation therapy regimes doses up to about 75 Gy are delivered over 5-8 weeks (1.8–3 Gy/day) but for palliative therapy doses are higher (3-8 Gy/treatment) and overall treatment duration is shorter (*Radiation Biology: A Handbook for Teachers and Students, 2010, p.57*). Here we adopted a standard irradiation dose of 6.2 Gy for *in vitro* study of HIF-1 α role in oxygen-dependent radiosensitivity of several human tumour cell lines.

The 6.2 Gy dose was chosen to allow for direct comparison with previous work in which an *in vitro* model of oxygen-dependent radiosensitivity using acute severe hypoxia had been established (Anoopkumar-Dukie S, PhD Thesis, 2006, p. 34-35, p. 46-47; Anoopkumar-Dukie *et al.*, 2009). Although 6.2 Gy is outside the range used for most radiotherapy (usually up to 3.4 Gy per single fraction irradiation), higher doses are sometimes used. For example doses of 15-24 Gy are given in single fraction radiosurgery of brain tumours depending on tumour diameter (Shaw *et al.*, 2000).

Moreover stereotactic irradiation therapy with high single doses is also reported for treatment of liver or lung tumours (16 Gy, Herfarth *et al.*, 2001; 30 Gy, Fritz *et al.*, 2006; Leeman *et al.*, 2012). Finally, high single doses are often used during palliative radiotherapy in patients with various cancers (6-20 Gy, *Radiotherapy Dose-Fractionation*, June 2006). Therefore the 6.2 Gy dose used here limits the extrapolation of results to intact tumours undergoing standard fractionated radiotherapy, but it is well within the range (2-20 Gy) used both clinically and in most *in vitro* studies (Palayoor *et al.*, 2008; Staab *et al.*, 2007; Liu *et al.*, 2010b; Ayrapetov *et al.*, 2011; Lagadec *et al.*, 2012).

Cell responses to ionizing radiation

The effect of ionizing radiation on biomolecules within the cell can be either direct or indirect. The nucleus, representing about 10% of the total volume of a typical somatic cell and containing most of the genetic material, is a major target. When energy, in the form of X- or γ -rays released from radioactive sources or a linear particle accelerator is absorbed in cells it can directly interact with the atoms and cause multiple lesions in the DNA (Hall & Giaccia, *Radiobiology for the Radiologist*, 2006). These can include base damage, single and double strand breaks, as well as DNA-protein and nuclear protein-protein cross-links (von Sonntag, 2007, p.21). Of these, DNA double strand breaks are the most disruptive and may lead to chromosomal rearrangements or changes including either loss or amplification of chromosomal material. If the deleted chromosomal region encodes a tumour suppressor, or an amplified region encodes a protein with oncogenic potential, then the DNA damage can lead to tumourigenesis (Khanna & Jackson, 2001). DNA damage is also induced indirectly when the radiation

ionizes other atoms or molecules in the cell (particularly water surrounding the DNA) to produce reactive hydroxyl radicals that are able to diffuse and damage critical cellular targets (Hall & Giaccia, *Radiobiology for the Radiologist*, 2006). Alternatively, primary radicals generated by ionizing radiation can also be amplified to produce further reactive oxygen (Oberley *et al.*, 1976) or nitrogen species (Leach *et al.*, 2002). Their generation contributes to activation of various radiation-induced signal transduction pathways that mediate complex cellular responses which can be either cytoprotective or cytotoxic (Schmidt-Ullrich *et al.*, 2000) and are often cell-type specific (Anoopkumar-Dukie *et al.*, 2009).

Ionizing radiation and oxygen effect

The biological effects of ionizing radiation on cells depend strongly on oxygen availability. Irradiation of cells directly ionizes genomic DNA to form DNA radical (DNA[•]) and/or generates various reactive species. In the absence of oxygen (hypoxia or anoxia) most radical damage can be repaired through donation of hydrogen from sulfhydryl-containing compounds in the cell. By contrast when present, molecular oxygen oxidizes DNA radicals to generate organic peroxide radicals (DNA-OO[•]), that prevent repair of the DNA damage. This mechanism is known as the “Oxygen Fixation” Hypothesis (Gray *et al.*, 1953; Alper & Howard-Flanders, 1956). The difference in surviving fraction of cells after irradiation in the absence and presence of oxygen is expressed in terms of the oxygen enhancement ratio (OER), which is defined as the ratio of doses required to obtain the same level of cell death in the absence or presence of oxygen. The OER for high ionizing radiation doses (usually > 3 Gy) is in the range

2.5-3.0 (Gray *et al.*, 1953) but at lower doses (< 3 Gy) it significantly decreases in a dose-dependent manner (Palcic *et al.*, 1982; Palcic & Skarsgard, 1984).

Many *in vitro* studies have shown that hypoxia of different severity (<0.1 – 5 % O₂) and duration (1-24 h) increases radioresistance of various tumour cell lines. These include derivatives from major types of human tumours such as cervical carcinoma (Liu *et al.*, 2010), breast (He *et al.*, 2012; Lagadec *et al.*, 2012), lung (Oike *et al.*, 2012), colon (Saalen *et al.*, 2012), prostate (Palayoor *et al.*, 2008) or liver (Zhang *et al.*, 2011) as well as less common cancers such as pharyngeal carcinoma and fibrosarcoma (Vordermark *et al.*, 2004). By contrast similar radiosensitivity of normoxic and hypoxic tumour cells (i.e. OER~1) was also reported for human fibrosarcoma (Zhang *et al.*, 2007) and lung cancer cells (Schilling *et al.*, 2012).

At the later stages of growth all solid tumours become hypoxic due to irregular vasculature development during angiogenesis and increased distance of some cells from blood vessels. This progressive and dynamic hypoxia in the tumour microenvironment directly influences cell radiosensitivity, which decreases rapidly when oxygen partial pressure is less than 25-30 mmHg (Fig. 3.1, adapted from Vaupel *et al.*, 2001). Hypoxia-associated resistance to irradiation and therefore poor prognosis has been documented for a wide range of human solid tumours (Fyles *et al.*, 1998, Brizel *et al.*, 1997, Vordermark *et al.*, 2003; Milosevic *et al.*, 2012).

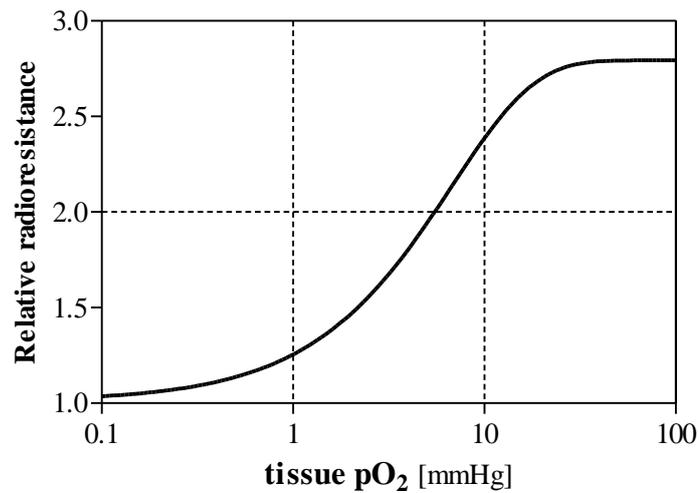


Figure 3.1. **Relative radioresistance of tumour cells as a function of tissue O₂**
[adapted from Vaupel et al., 2001].

Attempts to overcome hypoxia-induced radioresistance

Since the hypoxic microenvironment in solid tumours reduces the efficacy of radio- and chemotherapies, various strategies have been investigated to overcome this resistance. One of the earliest attempts to improve outcome of radiotherapy was by having patients breathe hyperbaric pure oxygen during irradiation (Watson *et al.*, 1978; Dische *et al.*, 1983; Henk, 1986) or oxygen/carbogen mixtures at normal atmospheric pressure (Inch *et al.*, 1970; Grau *et al.*, 1992; Overgaard & Horsman, 1996; Mendenhall *et al.*, 2005), to deliver extra oxygen for saturation of hemoglobin and therefore increase oxygenation of hypoxic tumour cells. The oxygen/carbogen method of enhancing tumor oxygenation showed a trend toward improved radiation response, but due to the many experimental variables (e.g. duration of breathing) the benefit was not universal among tumour types and has had no significant impact on general clinical practice (Overgaard,

2007). On the other hand, studies with hyperbaric oxygen are still continuing (Mayer *et al.*, 2005) although the method is not used routinely in clinical radiotherapy.

Another approach was to develop agents with selective toxicity to hypoxic cells. The general mechanism of action by which non-toxic prodrug is activated to a toxic form of free radical requires one-electron reduction. In the presence of oxygen, including in well-oxygenated healthy tissue surrounding the hypoxic tumour, the free radicals are oxidized back to the parental form of the compound. Tirapazamine, the most promising of the prodrugs showed selective toxicity towards hypoxic cancer cells (Brown, 1999), and in combination with radiotherapy (McKeown *et al.*, 1996; Lee, 1998) or chemotherapy (Dorie & Brown, 1993; Weitman *et al.*, 1999) improved the overall therapy outcome. However despite the promising results from pre- and early clinical studies (Rischin *et al.*, 2001; Quynh-Thu *et al.*, 2009), the Phase III trials failed to demonstrate the value of tirapazamine as an adjuvant to radiotherapy (Reddy & Williamson, 2009; Ghatage & Sabagh, 2012). Similarly the promising effects of several other hypoxia-selective prodrugs have been reported, including AQ4N, which has high DNA affinity and topoisomerase II inhibitor activity (Patterson *et al.*, 2000; Williams *et al.*, 2009) and NLCQ-1 which is a weak DNA-intercalating compound (Papadopoulou *et al.*, 2003). These bioreductive drugs and several other cytotoxins selective for hypoxic cells are either already being tested or being considered for clinical trials. Such attempts to enhance the efficacy of radiotherapy by improving tumour oxygenation or use of bioreductive hypoxia-selective drugs aim to counteract the radiochemical mechanisms behind tumour radioresistance under hypoxia.

In addition, radiobiological mechanisms that involve hypoxia-induced changes in DNA damage repair (Bindra *et al.*, 2007) and cell death/survival signaling pathways can also lead to increased radioresistance. Development of molecular biology in the past two decades has enabled the study of changes in signaling pathways and processes which specifically target the hypoxic microenvironment of tumour cells. The variety of tumour cell lines and animals in which either the protein or gene expression pattern can be modified, permits a selective focus on effects of specific genes, proteins or signal transduction pathways involved in hypoxia-induced radioresistance. Evidence is accumulating that hypoxia-mediated increase in radioresistance can occur through biological pathways, raising the possibility that activation of the transcription regulator hypoxia-inducible factor 1 (HIF-1) is important in this mechanism.

HIF-1 and radioresistance

Oxygen availability is inversely correlated with the level of hypoxia-inducible factor 1 α (HIF-1 α) (Wang *et al.*, 1995, Lee *et al.*, 2004) in most human tumours (Zhong *et al.*, 1999). Activation of the HIF-1 α pathway induces the expression of various genes related to angiogenesis, cellular adaptation to hypoxia, energy metabolism, metastasis, etc.. Thus HIF-1 α has become a major topic of interest in the last decade, as a potential target for increasing the efficacy of radiotherapy by disrupting multiple pathways crucial for tumour growth and invasion.

In addition to HIF-1 α stabilization under hypoxic conditions, ionizing radiation also increases HIF-1 α levels (Moeller *et al.*, 2004) and activity (Harada *et al.* 2007) in tumours 12-24 hours after irradiation, peaking at 48 hours. By contrast, the effects of

irradiation alone on HIF-1 α protein level in cultured tumour cells vary and are limited. Only one study found a minor increase in HIF-1 α protein level 1-2 hours after irradiation (Lund *et al.* 2004) whereas others observed no change in HIF-1 α protein level postirradiation (Moeller *et al.*, 2004; Zeng *et al.*, 2008), leading to the proposal that tumour microenvironment and/or tumour-host interface may be essential for the increase observed *in vivo* to occur.

Nevertheless the hypoxia-independent increase in HIF-1 α following irradiation has been attributed to two independent mechanisms secondary to radiation-induced tumour reoxygenation (Fig. 3.2, adapted from Dewhirst *et al.*, 2007). The first of these involves increased free radical formation following radiation-induced reoxygenation (Moeller *et al.*, 2004). The other proposed mechanism involves production of NO by tumour-associated macrophages (Li *et al.* 2007). More recently this radiation-induced increase in HIF-1 activity was suggested as a first step of the model describing HIF-1 role in oxygen-dependent radiosensitivity of solid tumours (Harada, 2011). This is suggested to lead to HIF-1-induced expression of VEGF and VEGF-induced radioprotection of endothelial cells which as a consequence are able to provide oxygen and nutrients to the tumour promoting its growth. Similar activation of the HIF-1 pathway after irradiation and consequent increase in radioresistance was also claimed in some tumour cell lines although in these *in vitro* experiments CoCl₂ treatment of cells was used to simulate postirradiation increase in HIF-1 level (Hennessey *et al.*, 2011).

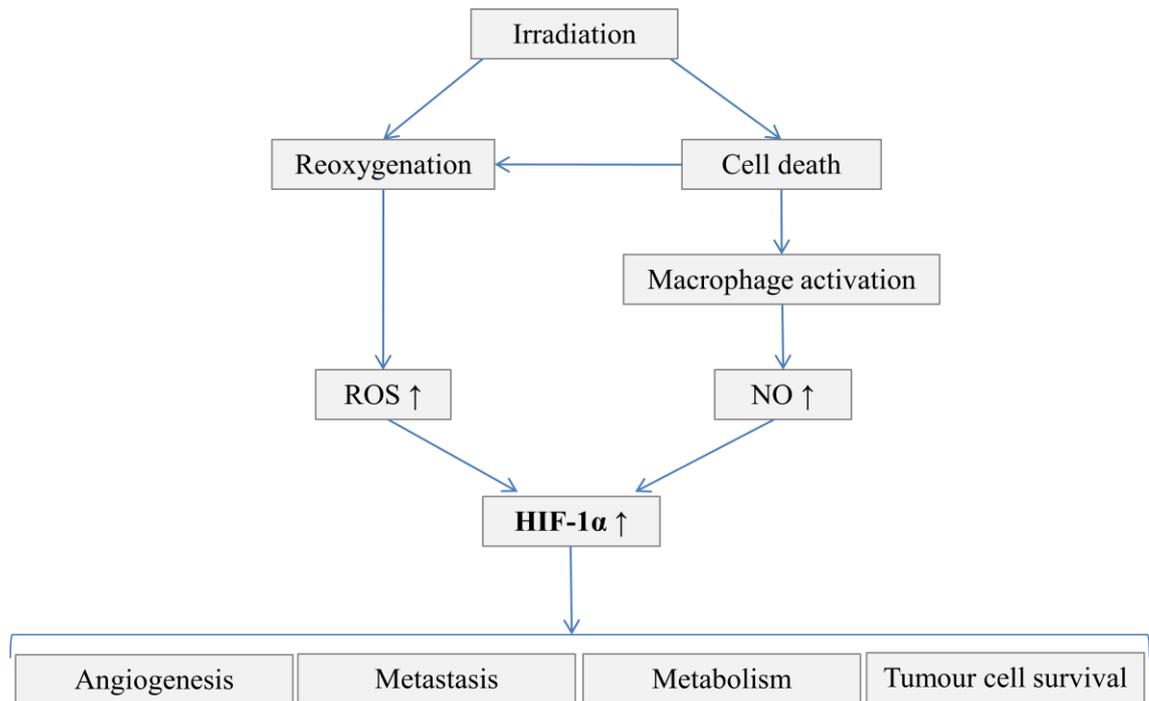


Figure. 3.2 **Hypoxia-independent mechanisms of HIF-1 α stabilisation following irradiation; suggested mechanisms** [adapted from Dewhirst *et al.*, 2007].

The connection between HIF-1 α and radiosensitivity of tumour cell cultures remains unclear. HIF-1 α inhibition was reported to enhance the radiosensitivity of some tumour cell types (Unruh *et al.*, 2003; Moon *et al.*, 2009; Hsieh *et al.*, 2010; Kessler *et al.*, 2010; Liu *et al.*, 2010b; Staab *et al.*, 2011), at least in some tumour cell types by promoting p53 activation and ATP metabolism (Moeller *et al.*, 2005). By contrast, others found no evidence that hypoxia-induced radioresistance of cell cultures depended on HIF-1 α (Arvold *et al.*, 2005; Williams *et al.*, 2005; Oike *et al.*, 2012; Schilling *et al.*, 2012). Despite elevated HIF-1 α in hypoxic tumours, there is also conflicting evidence in the literature about whether it plays a causal role. Some authors found an inverse correlation between HIF-1 α and oxygen-dependent radiosensitivity in HIF-1 α -deficient tumours (Zhang *et al.*, 2004; Williams *et al.*, 2005; Yasui *et al.*, 2008), but no impact of HIF-1 α status on radioresponsiveness was found in multicellular spheroids (Zou *et al.*,

2010). It may be that the role of HIF-1 α in radiosensitivity is context-dependent according to the experimental model, radiation treatment and experimental conditions used. In addition overexpression of HIF-1 α has been proposed as a novel prognostic and predictive parameter in the radiotherapy in patients with cervical (Dellas *et al.*, 2008), oropharyngeal (Aebersold *et al.*, 2001) and nasopharyngeal (Xueguan *et al.*, 2008) cancers.

Thus although there is evidence to suggest that HIF-1 may contribute to hypoxia-induced resistance of tumour cells, its role appears to be variable and apparent inconsistencies in the literature may reflect the different models used. Therefore the present study was designed to test the hypothesis that HIF-1 α plays a causal role in tumour cell resistance to radiotherapy and to evaluate at a tumour cell level the potential benefit of manipulating HF-1 α status by pharmacological and genetic means. To determine whether observed effects are likely to be cell type specific or more generalized, cell lines from three different common human tumour types were compared.

Materials and Methods

Chemicals and reagents

Ethyl-3,4-dihydroxybenzoate (EDHB), 3-[2-[4-(bis(4-fluorophenyl)methylene)-1-piperidinyl]ethyl]-2,3-dichloro-2-thioxo-4(1H)-quinazolinone (R59949) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were from the sources described in *Chapter 2*.

Stock solutions and storage

Stock solutions of EDHB (250 mM) and R59949 (50mM) were prepared in sterile DMSO and aliquots were stored at -20°C. Prior to use stock solutions were thawed, spun to collect then diluted in sterile culture medium containing FBS to a final concentration of 0.5 mM (EDHB) or 0.3 mM (R59949).

Optimisation of EDHB and R59949 exposure conditions

We chose EDHB and R59949 to increase and decrease respectively the HIF-1 α level in cells (Fig. 3.3). EDHB, a structural analog of α -ketoglutarate and ascorbate (Sasaki *et al.*, 1987) is a competitive inhibitor of prolyl hydroxylases (PHDs), with an IC₅₀ of 5 μ M for purified enzyme (Majamaa *et al.*, 1986). Treatment with EDHB (250-500 μ M) was reported to accumulate HIF-1 α protein in normoxic HeLa cells (Li *et al.* 2008). Conversely, R59949 is a direct diacylglycerol kinase (DGK) inhibitor, selective for type I DGK isoforms (Jiang *et al.*, 2000), with an IC₅₀ of 0.3 μ M for DGKs in isolated platelet membranes (de Chaffoy de Courcelles *et al.*, 1989). R59949 impairs HIF-1 α protein accumulation and activity (Aragones *et al.*, 2001), possibly by

promoting PHD activation through an indirect mechanism that involves inhibition of phosphatidic acid formation (Temes *et al.*, 2005).

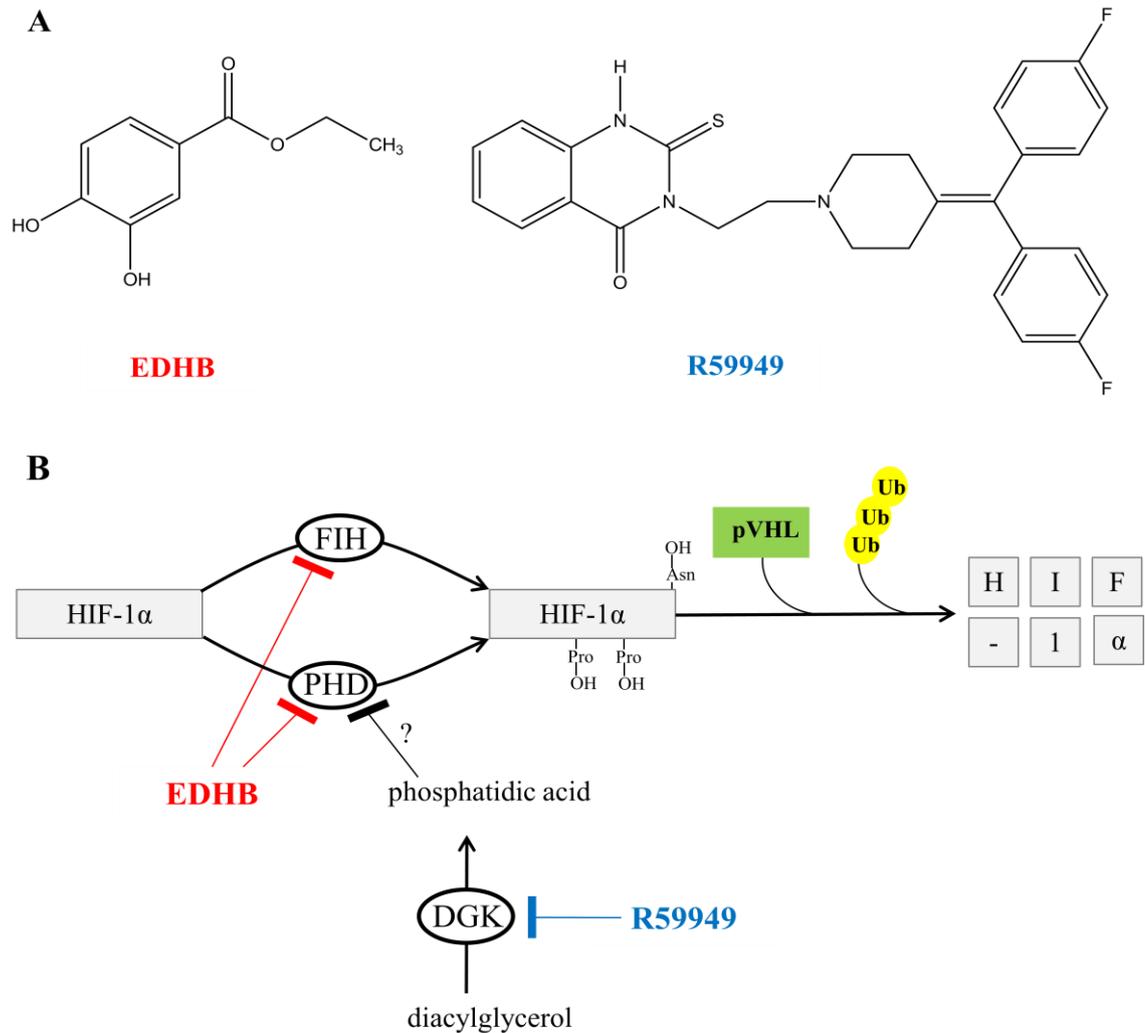


Figure 3.3 (A) **Structure** and (B) **mechanism of action** of EDHB and R59949. Abbreviations: HIF-1 α , hypoxia-inducible factor-1 α ; PHD, prolyl hydroxylases; FIH, factor inhibiting HIF; pVHL, von Hippel-Lindau protein; Ub, ubiquitin; DGK, diacylglycerol kinases.

Initial experiments were carried out to determine the optimal incubation conditions (concentration and exposure duration) for EDHB and R59949 which would significantly alter HIF-1 α protein levels across the three cell lines. Cell cultures were treated with EDHB at 0.25 mM or 0.5 mM for 1, 2 and 4 h, or R59949 at 0.15 mM or 0.3 mM for 3, 5 and 7 h. Figure 3.4 (*left*) shows that the increase in HIF-1 α protein level caused by EDHB was concentration-dependent, greatest at 0.5 mM. In HeLa, the HIF-1 α increase was maximal (5.2-fold) after 2 h exposure to the drug, while in LNCaP and MCF7 cells respectively, the difference between 2 h (4.8-fold and 6.6-fold) and 4 h (5.4-fold and 6.3-fold) exposures was minimal and could be disregarded. By contrast, the effect of R59949 was not strongly concentration-dependent in the range tested (Fig. 3.4, *right*). Exposures over 3-7 hours had rather similar effects, except for MCF7 cells in which HIF-1 α levels returned to near-control values after 7 h. Therefore based on these data 0.5 mM EDHB for 2 h or 0.3 mM R59949 for 3 h were chosen as standard treatments for subsequent experiments, expected to cause at least 5-fold increase or 2-fold decrease in HIF-1 α level respectively in all three cell lines.

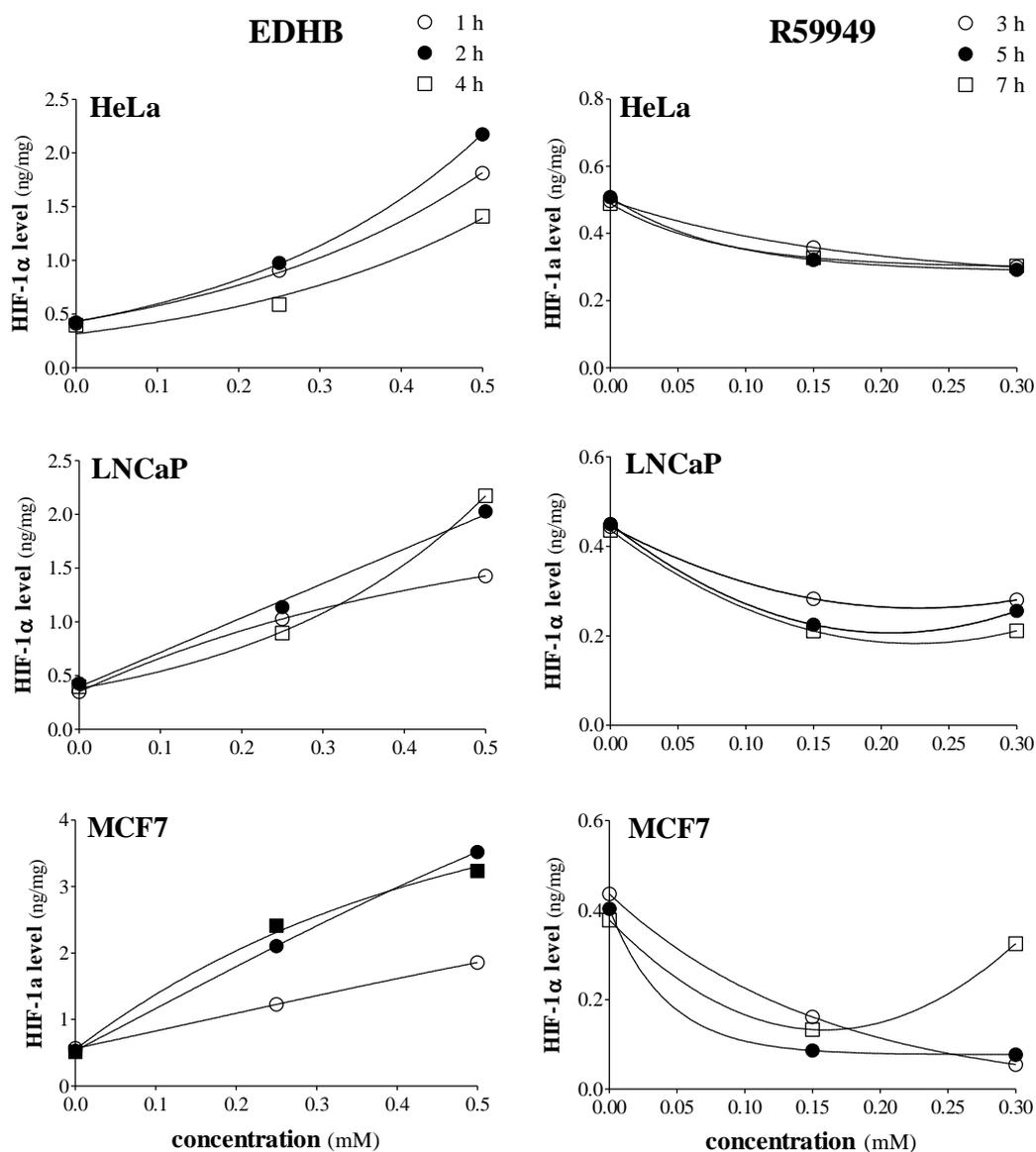


Figure 3.4. Changes in HIF-1α protein level in three cell lines after EDHB or R59949 exposure. Cell cultures seeded in glass Petri dishes at a final density of 1×10^6 cell/dish were treated with EDHB at 0.25 mM or 0.5 mM for 1, 2 or 4 hours, or R59949 at 0.15 mM or 0.3 mM for 3, 5 or 7 hours, then rapidly cooled, lysed and analysed for HIF-1α protein level by ELISA. Data shown are from a pilot experiment.

Solvent toxicity

Toxicity of the drug solvent, DMSO, was also tested. At the highest exposure used, 0.6% (v/v) for 3 hours, there was no effect on either HeLa or MCF7, but slight toxicity ($p=0.047$) to LNCaP cells (Fig. 3.5).

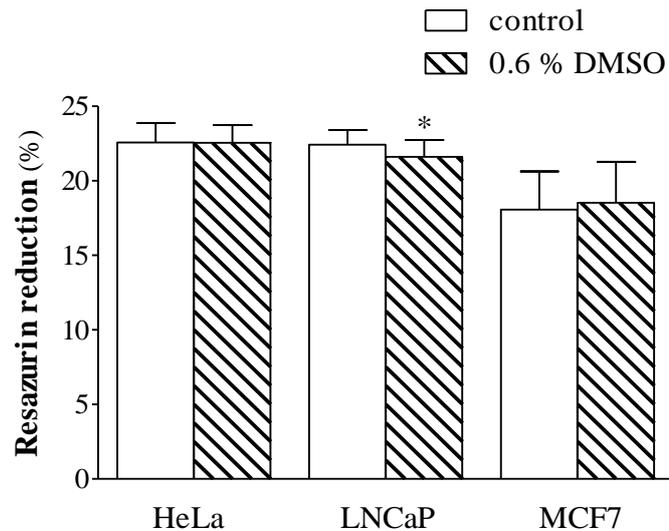


Figure 3.5. **Solvent (DMSO) effect on subsequent cell proliferation.** Cell cultures seeded into glass Petri dishes at a final density of 4×10^5 cells/dish were exposed to 0.6% (v/v) DMSO for 3 h, then harvested, counted and reseeded at low densities. Resazurin reduction was measured 7 days later. Data shown are means \pm SE from 3 separate experiments. Comparison: * treated vs. untreated control.

Induction of severe hypoxia

Subconfluent cultures seeded on borosilicate glass Petri dishes (55 mm or 70 mm diameter, at a final density of 4×10^5 or 1×10^6 cells/dish respectively) were subjected to hypoxia (hereafter referred to as *severe hypoxia* (SH) to distinguish it from *moderate hypoxia* (MH) used later) for 1 h at 37°C in a Modular Incubator Chamber

(diameter 30.5 cm; height 11.9 cm; MIC-101, Billups-Rothenberg, Del Mar, CA, USA). The chamber was flushed with oxygen-free nitrogen (BOC Gases, Dublin, Ireland) for 38 minutes (10 L/minute for 8 minutes, then 2 L/minute for 30 minutes) after which the inlet and outlet tubes were clamped for a further 22 minutes. At the end of this period dissolved oxygen concentration in the culture medium remained below 0.2% (the limit of detection by the oxygen probe), and this extreme hypoxia was maintained for at least two hours while inlet and outlet tubes remained clamped (S. Anopokumar-Dukie, *PhD thesis*, 2006). Effects on the culture medium of gassing for 38 minutes with dry O₂-free N₂ had been investigated previously. Medium evaporation, measured gravimetrically, was less than 0.2% (w/w) and pH was almost unchanged (Δ pH=0.02) comparing to normoxic conditions (Anoopkumar-Dukie S., *PhD thesis*, 2006). Figure 3.6 shows schematically the arrangement used to induce SH.

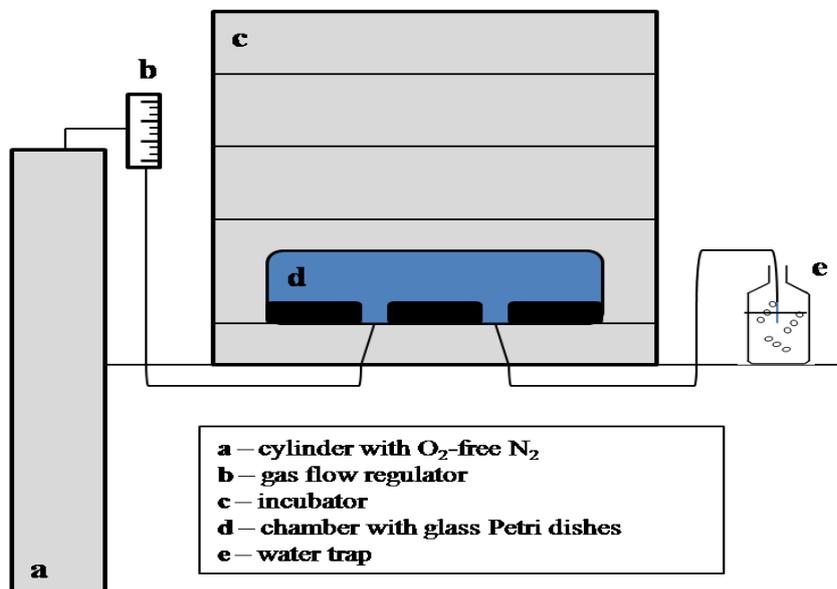


Figure 3.6 System for induction of SH in cell cultures within a controlled-atmosphere chamber (*schematic, not to scale*).

Irradiation of cell cultures

When a photon beam propagates through an irradiated object the dose amplifies to a certain depth that depends on the beam energy and the object itself before decreasing again at greater depths (Fig. 3.7). The region between the surface where photon beams are incident on the target and the depth at which dose reaches a maximum is known as the *dose build-up region* and depends on photon beam energy. In experiments described here cell cultures were irradiated at 6 megavolts (MV), for which the absorbed dose maximum is attained at a depth of 1.5 cm in biological tissue or its equivalent. Irradiation was performed with a Siemens Oncor Linear Accelerator (Erlangen, Germany) in the Radiation Oncology Department at Cork University Hospital. Following irradiation cells were then returned to the laboratory within 5 minutes.

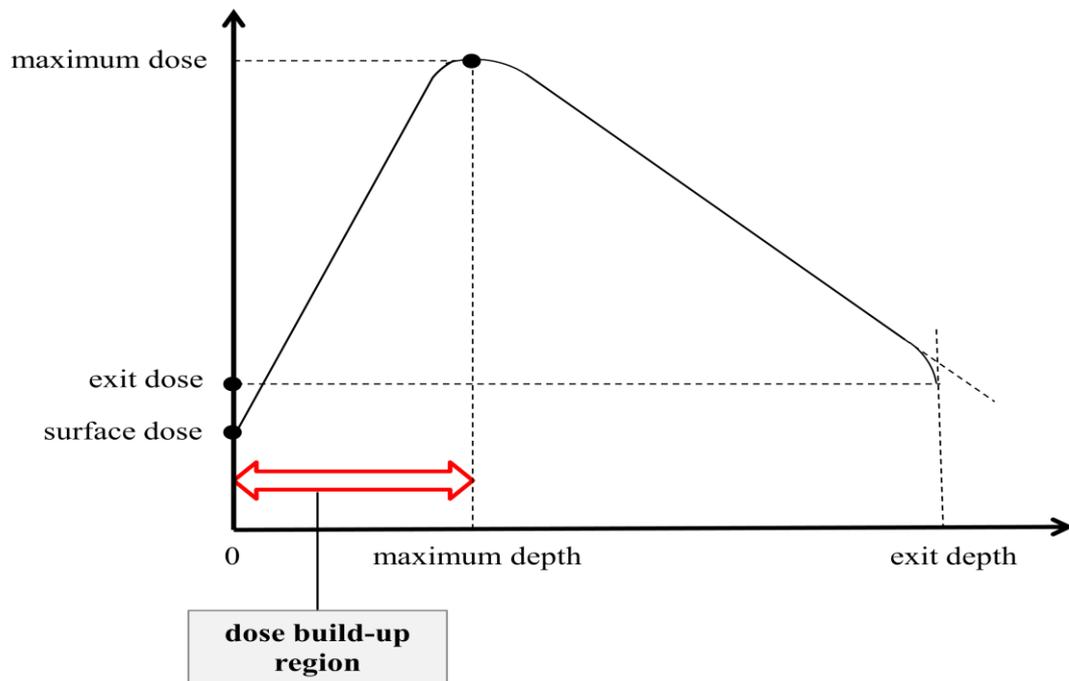


Figure 3.7. **Dose deposition from a megavolt photon beam** (adapted from Podgorsak, 2005, Figure 6.3).

Cell cultures in glass Petri dishes (55 mm or 70 mm diameter, at a final density of 4×10^5 or 1×10^6 cells/dish, respectively) received a dose of 6.2 Gy at 3Gy/minute under normoxia or SH. The cells in controlled-atmosphere MIC-101 chambers were placed on a tissue equivalent slab (1.5 cm high) located on the treatment couch and centered within the 40 x 40 cm irradiation field using the laser position guides. The linear accelerator gantry was rotated 180° and cells were irradiated from below. Depending on the experiment, cells were either returned within 5 minutes to the laboratory for subculture or when cell lysates were to be prepared, plates were placed on ice within 1 minute of irradiation. The settings for irradiation were established previously (Anoopkumar-Dukie S, PhD Thesis, 2006). In general the dose measurements were confirmed using thermoluminescent dosimetry (Anoopkumar-Dukie *et al.*, 2005). The total dose uncertainty was estimated as $\pm 5\%$. Figure 3.8 shows the physical arrangement used.

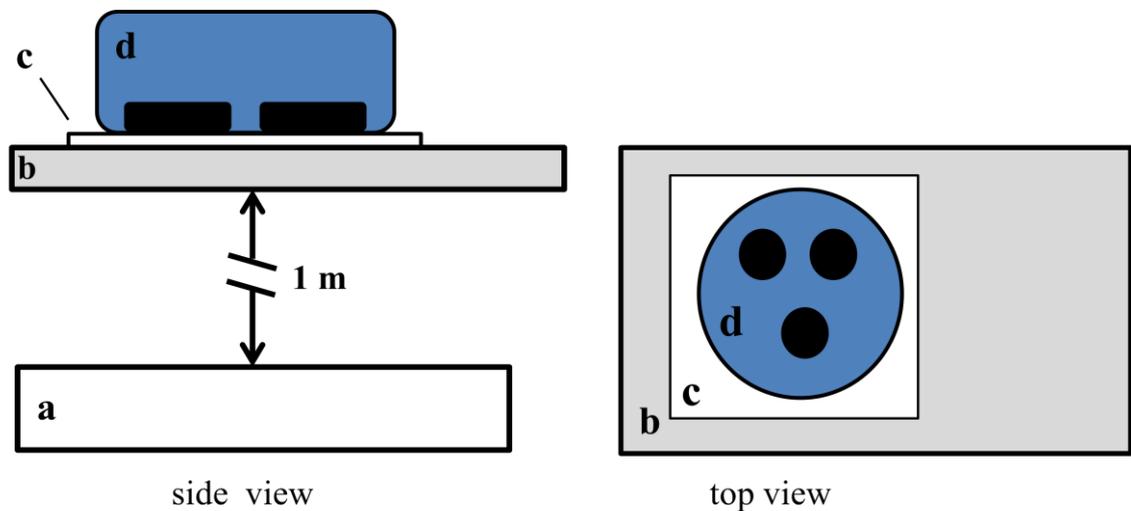


Figure 3.8 **Configuration used for irradiation of cell cultures in controlled atmosphere chambers** (*not to scale*) **a** – linear accelerator gantry, **b** – treatment table, **c** – tissue equivalent pad (1.5 cm high) centered around 40 cm x 40 cm radiation field, **d** – MIC-101 controlled-atmosphere chamber with glass Petri dishes containing cultures.

Experimental design

The standard model we adopted for studying oxygen-dependent radiosensitivity involved cell irradiation under normoxia or SH, followed by subculturing and measurement seven days later of irradiation impact on cell proliferation. In general cells were seeded according to their doubling time and plating efficiency the day before either being transfected or exposed to drug, SH or irradiation. Transfected cells were reseeded into glass Petri dishes 24 hours before irradiation so that at that time (see later) the HIF-1 α knockdown was maximal. Irradiation was always the last part of the treatment after which, cells were either rapidly cooled (< 1 minute) by placing the Petri dish on ice and lysed, or else harvested (< 10 minutes), counted and reseeded at low densities then allowed to grow for seven days before measurement of cell proliferation. The experimental design is summarized in Figure 3.9.

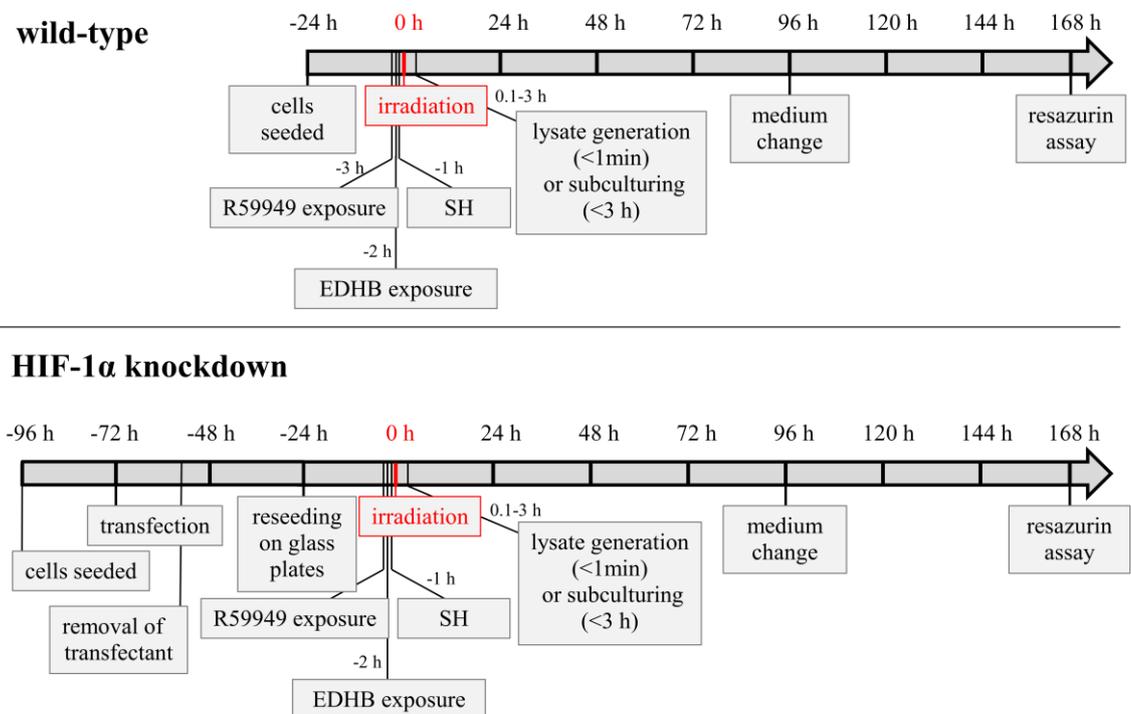


Figure 3.9. Timelines for hypoxic irradiation experiments (not to scale).

Results

Effect of SH on subsequent cell proliferation

Acute and temporary oxygen depletion is one of the two hypoxia types that occur commonly in human solid tumours. A standard procedure of 1 hour profound hypoxia (SH) was used as a first approximation to the highly variable oxygen depletion which tumour cells experience *in vivo*, in terms of severity, duration and fluctuation. In this *in vitro* model cell proliferation capacity following acute SH insult in the absence or presence of drugs was measured in terms of resazurin reduction seven days later after 5-7 cell divisions had occurred. This model was chosen to reflect longer-term responses of tumour cells to non-fractionated radiotherapy.

Ideally the impact of the hypoxic insult should be minimal if the effect of the other interventions is not to be obscured. In order to test the effect of SH on subsequent proliferation of the three cancer cell lines used we subjected cell cultures (plated in glass Petri dishes and placed in the environmentally controlled chambers) either to standard normoxic conditions (5% CO₂ in air, \geq 95% humidity) or to 1 hour of severe oxygen depletion ($<$ 0.2 % O₂). Figure 3.10 shows that acute SH insult did not significantly affect the proliferative capacity of any of the three cell lines ($p > 0.05$) as measured 7 days later in terms of resazurin reduction.

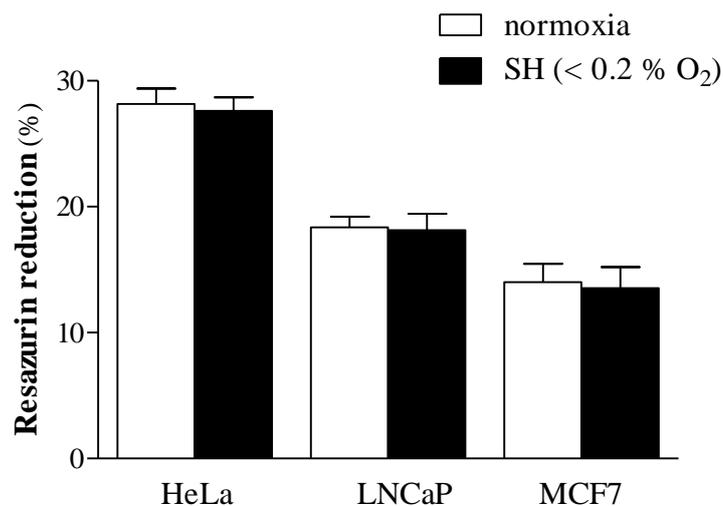


Figure 3.10. **Lack of effect of acute SH on subsequent cell proliferation.** Cells cultures were seeded in glass Petri dishes (55 mm diameter) at a final density of 4×10^5 cells/dish. After incubation overnight cells were subjected to acute SH (< 0.2% O₂) for 1 hour then reoxygenated and subcultured for 7 days under standard conditions before cell proliferation was measured in terms of resazurin reduction. Data are means \pm SE from 5 separate experiments. Comparison SH vs. normoxia, $p > 0.05$.

Oxygen-dependent radiosensitivity

Previous work in our laboratory had shown that proliferation of HeLa cells irradiated at 6.2 Gy under normoxia or SH began to diverge measurably after 3 days or approximately 2-3 divisions, as measured in terms of resazurin reduction (Anoopkumar-Dukie S., *PhD Thesis*, 2006, p.45). By 4 days the difference was highly significant ($p < 0.001$). Therefore we routinely measured cell response 7 days after irradiation, by which time cells had had time to undergo 5 (MCF7) – 7 (HeLa, LNCaP) division cycles and more subtle differences in proliferative capacity should become evident.

In order to establish a standard *in vitro* model of oxygen-dependent radiosensitivity the cells cultures were irradiated at 6.2 Gy either in the presence or

virtual absence of oxygen. Proliferation capacity of all three cell lines was significantly reduced after irradiation under normoxia (Fig 3.11). However this injury was significantly attenuated if cells were irradiated under SH, confirming that each cell line showed clear oxygen-dependent radiosensitivity under these conditions.

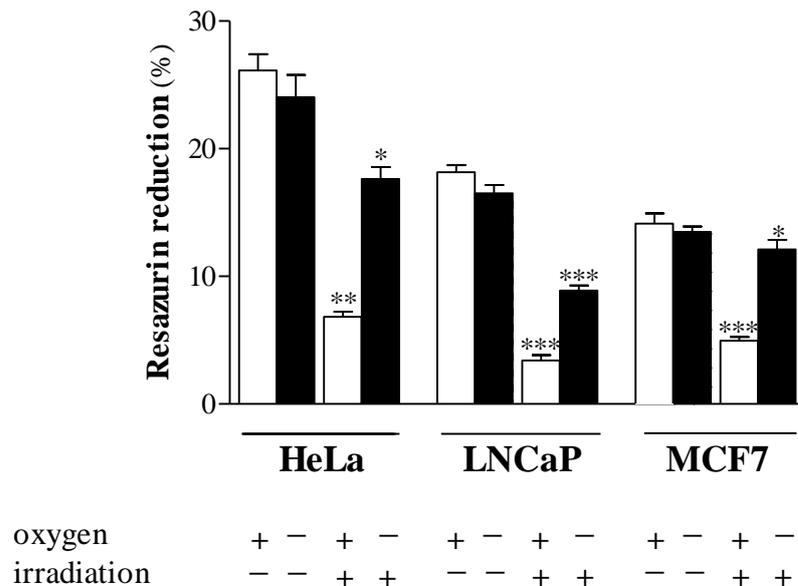


Figure 3.11. **Cell proliferation seven days after irradiation under normoxia or SH.** Cultures seeded overnight at a density of 4×10^5 cells/55 mm Petri dish then incubated for 1 h under normoxia or SH ($< 0.2\% O_2$) were irradiated at 6.2 Gy and subcultured in 96-well plates. After seven days resazurin reduction was measured. Data are means \pm SE from 6 separate experiments. Comparison: *, irradiated vs. unirradiated controls.

To facilitate comparison between the cell lines oxygen-dependent radiosensitivity data were normalized and presented as a percentage of unirradiated controls (Fig. 3.12). Hypoxic MCF7 cells were the most resistant to irradiation (10% cell death), followed by HeLa and LNCaP cells with 26 % and 46 %, respectively. LNCaP cells were also the most sensitive to irradiation under normoxia (81% cell

death) as compared to HeLa (74%) or MCF7 (65%) but *relative* sensitivity (SH vs. normoxia) was similar in all three cell lines.

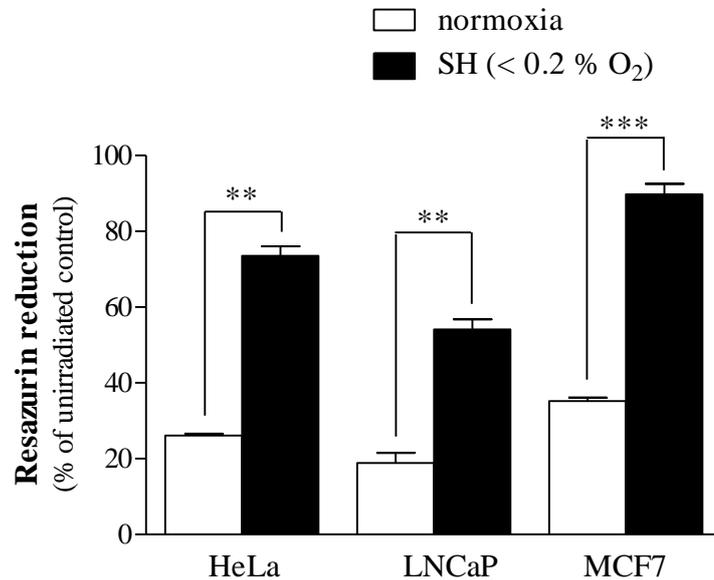


Figure 3.12. **Oxygen-dependent radiosensitivity of 3 cancer cell lines.** Cultures seeded at a final density of 4×10^5 cells/55 mm Petri dish then incubated for 1 h under normoxia or SH (< 0.2% O₂) were irradiated at 6.2 Gy and subcultured in 96-well plates. After 7 days resazurin reduction was measured. Data are means \pm SE from 6 separate experiments. Comparison: *, SH vs. normoxia.

Impact of passage number on oxygen-dependent radiosensitivity

To determine whether passage number affected oxygen-dependent radiosensitivity we compared early (≤ 10), intermediate (11-29) and late (≥ 30) passages of HeLa cells. Surprisingly, under normoxia early passages appeared to be more sensitive to irradiation relative to intermediate passages, whereas under SH the high passages were more radioresistant (Fig. 3.13). Furthermore, the absolute resazurin reduction rate was increased in high passages by almost 10 % suggesting passage number-dependent stimulation of cell proliferation. Therefore to reduce this source of

variability all subsequent experiments were carried out using passages 10-30 only. However significant oxygen-dependent radiosensitivity was observed across the full range of passages tested.

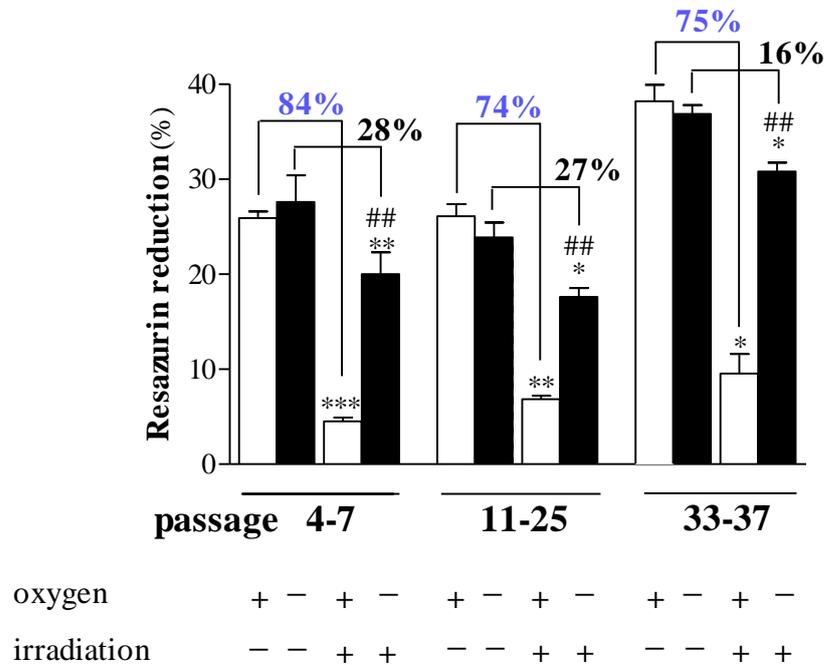


Figure 3.13. **Impact of passage number on oxygen-dependent radiosensitivity of HeLa cells.** Cultures from passages between 4 and 37 seeded at a final density of 4×10^5 cells/55 mm Petri dish then incubated for 1 h under normoxia or SH ($< 0.2\% O_2$) were irradiated at 6.2 Gy and subcultured in 96-well plates. After 7 days resazurin reduction was measured. Data are means \pm SE from 3 separate experiments. Comparisons: *, irradiated vs. corresponding unirradiated control; #, SH vs. normoxic.

Changes in HIF-1 α protein level

Although HIF-1 α is stabilized in cells by hypoxia, relatively little is known about how levels change over time following either acute SH insult or exposure to ionizing radiation. Therefore we investigated how these stimuli, separately or together, affected HIF-1 α level over the 48-hour period after treatment since consequent changes in gene expression might influence cell proliferation as measured after 7 days.

Effect of irradiation

Figure 3.14 shows that HIF-1 α was readily measurable in all three cell lines under normoxia (*control*), but was not greatly affected by irradiation at 6.2 Gy. Only in LNCaP cells over the period 24-48 h and in MCF7 at 24 h but not 4 or 48 h were small deviations found. Therefore irradiation at 6.2 Gy under standard normoxic conditions had little effect on HIF-1 α protein level over the following 48 hours in any of the cell lines tested.

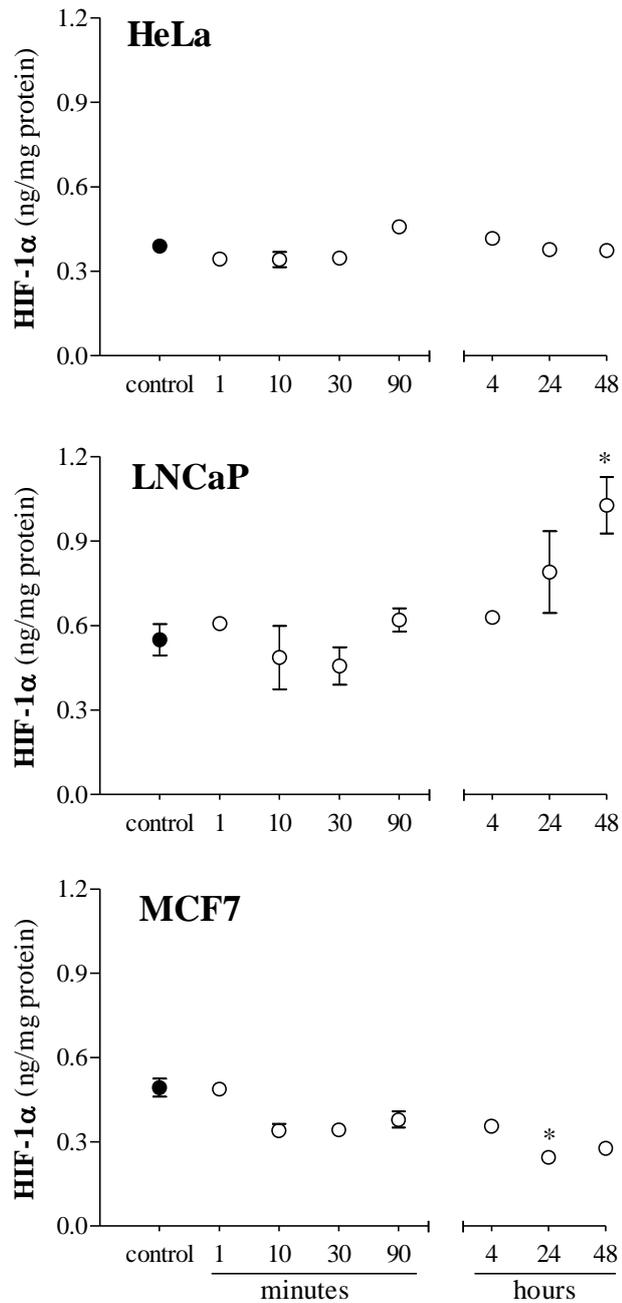


Figure 3.14 **HIF-1 α** protein level in total cell extracts following irradiation under **normoxia**. Normoxic cultures (10^6 cells/dish) were irradiated at 6.2 Gy, then at defined time points cooled to 4°C and lysed for measurement of HIF-1 α protein level by ELISA. Data are mean \pm SE from 3 separate experiments. Comparison: *, irradiated vs. unirradiated control.

Effects of reoxygenation after SH without and with irradiation

Although it is well established that oxygen depletion *in vitro* and *in vivo* increases HIF-1 α protein level, we tested how this was reversed following reoxygenation in each cell line. Figure 3.15 (*left column*) shows that SH (<0.2 % O₂ x 1 h) induced a transient increase in HIF-1 α protein level that persisted for at least 10 minutes after reoxygenation but was then followed by rapid HIF-1 α degradation below control levels by 30 minutes in two of the three cell lines. This decrease in HIF-1 α protein level was sustained for at least 48 hours in HeLa cells. By contrast, in LNCaP cells HIF-1 α level had returned to control levels by 48 hours. In MCF7 cells HIF-1 α level had recovered to control levels within 30 minutes of reoxygenation and remained stable thereafter.

SH-treated cells also subjected to irradiation showed patterns of HIF-1 α changes generally similar to those of unirradiated controls (Figure 3.15, *right column*). A transient increase at 1 and 10 minutes, most marked in HeLa and MCF7, was followed by a return to control levels (LNCaP and MCF7 cells) or below (HeLa) from 30 minutes onward. Irradiation caused a smaller delayed rise in HIF-1 α protein level at later time points (≥ 4 h) in HeLa and LNCaP but not in MCF7 cells.

SH (<0.2 % O₂)

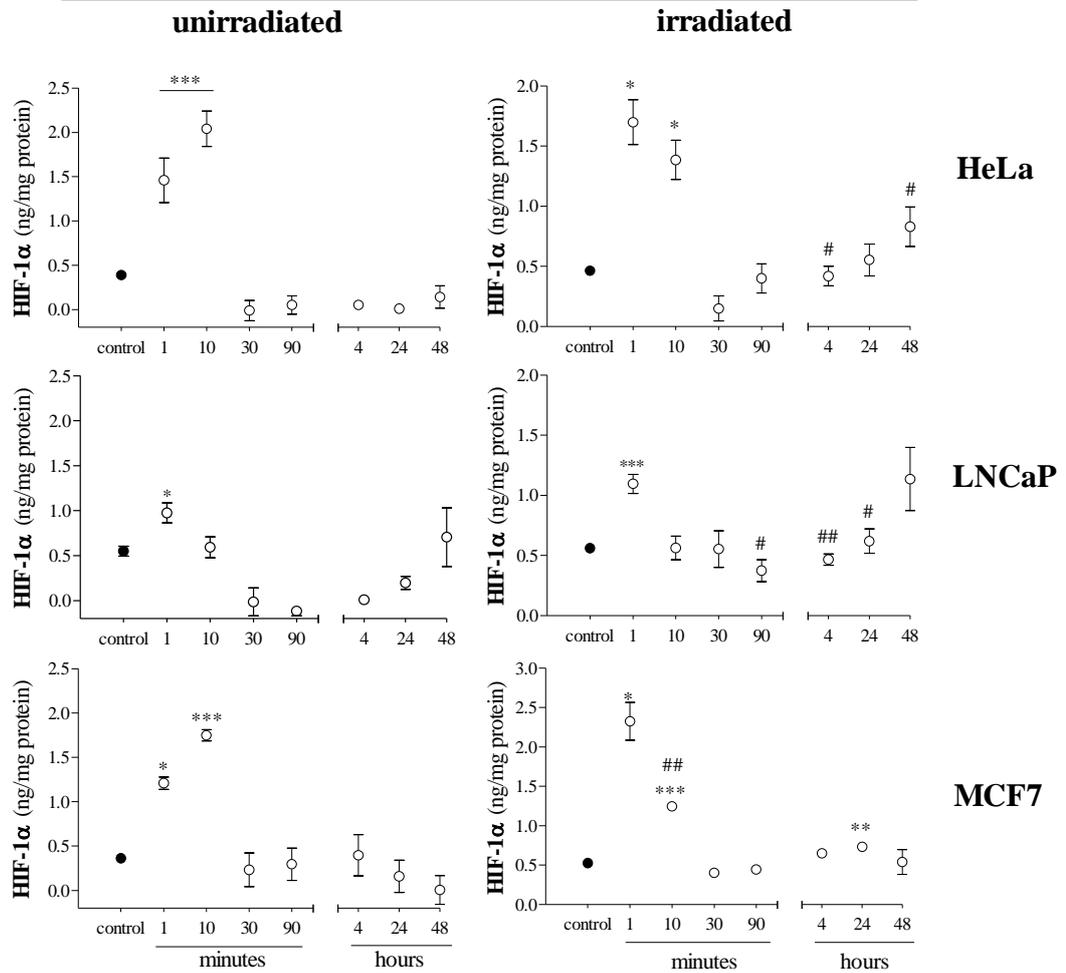


Figure 3.15. **Time course of changes in HIF-1α protein level following hypoxia and irradiation and the effect of irradiation.** SH(<0.2% O₂ x 1 h) cultures (10⁶ cells/dish) were irradiated at 6.2 Gy, then reoxygenated at the indicated time points cooled to 4°C and lysed for measurement of HIF-1α protein level by ELISA. Data are means ± SE from 3 separate experiments. Comparisons: *, SH vs. normoxic controls; #, irradiated vs. unirradiated. Note difference in scale of vertical axes between left and right panels.

For all subsequent experiments cells were cooled within 1 minute of reoxygenation to reflect as closely as possible HIF-1 α level and DNA binding during the preceding period of hypoxia. Figure 3.16 shows combined data for HIF-1 α protein level as measured immediately (≤ 1 min) after reoxygenation.

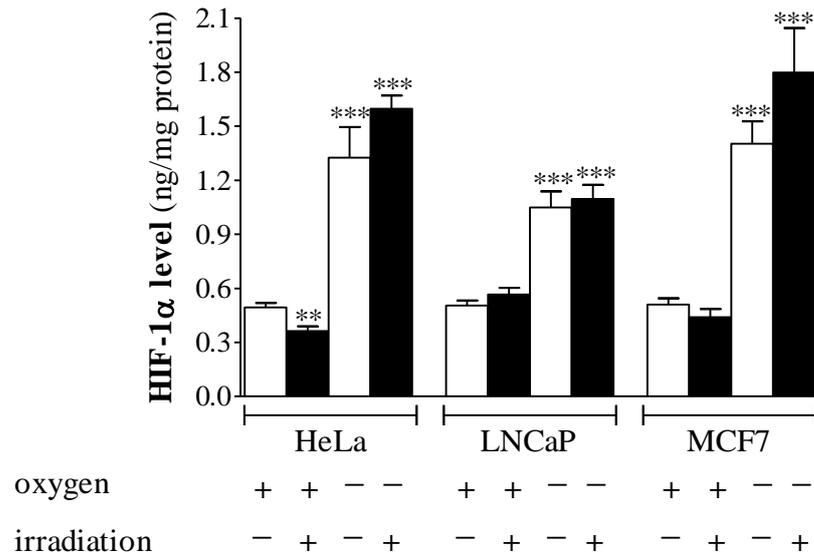


Figure 3.16. **Acute impact of SH and/or irradiation at 6.2 Gy on HIF-1 α protein level in three cell lines.** Cells cultures were plated at 1×10^6 cells/55 mm dish, incubated overnight then exposed to normoxia or SH (0.2% O₂) for 1 hour before irradiation. Then within less than one minute they were rapidly cooled to 4°C, lysed and analysed for HIF-1 α level by ELISA. Data shown are from 9 separate experiments. Comparison *, treated vs. untreated controls.

Collectively these experiments confirm that all three cell lines showed oxygen-dependent radiosensitivity under the conditions used. Moreover, all three cell lines showed oxygen-dependent changes in HIF-1 α level but little or no further effect of irradiation at 6.2 Gy.

Effects of EDHB and R59949 on oxygen- and irradiation-dependent changes in HIF-1 α protein level

An inhibitor (EDHB) and an indirect activator (R59949) of prolyl hydroxylases were used to pharmacologically alter HIF-1 α protein level in each cell line in order to test whether this affected oxygen-dependent radiosensitivity. It had already been shown that EDHB and R59949 caused the most marked effect after cell exposure to either 0.5 mM x 2 h or 0.3 mM x 3 h respectively (Fig. 3.4). Therefore next we pre-treated cells with either EDHB or R59949 under these conditions, then exposed them to 1 h SH without or with concomitant irradiation in order to determine acute effects on HIF-1 α level.

Effects of EDHB and R59949

EDHB significantly increased HIF-1 α protein level in non-irradiated and irradiated cells under both normoxia and SH (Fig. 3.17). Conversely, R59949 treatment reduced HIF-1 α protein levels in normoxic HeLa, LNCaP and MCF7 cells by 66, 51 and 27 %, and in SH cells by 63, 50 and 70%, respectively. In irradiated normoxic cells R59949 exposure did not affect HIF-1 α protein levels as compared to the untreated controls, whereas levels elevated under SH were significantly reduced by 43, 57 and 75 % in HeLa, LNCaP and MCF7 cells respectively (#, Fig. 3.17).

Effect of SH

As previously shown, a 1 h period of severe oxygen deficiency caused significant elevation in HIF-1 α protein levels across all three cell lines (Fig 3.15).

However the increase of HIF-1 α protein level caused by EDHB in normoxic unirradiated cells was not further modulated by SH.

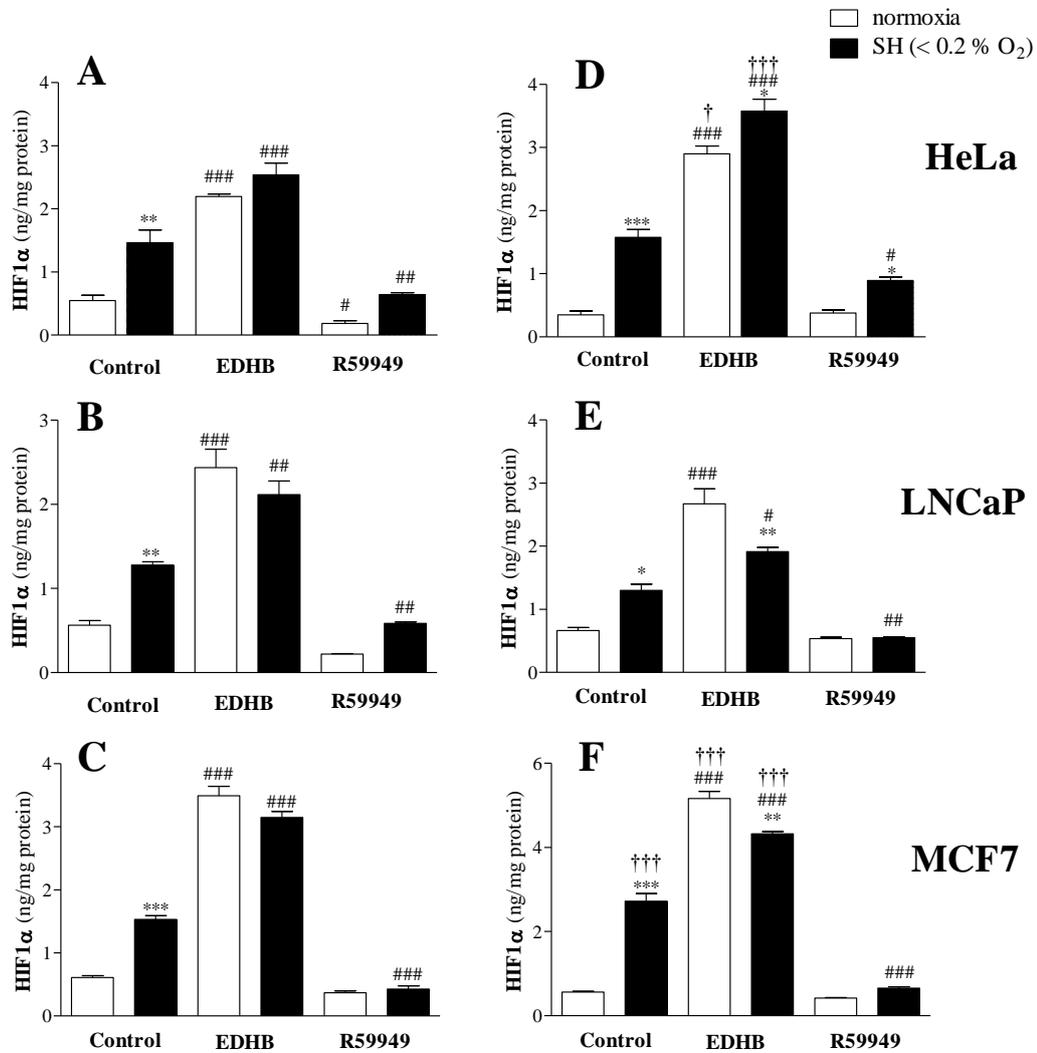


Figure 3.17. Impact of EDHB or R59949 on HIF-1 α level in total cell lysates of unirradiated (A-C) and irradiated (D-F) HeLa, LNCaP and MCF7 cells. Cells pretreated with EDHB (0.5 mM x 2 h) or R59949 (0.3 mM x 3 h) together with SH (<0.2% O₂) for the last hour were irradiated (6.2 Gy) as indicated (D-F), then harvested and HIF-1 α levels were measured by ELISA. Data are means \pm SE from 3 experiments. Comparisons: *, SH vs. normoxia; #, drug-treated vs. untreated; †, irradiated vs. unirradiated. Note differences in scale of the vertical axes.

EDHB also caused HIF-1 α accumulation in all normoxic irradiated cells but SH raised it further only in HeLa cells, whereas in LNCaP and MCF7 cells SH decreased it. When comparing the effect of SH on R59949 treatment in irradiated cells, HIF-1 α protein level was increased in all cell lines except irradiated LNCaP and unirradiated MCF7 cells (*, Fig 3.17). In all cases combination of SH with R59949 pretreatment brought HIF-1 α back to control levels i.e. the opposing effects largely cancelled one another out.

Effect of irradiation

The acute effect of irradiation on HIF-1 α level was little altered by the oxygen status of the cells. Under normoxia irradiation at 6.2 Gy did not significantly alter HIF-1 α protein level, while irradiation of SH cells did not affect HIF-1 α protein level as compared to unirradiated conditions (consistent with data in Fig 3.14). However irradiation caused a further increase in HIF-1 α protein level in normoxic and SH EDHB-treated HeLa and MCF7 cells, whereas it has no effect on HIF-1 α level modulation in EDHB-treated LNCaP cells (†, Fig. 3.17). In R59949 pre-treated normoxic cells there was no effect of modulation, but under SH irradiation increased HIF-1 α protein level in HeLa and MCF7 cells.

Overall therefore, HIF-1 α level was generally increased by EDHB, SH and irradiation, and decreased by R59949. These effects with small exceptions were consistent across all three cell lines.

HIF-1 DNA binding activity

To test how previously observed changes in HIF-1 α level correlated with ability to interact with its DNA target motif, cells incubated under normoxia or SH were exposed to either EDHB or R59949 and the HIF-1 DNA binding activity in nuclear extracts was measured. Samples for DNA binding analysis were prepared like those for measurement of protein level, in that hypoxic cultures were cooled to 4° C within 1 minute of reoxygenation before further processing.

Figure 3.18 shows that following 1 h SH the HIF-1 DNA binding activity was only increased slightly in each of the cell lines tested, most clearly in HeLa. This does not correlate well with HIF-1 α protein level which was significantly raised in all three cell lines after 1 h SH (Fig. 3.16). However pretreatment with EDHB caused larger increases in HIF-1 DNA binding in all three cell lines under normoxia, most markedly in MCF7 cells. Combination of EDHB pretreatment with SH caused further increase in HIF-1 DNA binding only in LNCaP cells, whereas in HeLa and MCF7 binding was decreased as compared to cells treated with EDHB under normoxia. By contrast in both normoxic and SH cells pretreated with R59949 HIF-1 DNA binding activity remained at about normoxic control levels (Fig. 3.18), whereas total HIF-1 α levels had been significantly reduced in all cell lines treated with R59949 both under normoxia and SH (Fig.3.17).

Overall, although SH- or EDHB-induced rise in HIF-1 α protein level is significant in all three cell lines tested, a marked increase in HIF-1 DNA binding activity was evident only in EDHB-treated cells. Conversely, R59949 reduction of HIF-1 α level was not found to correlate with any measurable decrease in HIF-1 DNA

binding activity. Therefore in comparison to HIF-1 α protein level, HIF-1 DNA binding is either less responsive to the conditions used or responds more slowly.

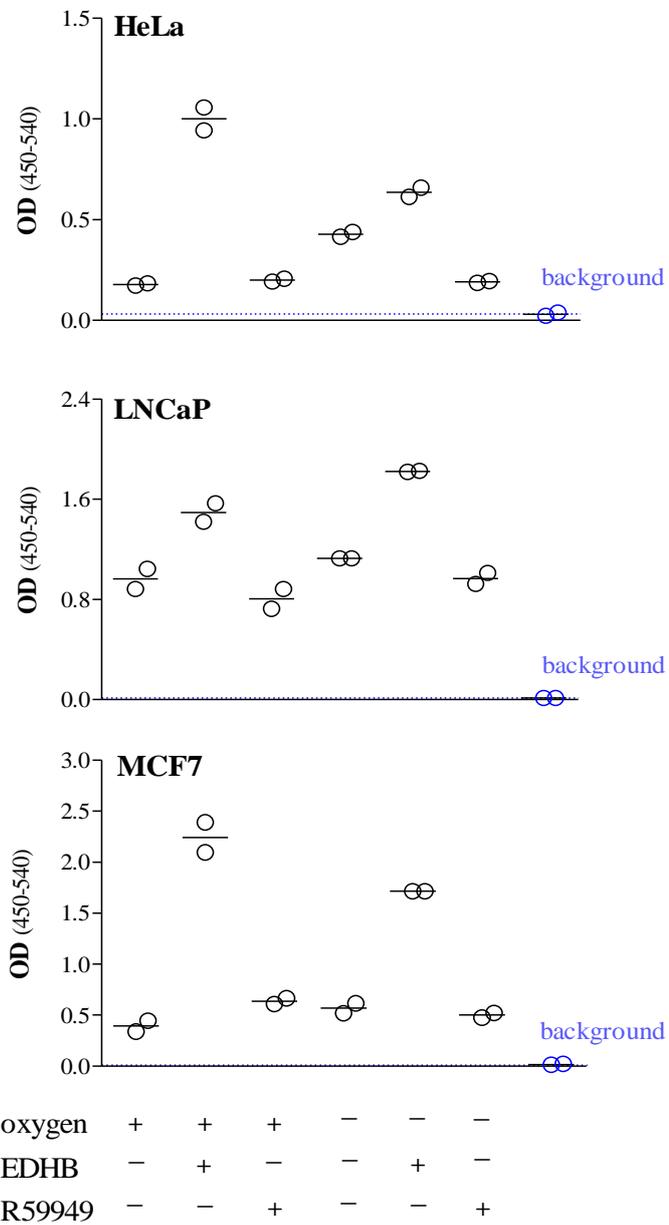


Figure 3.18. **HIF-1 DNA-binding activity.** Cell cultures pretreated with EDHB (0.5 mM x 2 h) or R59949 (0.3 mM x 3 h) under normoxia or SH (<0.2% O₂) conditions for the last hour were then reoxygenated and immediately cooled to 4°C (<1 min) before being lysed for preparation of nuclear extracts and analysed for HIF-1 DNA-binding activity. Data shown are individual values from 2 independent experiments.

HIF-1 α distribution between cytoplasmic and nuclear fractions

Stabilisation of HIF-1 α protein in the cytoplasmic fraction is followed by its translocation to the nucleus in order to dimerise with HIF-1 β subunit and subsequently bind to HREs of target genes. Since HIF-1 α protein level measured in whole cell extracts did not fully correlate with HIF-1 DNA binding we also examined HIF-1 α protein distribution between the cytoplasmic and nuclear fractions.

Under normoxia total HIF-1 α level was similar in HeLa, LNCaP and MCF7 cells (Fig. 3.16) but HIF-1 DNA binding varied. Therefore the two cell lines (HeLa and LNCaP) which differed most in terms of basal HIF-1 DNA binding activity were compared. Distribution of HIF-1 α protein between the cytoplasmic and nuclear fractions of these normoxic cells varied (Table 3.1). However, although more HIF-1 α was present in the nuclear fraction of LNCaP (50% versus 36% in HeLa), this was far less than the almost 3-fold difference in HIF-1 DNA binding signal between the two cell lines (Fig. 3.17). Following 1 h SH HIF-1 α protein predominated in nuclear fraction (70%) of HeLa cells, whereas its distribution was more equal between cytoplasmic and nuclear fraction in LNCaP and MCF7 cells (Table 3.1). Furthermore, although 24 h MH significantly increased HIF-1 DNA binding activity in HeLa cells (Fig. 4.20) its effect on HIF-1 α protein subcellular distribution was less than that caused by 1 h SH (Table 3.1). In conclusion, although the data are limited (n=1) it appears that differences in HIF-1 DNA binding were not consistent with HIF-1 α protein distribution between nuclear and cytoplasmic fractions of the cells.

Cell type	Oxygenation status	HIF-1 α (% of total) / Fraction	
		Cytoplasmic	Nuclear
HeLa	normoxia	64	36
LNCaP	normoxia	50	50
HeLa	SH	30	70
LNCaP	SH	45	55
MCF7	SH	55	45
HeLa	MH	43	57

Table 3.1 **Subcellular distribution of HIF-1 α protein in wild-type HeLa, LNCaP and MCF7 cells.** *SH* (<0.2% O₂ x 1 h), *MH* (0.5% O₂ x 24 h) or normoxic cultures were immediately cooled to 4°C then fractionated for measurement of HIF-1 α level by ELISA. Data are from a pilot set of measurements (n=1).

Effects of EDHB and R59949 on subsequent cell proliferation

The next series of experiments tested whether and how EDHB and R59949 exposures previously shown to alter HIF-1 α protein status also affected subsequent cell proliferation. Cell cultures pretreated with EDHB or R59949 then subjected to standard normoxia or to SH for 1 hour were subcultured for 7 days after which cell proliferation was measured in terms of resazurin reduction. Figure 3.19 shows that although EDHB had not impaired the proliferative capacity of any cell line under SH, it reduced that of normoxic LNCaP cells (90 % of untreated control). By contrast, R59949 exposure significantly decreased subsequent proliferation of all three cell lines. The effect was most marked in SH LNCaP cells in which resazurin reduction was reduced by 49 %, whereas proliferation of SH R59949-treated HeLa and MCF7 cells was impaired by 15 and 21 % respectively. It had previously been established that solvent (DMSO) at the highest concentration used (0.6%) was not toxic to HeLa or MCF7 cells but showed

minor toxicity (3 %) to LNCaP cells (Fig 3.4). Therefore toxicity observed here was drug-related, potentially due to non-specific effects of R59949 at the high concentration used relative to its IC₅₀ for purified enzyme.

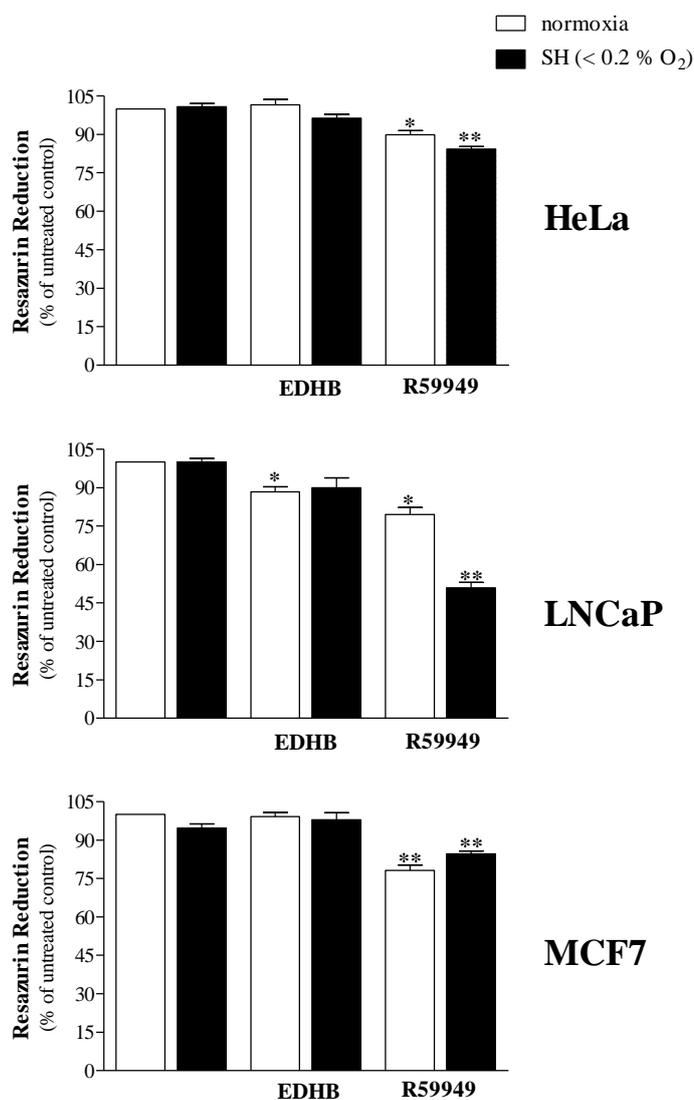


Figure 3.19. **Effect of EDHB or R59949 pretreatment on subsequent proliferation of HeLa, LNCaP and MCF7 cells.** Cells treated with EDHB (0.5 mM x 2 h) or R59949 (0.3 mM x 3 h), together with SH (<0.2% O₂) for the last hour, were subcultured in 96-well plates. After 7 days resazurin reduction was measured. Data are means ± SE from 3 experiments. Comparisons: * treated vs. untreated controls.

Effect of EDHB or R59949 pretreatment of cells on oxygen-dependent radiosensitivity

Having established the functional effect of altered HIF-1 α status on subsequent cell proliferation, the effect on relative sensitivity to irradiation under normoxia or SH was tested. Cell cultures pre-exposed to EDHB or R59949 were subjected to normoxia or 1 hour SH then irradiated at 6.2 Gy and subcultured for 7 days, after which cell proliferation was measured in terms of resazurin reduction.

Clear oxygen-dependent radiosensitivity remained evident in all cases, except EDHB-treated LNCaP (*, Fig. 3.20). Both EDHB and R59949 pretreatments partly protected normoxic HeLa and MCF7 cells (#) against irradiation injury, while having no effect on LNCaP cells. Under SH the effects were more complex. Namely, EDHB had no effect on irradiation-induced cell damage in LNCaP and MCF7 cells, whereas it caused protection in HeLa cells. By contrast, R59949 partly radiosensitised HeLa and MCF7 cells, while again having no effect on LNCaP cells (#, Fig. 3.20).

Overall, pharmacological manipulation of HIF-1 α level had limited and mixed effects on oxygen-dependent radiosensitivity across the three cell lines tested. However some protection by EDHB and sensitization by R59949 was evident, consistent with a major causal role of HIF-1 α in oxygen-dependent radiosensitivity.

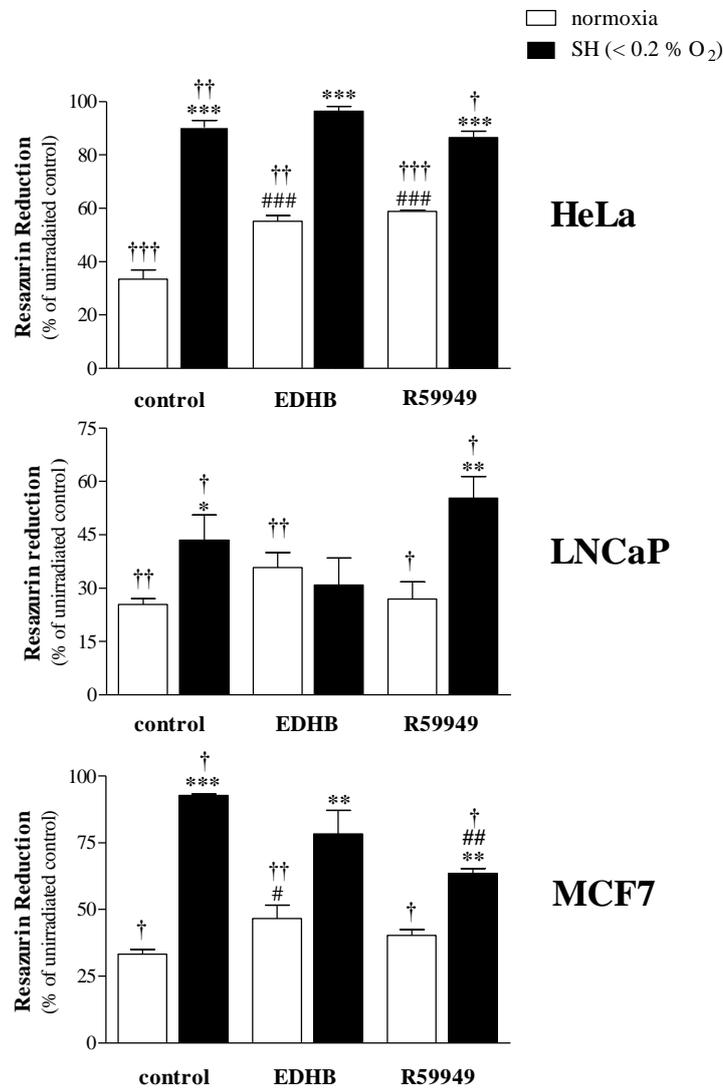


Figure 3.20. **Functional effects of EDHB or R59949 pretreatment on oxygen-dependent radiosensitivity of HeLa, LNCaP and MCF7 cells.** Cells treated with EDHB (0.5 mM x 2 h) or R59949 (0.3 mM x 3 h), together with SH (<0.2% O₂) for the last hour, were irradiated (at 6.2 Gy) and subcultured in 96-well plates. After 7 days resazurin reduction was measured. Data are mean ± SE from 3 experiments. Comparisons: *, SH vs. normoxia; #, drug-treated vs. untreated; †, irradiated vs. corresponding unirradiated controls.

Transient reduction in HIF-1 α expression using siRNA

To further investigate the role of HIF-1 α in oxygen-dependent radiosensitivity of cancer cells, independent experiments using specific siRNA targeted against HIF-1 α were undertaken. As previously shown (Fig. 2.8) the greatest HIF-1 α protein knockdown occurred 72 (HeLa, LNCaP) or 96 (MCF7) h after transfection. At these times HIF-1 α protein level in normoxic HeLa, LNCaP and MCF7 cells was reduced by 87, 72 and 82% of control levels, respectively (Fig. 3.21, *left*). Furthermore the ability of SH (<0.2% O₂ for 1 h) to raise HIF-1 α level was strongly attenuated in HeLa, LNCaP and MCF7 cells transfected with targeted siRNA, and were reduced by 85, 60 and 82 % respectively relative to wild-type SH controls (Fig. 3.21, *right*). Exposure to either transfection reagent alone or together with non-targeting siRNA did not significantly affect HIF-1 α protein level under normoxic conditions in any cell line. Moreover, SH-induced HIF-1 α stabilisation was not altered by either treatment, except in the case of LNCaP cells where pre-exposed to transfection reagent unexpectedly increased HIF-1 α protein level (Fig. 3.21, *right*). Furthermore, exposure of transfected cells to PHD inhibitor EDHB failed to increase HIF-1 α level (Fig.3.21, *left*) in contrast to the 2-3 fold increase it produced in wild-type cells under normoxia (Fig. 3.17). The effect of R59949 was not tested because it was unlikely to reduce measurably the already low HIF-1 α level in knockdown cells (Fig. 3.20, *left*).

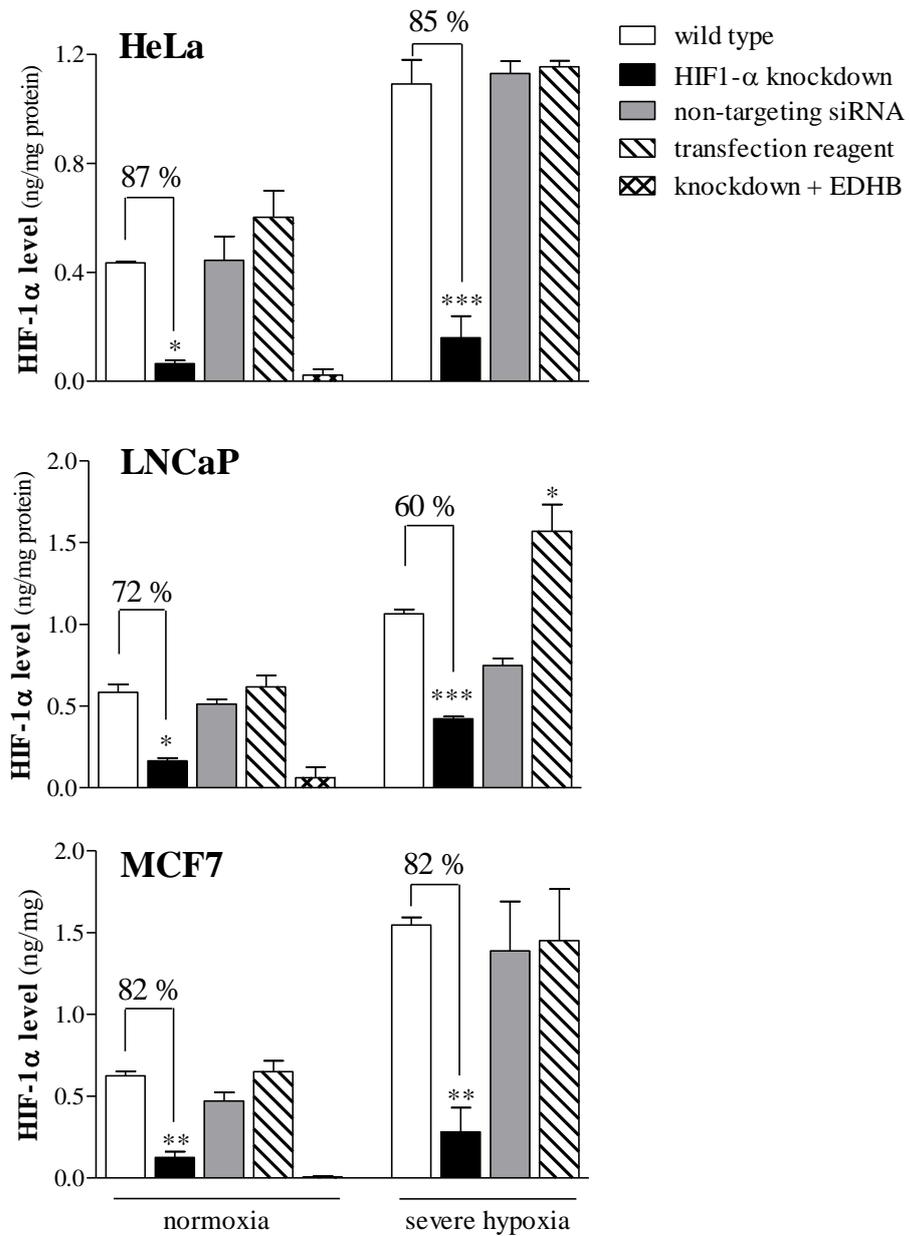


Figure 3.21. **Effect of transfection with siRNA on HIF-1 α level.** Cells transfected with HIF-1 α targeting siRNA, non-targeting siRNA or vehicle either 72 (HeLa, LNCaP) or 96 (MCF7) hours earlier were subjected either to normoxia or SH (<0.2% O₂) for 1 h then cooled to 4°C and lysed for measurement of HIF-1 α level by ELISA. HIF-1 α knockdown cells were also exposed to EDHB (0.5 mM for 2 h) under normoxia then analysed for HIF-1 α by ELISA. Data are means \pm SE from 3 separate experiments except for EDHB-treated cells (n=1). Comparison: *, treated vs. wild type controls.

Effect of transient HIF-1 α knockdown on oxygen-dependent radiosensitivity

Having established that transfection with targeted siRNA significantly reduced HIF-1 α protein level in all three cell lines under both normoxia and SH, the functional effect on oxygen-dependent radiosensitivity was investigated. First, the effect of HIF-1 α knockdown on oxygen-dependent radiosensitivity in each of the three cells lines was analysed by comparing cell proliferation (measured as resazurin reduction) 7 days after irradiation at 6.2 Gy to that of wild-type cells. Figure 3.22 shows that HIF-1 α knockdown conferred significant protection on HeLa and MCF7 (but not LNCaP) cells irradiated under normoxia. By contrast, HIF-1 α knockdown significantly sensitized SH cultures of all three cell lines to irradiation injury.

Overall, HIF-1 α knockdown reduced (HeLa, LNCaP) or abolished (MCF7) the disparity in response to ionizing radiation between normoxic and SH cells. This marked change in oxygen-dependent radiosensitivity reflected either lower radioresistance of SH cells rather than increased radioresistance of normoxic cells (LNCaP), or a combination of both effects (HeLa, MCF7).

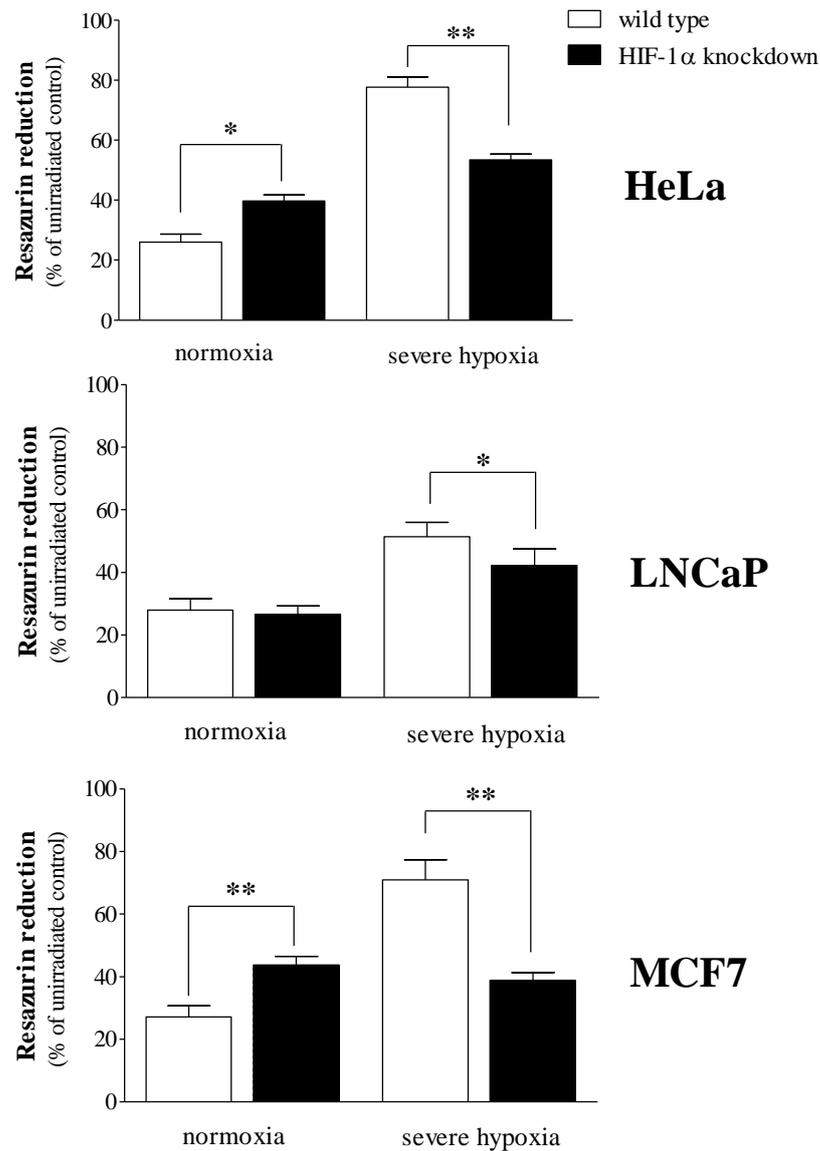


Figure 3.22. **Oxygen-dependent radiosensitivity in HIF-1 α knockdown cells.** Cells were transfected with siRNA either 72 (HeLa, LNCaP) or 96 (MCF7) h before irradiation (at 6.2 Gy) under normoxia or hypoxia (<0.2% O₂ for the last hour), then harvested and subcultured. Cell proliferation was measured 7 days later in terms of resazurin reduction. Data shown are means \pm SE from 3 separate experiments,. Comparisons: *, knockdown vs. wild type.

To further analyse HIF-1 α protein role in oxygen-dependent radiosensitivity of cancer cells we tested the functional effect of combining pharmacological and genetic manipulation of HIF-1 α protein level. In principle this would be expected to reflect drug effects not mediated by HIF-1 α . As shown previously (Fig. 3.21) EDHB treatment did not increase HIF-1 α protein level in knockdown cells. This is consistent with its lack of effect on response of normoxic knockdown cells to irradiation as compared to knockdown control cells (Fig. 3.23), and in contrast to its radioprotective effect on normoxic wild type cells (Fig. 3.20). However EDHB abolished the increased radiosensitivity of SH HeLa and MCF7 knockdown cells. Although the effect of EDHB on HIF-1 α protein level was not measured in knockdown cells under SH, since it did not affect HIF-1 α protein level under normoxia its ability to restore hypoxic radioresistance in HeLa and MCF7 may reflect a non-specific effect independent of HIF-1 α under these conditions.

The effect of R59949 on HIF-1 α level was not measured in knockdown cells because it was unlikely to reduce HIF-1 α much further. Nevertheless R59949 treatment caused strong radioprotection in all three knockdown cell lines both under normoxia and SH (except in normoxic LNCaP cells where no effect was observed, Fig. 3.23). This reduction by R59949 of oxygen-dependent radiosensitivity seen in wild type cells (Fig. 3.20) was at least as evident in knockdown HeLa, MCF7 and LNCaP cells under SH (Fig. 3.23). Therefore the R59949 effect may be independent of HIF-1 α , perhaps not surprising due to the high concentration used.

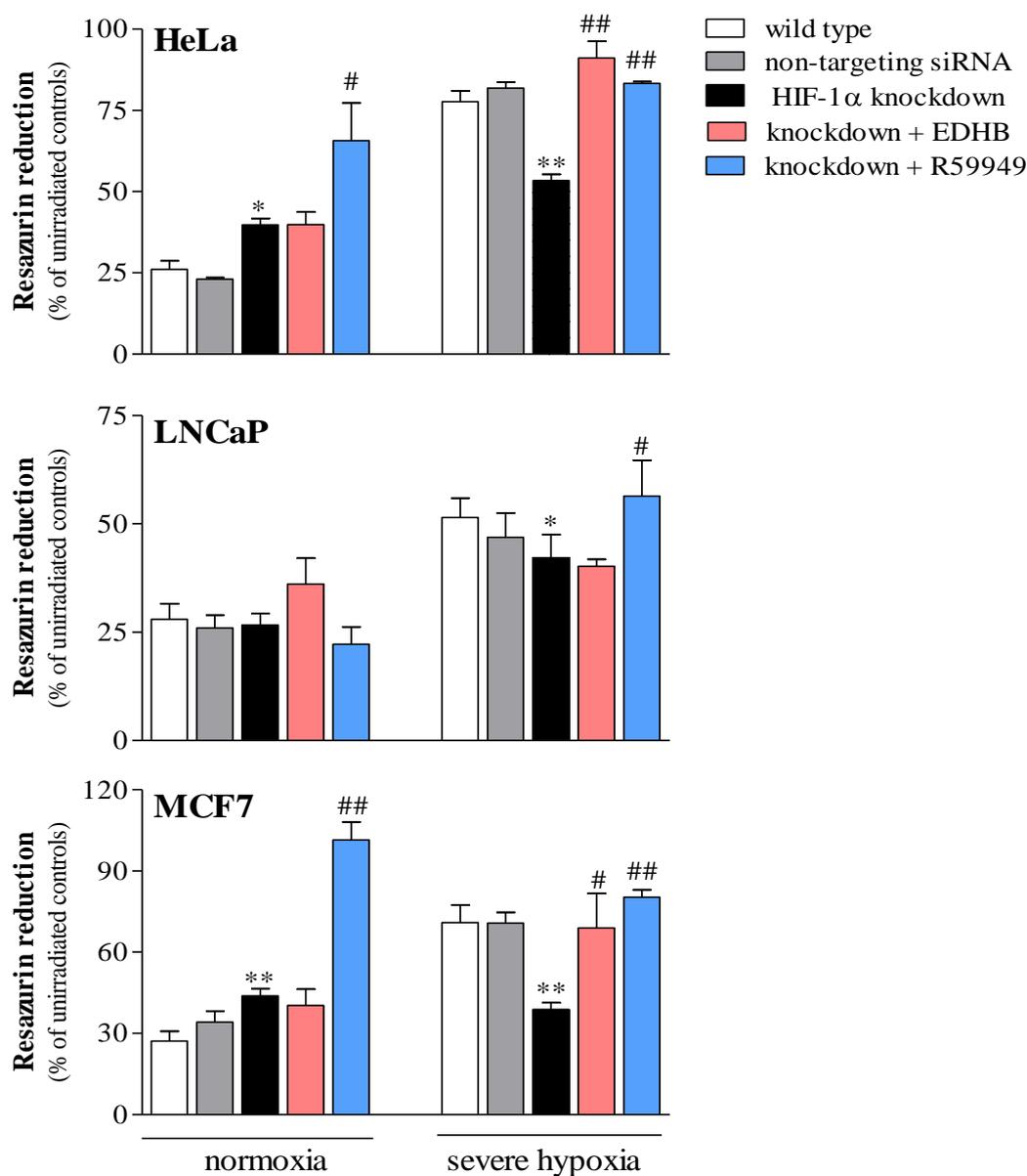


Figure 3.23. **Effect of EDHB or R59949 on oxygen-dependent radiosensitivity in HIF-1 α knockdown cells.** Cells were transfected with siRNA directed against HIF-1 α either 72 (HeLa, LNCaP) or 96 (MCF7) h before irradiation. Cultures were pretreated with EDHB (0.5 mM x 2 h) or R59949 (0.3 mM x 3 h) combined with either normoxia or SH (< 0.2% O₂) for the last hour, prior to irradiation at 6.2 Gy and then being subcultured. Cell proliferation was measured 7 days later using the resazurin reduction assay. Data are mean \pm SE from 3 separate experiments. Comparisons: *, knockdown vs. wild type; #, knockdown drug-treated vs. knockdown untreated.

In conclusion, SH-induced radioresistance and the effects of HIF-1 α knockdown are fully consistent with a major contribution of HIF-1 α to oxygen-dependent radiosensitivity in all cell lines tested. However both EDHB and R59949 effects are complicated by non-specific effects that possibly include compensatory adaptations affecting radiosensitivity. Interestingly both drug effects through HIF-1 α occur when major change in protein level is possible, but when this is not the case (EDHB in hypoxia, R59949 in normoxia) the dominant drug effect is independent of HIF-1 α . In addition, factors other than HIF-1 α may also be involved in radioresistance or radiosensitisation of hypoxic cancer cells. It is possible that the balance between several opposing mechanisms determines the net effect of ionizing radiation injury in hypoxic cells, so that the contribution of HIF-1 α to oxygen-dependent radiosensitivity varies. Furthermore these *in vitro* observations in cell lines do not exclude the possibility that HIF-1 α might play a different overall role *in vivo*, perhaps at higher levels of organisation within the tumour.

Discussion

Hypoxia-induced activation of HIF-1 α is common in human solid tumours and is associated with cell survival and tumour radioresistance. However the role of HIF-1 α in this resistance is complex and its causality has not been established. In the present study we used genetic and pharmacological methods to test HIF-1 α contribution to radioresistance in three cancer cell lines derived from different human tumours. Expected changes in HIF-1 α protein level were observed in wild type and HIF-1 α knockdown cells incubated either under normoxia or SH (<0.2 % O₂ x 1h) and co-exposed to EDHB or R59949, as measured by ELISA in samples taken acutely (within less than 1 minute of reoxygenation). HIF-1 α DNA-binding activity was also increased in SH- and EDHB-treated cells, and lowered by R59959 under hypoxic conditions. Next, the functional role of HIF-1 α in oxygen-dependent radiosensitivity was investigated by comparing EDHB or R59949-treated wild-type cells with cells in which HIF-1 α expression was inhibited transiently using targeted siRNA. SH-induced resistance to irradiation measured as cell proliferation seven days after treatment was evident in all cell lines, in wild-type cell lines, while it was reduced in HIF-1 α knockdown cells. The EDHB and R59949 effects were consistent with an overall contribution of HIF-1 α to radioresistance, but also reflected some HIF-1 α -independent actions which obscured predicted radioprotection or radiosensitisation.

In this study we showed for the first time that HIF-1 α plays a direct causal role in mediating oxygen-dependent radiosensitivity of tumour cells. Pharmacological inhibition of HIF-1 α by R59949 sensitised hypoxic cells to irradiation injury. Although the mechanisms remain to be identified, this study suggests a potential role for R59949

or similar compounds as radiosensitising agents for antitumour therapy. Moreover stabilising HIF-1 α pharmacologically with EDHB conferred radioprotection on normoxic cells. This may be relevant to reducing collateral injury to healthy tissue adjacent to hypoxic tumour masses during radiotherapy. In addition we showed that HIF-1 α contribution to oxygen-dependent radiosensitivity varies among three cell lines derived from different human solid tumours. This suggests that interventions directed at HIF-1 α as an adjunct to radiotherapy are likely to be more effective in some tumour types than others. Finally in contrast to many other studies we showed that HIF-1 α is present under normoxia in all cell lines tested, suggesting that it may play a constitutive role and thereby contribute to the side-effect profile of drugs used to manipulate the HIF-1 α pathway for therapeutic purposes.

HIF-1 α level

Under standard normoxic conditions many cancer cell lines have been reported to express very low or even undetectable levels of HIF-1 α protein. Most of these reports are based on densitometric analysis of Western blots whereas quantitative ELISA or assays that use reporter constructs in which expression of fluorescent proteins is under the control of HIF-1 α are less commonly used. Using Western Blotting, weak or undetectable HIF-1 α bands were reported under normoxia for a variety of cancer cell lines derived from human tumours. These include representatives of digestive (oral (Yoshida *et al.*, 2009), gastric (Stoeltzing *et al.*, 2004), liver (Mottet *et al.*, 2003), pancreatic (Akakura *et al.*, 2001), colon (Tammali *et al.*, 2011)), respiratory (lung (Uchida *et al.*, 2004), pharynx (Vordermark *et al.*, 2004)), reproductive (breast (Welsh *et al.*, 2003), cervix (Triantafyllou *et al.*, 2006, Ioannou *et al.*, 2010), ovary (Akakura *et*

al., 2001)) and nerve (glioma (Kessler *et al.*, 2010)) tissues. Applying Western Blotting to a panel of human prostate cancer cells Zhong *et al.* (1998) found that some cell lines (e.g. PC-3) constitutively express high levels of HIF-1 α under standard normoxic conditions while in others (DU-145, LNCaP, PPC-1) the protein was undetectable. In a subsequent study, this group used Western blotting to show a highly heterogeneous pattern of HIF-1 α expression under normoxic conditions among 17 human tumour cell lines (Zhong *et al.*, 2002). These ranged from cell types in which HIF-1 α was undetectable (e.g. colon adenocarcinoma, leukemia cells) to others that constitutively expressed HIF-1 α at high levels (breast carcinoma, choriocarcinoma, osteosarcoma).

Similarly in studies that used ELISA to determine HIF-1 α level the protein was found to be either undetectable under normoxia in breast, cervical, colon, head and neck cancer cells (Formento *et al.*, 2005) or measurable but lower than positive controls in colon (Tammali *et al.*, 2011) or non-small cell lung cancer cells (Xu *et al.*, 2010). However others found that HIF-1 α level may also be high under normoxic conditions in some lung cancer cells (Schilling *et al.*, 2012). Finally, studies using fusion proteins where their expression is under the regulation of HIF-1 found fluorescent intensity to be low or undetectable in various normoxic cancer cells including liver (Zheng *et al.*, 2006), glioma (Moroz *et al.*, 2009) and cervix (Zhou *et al.* 2011).

In the present study we used ELISA to measure HIF-1 α level in total cell extracts. We tested three human cancer cell lines derived from distinct tissue origins (cervical, breast and prostate) and found that all expressed similar and low but still detectable HIF-1 α protein levels (~0.5 ng/mg total cell protein) under normoxic conditions. This is consistent with other reports in the literature that used quantitative

ELISA to measure HIF-1 α level. Schilling *et al.* (2012) found basal HIF-1 α levels in some lung and squamous carcinoma of head and neck cancer cells to be in the range 0.25-0.7 ng/mg, while Guo *et al.* (2009) reported ~0.6 ng/mg in a human neuroblastoma cell line. Another study using the same ELISA kit as the one used here (R&S Systems, Minneapolis, USA), showed that HIF-1 α level expressed as OD values (450nm) was ~0.2 for normoxic colon cancer cells (Tammali *et al.*, 2011), consistent with the OD values of 0.25-0.35 we observed in cervical, prostate and breast cancer cell extracts. Moreover our low normoxic HIF-1 α levels (~0.5 ng/mg) are also broadly consistent with those reported for non-small cell lung cancer cells (0.9 ng/mg; Xu *et al.*, 2010) and bone marrow stem cells (0.01 ng/mg; Dai *et al.*, 2007). Nevertheless, these values in normoxic cells were all significantly lower than the respective positive controls. Variation in HIF-1 α levels measured by ELISA probably reflects use of different detection antibodies (Ioannou *et al.*, 2010), kits, protocols and cell lines. Thus our measurements contrast with those Western Blot studies which failed to detect the protein under normoxia, but are consistent with reports that found the levels of HIF-1 α to be low but detectable. These latter include the cervical (HeLa; Zhong *et al.*, 2002; Bracken *et al.*, 2006), breast (MCF7; Zhong *et al.*, 2002) and prostate (LNCaP; Mabweesh *et al.*, 2003) cell lines used here.

In summary, both Western Blot and ELISA methods indicate that HIF-1 α protein level is low but usually measurable under normoxia. Whatever discrepancies appear to exist in the literature may reflect differences in sensitivity of the methods, the cell line(s) used and whether HIF-1 α expression is measured in nuclear or whole-cell extracts. In addition, factors such as cell density (Zhong *et al.*, 1998) or passage number may affect HIF-1 α expression under normoxia.

Stabilization of HIF-1 α protein in response to hypoxic insults of various severity and duration has been reported in many cancer cell lines. However the time course and extent of this response varies. Although HIF-1 α is usually detectable under normoxia (21% O₂) in many cancer cell lines, levels increase with severity of hypoxia imposed. Bracken *et al.* (2006) found HIF-1 α to be barely detectable at 5% O₂ or higher but elevated (~3-fold) at 2% O₂ and strongly increased (~3 to 6-fold) at <1% O₂ in cervical (HeLa), hepatoma (HepG2) and colon (CaCo2) cancer cells. Similarly in lung cancer cells (A549) hypoxia-induced HIF-1 α was measurable at 3% O₂ or higher, but increased 3- and 8-fold at 1 and 0.5% O₂ respectively (Uchida *et al.*, 2004). Both studies used a 4 h exposure to stabilise HIF-1 α , and whole cell extracts to measure it. Detailed analysis of HIF-1 α level in nuclear extracts of cervical cancer cells (HeLa) revealed a 3-fold increase in protein level as oxygen level was progressively reduced from 20 to 6 % (4 h incubation), while further oxygen depletion increased HIF-1 α level more dramatically to a maximum of ~14-fold at 0.5% O₂ (Jiang *et al.*, 1996).

HIF-1 α stabilization depends also on the duration of hypoxia used. Short exposures (<1 h) to 0.02-5% oxygen caused rapid accumulation of HIF-1 α in the nucleus of HeLaS3 cells within 2 minutes (Jewell *et al.*, 2001). By contrast, in nuclear extracts of human pharyngeal carcinoma and fibrosarcoma cells HIF-1 α expression was still weak after 10 minutes of exposure to 0.1-5% O₂, but increased progressively over 1 hour and remained stable over the following 24 hours (Vordermark *et al.*, 2004). Part of this disparity may be due to differences in the rate of onset of hypoxia due to differences in methodology. In addition others using whole cell extracts reported that subjection of HeLa cells to 1.5 % O₂ for 1-48 h caused measurable stabilisation of HIF-1 α protein by 1 h, with maximal expression at 4-8 h then a decrease to undetectable levels by 48 h

(Triantafyllou *et al.*, 2006). In general, moderate to maximal accumulation of HIF-1 α protein is widely reported for various cancer cell lines subjected to longer periods of hypoxia (≥ 1 h). Nevertheless, although most studies use longer exposures, hypoxic insults of 1 h or less are sufficient to increase HIF-1 α level significantly, even in whole cell extracts, as shown by Oike *et al.*, (2012) for A549 (human lung cancer cells) and confirmed here (Fig. 3.16) for HeLa, LNCaP and MCF7 cells.

Hypoxia-induced stabilisation of HIF-1 α protein can also vary between cell types. While in some cell lines HIF-1 α protein is detectable at 5% O₂ or higher, others require more severe oxygen depletion ($\leq 2\%$ O₂) for significant HIF-1 α stabilization (Bracken *et al.*, 2006). Cell plating density may also contribute to HIF-1 α protein level under hypoxia although the relationship and mechanism(s) are not clear. Under the same hypoxic conditions some authors reported that HIF-1 α levels were inversely related to plating density in prostate cancer cells (Zhong *et al.*, 1998), while others found the opposite in lung cancer cells (Uchida *et al.*, 2004).

Although it is widely reported that both moderate and severe oxygen deprivation activate and stabilise HIF-1 α protein, less is known about the reversal of this effect upon reoxygenation. Under normoxia HIF-1 α protein has a half-life of approximately 5 minutes (Huang *et al.*, 1996). Vordemark *et al.* (2004) reported that elevated HIF-1 α levels in several types of cancer cells return to baseline values within 15 minutes of reoxygenation. Others found that HIF-1 α protein in HeLa cells is undetectable within 30 minutes of reoxygenation (Jewell *et al.*, 2001; Tomes *et al.*, 2003). Here we observed that HIF-1 α protein level remained high for at least 10 minutes after reoxygenation but then fell rapidly over the next 20 – 80 minutes and returned to control levels within 48 h

(Fig. 3.14). This pattern was marked in HeLa and LNCaP but not in MCF7 cells, and resembles that reported previously in human pharyngeal carcinoma and fibrosarcoma cells (Vordermark *et al.*, 2004). The small rebound of HIF-1 α below control levels we observed in HeLa and LNCaP cells might reflect enhanced targeting of HIF-1 α for degradation due to higher prolyl hydroxylase expression (Epstein *et al.*, 2001) induced by the preceding hypoxic insult.

Before discussing the role of HIF-1 α in anoxia-induced radioresistance of cancer cells first we must first consider how HIF-1 α level is affected by ionizing radiation itself. Lund and colleagues (2004) reported a small increase 1-2 h after irradiation of normoxic cell cultures at 20 Gy under normoxia, whereas others found no change following irradiation at 5-15 Gy (Moeller *et al.*, 2004; Zeng *et al.*, 2008). This latter finding contrasts with reports of an increase in HIF-1 α levels following *in vivo* irradiation of tumours (Moeller *et al.*, 2004; Dikmen *et al.*, 2008). However in the present study we found no change in HIF-1 α level in either HeLa or MCF7 following irradiation at 6.2 Gy. The only increase observed was at 48 h in LNCaP cells which might reflect stress due to medium depletion, because in contrast to the other cell lines used the medium over LNCaP cultures was not changed because the cells were poorly attached.

As far as we are aware, the combined effect of SH and ionizing radiation on HIF-1 α level has not been studied before. By contrast with the lack of effect of ionizing radiation on HIF-1 α level under normoxia, irradiation at 6.2 Gy of cells subjected to SH for 1 h caused a further increase in HIF-1 α level in HeLa and MCF7 but had no effect in LNCaP cells. Therefore this further stabilisation of HIF-1 α by ionizing radiation under

SH might be due to indirect oxygen-dependent mechanisms. However the pattern of HIF-1 α decrease upon reoxygenation was little affected by irradiation in two of the three cell lines used. Therefore we conclude that HIF-1 α -dependent consequences for cell proliferation in the longer term (here 7 days) are influenced mainly by hypoxia rather than by concurrent irradiation.

HIF-1 DNA-binding activity

HIF-1 α protein stabilisation and accumulation is followed by its nuclear translocation and heterodimerisation with HIF-1 β subunit. Then after binding to specific DNA sequences, HIF-1 activates transcription of genes that contribute to cellular adaptation to hypoxia or influence other processes leading to cell survival or death. Using a panel of different mammalian cell lines Wang & Semenza (1993) first reported that HIF-1 DNA binding was induced in cells subjected to hypoxia (1% O₂ x 4 h), while under normoxia DNA binding activity was undetectable. This observation was confirmed and extended by later studies that found HIF-1 DNA binding activation to be undetectable in the nuclear fraction of normoxic prostate (Zhong *et al.*, 1998), cervical (Jewell *et al.*, 2001), brain (Berchner-Pfannschmidt *et al.*, 2004; Guo *et al.*, 2009), lung and colon (Weinmann *et al.*, 2004), liver and lung (Cosse *et al.*, 2007) and breast (Flamant *et al.*, 2010) cancer cells, but increased under hypoxia.

Here we tested how changes of HIF-1 α level in whole cell extracts correlated with HIF-1 DNA binding activity in the nuclear fraction. Under normoxia HIF-1 α protein level was low and similar among the three cell lines tested, but basal HIF-1 DNA-binding activity differed (LNCaP>MCF7>HeLa; Fig. 3.17). Although this might reflect the subcellular localization of HIF-1 α , its distribution between the cytoplasmic

and nuclear fractions varied only marginally between normoxic HeLa and LNCaP cells (Table. 3.1), albeit based on single measurements. Furthermore, although SH (<0.2 % O₂ x 1 h) significantly elevated HIF-1 α level in total cell extracts of all three cell lines tested, this correlated only loosely with HIF-1 DNA binding measured in nuclear extracts under the same conditions. Namely, SH raised HIF-1 DNA-binding activity in HeLa, whereas in MCF7 and LNCaP cells there was no measurable effect. This pattern reflected the subcellular distribution of HIF-1 α in SH cells (Table.3.1). While in MCF7 and LNCaP cells HIF-1 α was almost equally distributed between the cytoplasmic and nuclear fractions, in HeLa cells most of the protein was localized in the nucleus. However although less severe hypoxia (MH) over a longer period (0.5 % O₂ x 24 h) resulted in greater DNA-binding signal (approximately 10-fold increase, Fig. 4.20), less marked relative differences in nuclear/cytoplasmic HIF-1 α distribution were found in HeLa cells. Thus based on these limited data less severe but longer hypoxia promotes a greater increase in HIF-1 DNA-binding which is not related in any obvious way to HIF-1 α distribution between cytoplasm and nucleus. Therefore it is possible that other factors in addition to subcellular localisation are also involved.

Poor correlation or apparent discrepancies between HIF-1 α protein levels and HIF-1 DNA binding activity could have several explanations. The simplest may be related to cell type and hypoxia conditions. Since SH stabilised HIF-1 α , events leading to its degradation (prolyl hydroxylation (Hon *et al.*, 2002) and sumoylation (Cheng *et al.*, 2007)) should be limited. However as discussed above, under these conditions (<0.2% O₂ x 1 h) different HIF-1 DNA-binding activities did not correlate with rather similar total protein levels. Other studies based on the same ELISA kit for detection of DNA-bound HIF-1 complexes (R&S Systems, Minneapolis, USA) used different cell

types and longer (2-30 h) but less severe hypoxia (0.5-5 % O₂) (Frede *et al.*, 2006; Dai *et al.*, 2007; Gerber & Pober, 2008; Guo *et al.*, 2009; Hsieh *et al.*, 2010; van de Sluis *et al.*, 2010). In most of these reports (except one which lacks the normoxia control) the relative difference in signal between normoxic and hypoxic cells was detectable and significant. Thus although the SH protocol used here increased total HIF-1 α level its short duration may have been insufficient to allow measurable HIF-1 α /oligonucleotide complex formation later. Furthermore the positive control used for validation of the ELISA kit was a nuclear fraction from normoxic cells treated with CoCl₂ to mimic HIF-1 α stabilisation by hypoxia. Thus oxygen-dependent HIF-1DNA-binding seems to be strongly related to severity and duration of oxygen depletion, and to develop at a rate that is cell type specific.

Detectable hypoxia-induced HIF-1 DNA-binding activity depends not only on HIF-1 α stabilisation but also on protein translocation to the nucleus, the rate of which process is unknown. Since HIF-1 α was present in the nuclear fractions protein transport via the classical importin α/β pathway (Depping *et al.*, 2008) and/or other importins (Chachami *et al.*, 2009) had clearly taken place. However post-translocation events that lead to HIF-1 DNA binding can also influence HIF-1 α localisation. For example, CMR1 exports HIF-1 α back to the cytoplasm by a mechanism that depends on phosphorylation of the HIF-1 α nuclear export signal domain (Mylonis *et al.*, 2006, 2008). Here we found that similar relative distributions of HIF-1 α between cytoplasm and nuclear fractions produced substantially different DNA binding signals (e.g. SH MCF7 vs. SH LNCaP cells), so HIF-1 α export is unlikely to explain the limited DNA binding in some samples. Alternatively, poor HIF-1 DNA binding might reflect different concentrations of HIF-1 α protein (ng/mg total protein) present in nuclear fractions (range > 10-fold),

but these two parameters showed weak correlation ($r^2 = 0.34$). In order to dimerise with HIF-1 β , HIF-1 α must escape prolyl hydroxylation which may also occur in the nucleus of hypoxic cells (Berchner-Pfannschmidt *et al.*, 2008; Pientka *et al.*, 2012) and result in proteasomal degradation (Berra *et al.*, 2001). Another obstacle to successful HIF-1 DNA binding is asparaginyl hydroxylation of HIF-1 α in hypoxic cells which inhibits its transcriptional activation by preventing interaction of the HIF-1 α C-terminal activation domain with p300/CBP co-activators (Tian *et al.*, 2011). That study showed residual HIF-1 α asparaginyl hydroxylation after 5 h exposure to 0.2 % O₂ in extracts from various cell types. In our case oxygen level was below 0.2 % but the duration of SH was shorter (1 h). Although our cell cultures were briefly reoxygenated (< 1 minute) while being cooled to 4°C for fractionation, hydroxylation of HIF-1 α is likely to have been slight and to apply equally to all samples. Therefore residual asparaginyl hydroxylation is unlikely to account for the limited HIF-1 DNA binding activity that we could detect. However it remains possible that some other post-translational modification of HIF-1 α (e.g. phosphorylation) could limit formation of HIF-1 α /oligonucleotide complexes in the assay.

Therefore the limited correlation we observed between HIF-1 α level and subsequent HIF-1 DNA-binding under SH conditions could be related to methodology rather than to lack of HIF-1 α DNA binding activity *per se*. Neither nuclear export nor residual asparaginyl hydroxylation of HIF-1 α are likely to be responsible for the low level of HIF-1 DNA binding measured under conditions used here. Nevertheless SH-induced HIF-1 α stabilisation leads to HIF-1 α -dependent changes in cell proliferation measurable at 7 days (Fig. 3.20), so may entail some level of DNA binding and transcriptional regulation below the threshold for detection by the assay used.

Alternatively or in addition, HIF-1 α might act via a mechanism independent of DNA-binding and transcriptional control. For example hypoxia-induced stabilisation of HIF-1 α protein, independent of its transcriptional activity, was shown to induce cell cycle arrest by functionally counteracting Myc activity in colon cancer cells (Koshiji *et al.*, 2004). Moreover IFN- α -inducible expression of HIF-1 α protein was found to contribute to the antiproliferative activity of IFN- α in endothelial cells (Gerber & Pober, 2008), while HIF-1 α -induced differentiation of leukemic cells was also reported to be independent of HIF-1 transcriptional activity (Song *et al.*, 2008).

Oxygen-dependent radiosensitivity

The term hypoxia denotes an oxygenation status of the cell, tissue or organism that is below the norms established for it. Hypoxia-induced cellular response depends on the severity of oxygen depletion, its duration and the cell type involved. However, in general there is evidence that proliferation capacity decreases with increasing severity and duration of oxygen depletion. In most *in vitro* studies of the effect of hypoxia on cancer cell proliferation the duration of oxygen depletion is 24 h or more. Extreme hypoxia (<0.028 % O₂ x 24 or 72 h) significantly slows the cell doubling time of cancer cells (Zolzer & Streffer, 2002). Severe hypoxia (<0.1 % O₂ x 24-72 h) also caused growth arrest in some cancer cell lines, but under more moderate hypoxic conditions (1% O₂ x 30-60 h) cell doubling-times and viability were reported to be unaffected (Weinmann *et al.*, 2004). By contrast, moderate hypoxia (1% O₂ x 72 h) slowed or stopped the growth of other tumour cells (Koch *et al.*, 2003). Furthermore, proliferative capacity of cancer cells reoxygenated after a period of hypoxia (<1.4% O₂ x 24 h) was reduced by 20-65% compared to normoxic controls (Krtolica & Ludlow, 1996). Finally,

the survival of human cancer cells was found to depend on the duration of moderate hypoxia (1% O₂); short (≤ 6 h) insults increased cell survival whereas long exposures (≥ 24 h) decreased cell proliferation and induced cell death (Dai *et al.*, 2011).

Here we found that short exposure of three human cancer cell lines to severe hypoxia (<0.2% O₂ x 1 h) had no effect on their subsequent proliferation (Fig. 3.10). This lack of effect of hypoxia alone made it easier to discern the long-term effect of ionizing radiation and to study the role of HIF-1 α in oxygen-dependent radiosensitivity. Under these conditions we observed clear oxygen-dependent radiosensitivity in wild type HeLa, LNCaP and MCF7 cells (Fig. 3.19), consistent with reports for several other cell lines (Shibamoto *et al.*, 2004; Vordermark *et al.*, 2004; Weinmann *et al.*, 2004; Zhang *et al.*, 2007; Liu *et al.*, 2010b).

Does HIF-1 α knockdown affect oxygen-dependent radiosensitivity?

The most direct way to test whether HIF-1 α contributes to oxygen-dependent radioresistance is with cells in which HIF-1 α expression is abrogated. Use of small interfering RNA (siRNA) to directly suppress HIF-1 α expression has been reported for various normoxic as well as hypoxic tumour cells including oral (Sasabe *et al.*, 2006); cervical (Lu *et al.*, 2008); breast (Sullivan & Graham, 2009); neuroblastoma (Guo *et al.*, 2009); pancreatic (Chen *et al.*, 2009a); liver (Daskalow *et al.*, 2010) and fibrosarcoma (Staab *et al.*, 2011) cell lines. Here we found that transfection with HIF-1 α targeted siRNA transiently but markedly reduced expression of HIF-1 α in HeLa, LNCaP and MCF7 cells under both normoxic and SH conditions (Fig. 3.21).

It is commonly reported that HIF-1 α deficiency enhances radiosensitivity of various tumour cells regardless of severity or duration of hypoxia before irradiation (Arvold *et al.*, 2005; Liu *et al.*, 2010b; Staab *et al.*, 2011; Stroefel *et al.*, 2011). In the present study we also found that transient HIF-1 α knockdown significantly increased radiosensitivity in all three cell lines under SH conditions (Fig. 3.22). By contrast under normoxia where HIF-1 α levels are lower we found that HIF-1 α knockdown conferred small protection against ionizing radiation in HeLa and MCF7 cells but not LNCaP. These variable effects under normoxia are consistent with those reported by others. Some malignant glioma cells in which HIF-1 α expression was abrogated showed increased survival after irradiation under normoxia (Kessler *et al.*, 2010), while no effect on radiosensitivity under normoxia was reported for fibrosarcoma cells (Staab *et al.*, 2011) or lung cancer cells grown either as monolayers or spheroids (Zou *et al.*, 2010). Similarly no significant difference was observed in the radiosensitivity of mouse hepatoma wild type and HIF-1 α knockout cells under normoxia (Williams *et al.*, 2005). These apparently contradictory findings regarding HIF-1 α role in normoxic radiosensitivity may reflect low basal levels of the protein in wild type cells as well as use of different experimental models and conditions. To summarise, we found that transient HIF-1 α knockdown sensitised all three cell lines to ionizing radiation injury under SH conditions but produced variable effects under normoxia. Consequently the difference between normoxic and hypoxic radiosensitivity was decreased in HeLa and LNCaP cultures and indeed abolished in MCF7. These observations are fully consistent with a causal role for HIF-1 α in protecting tumour cells against oxygen-dependent radiosensitivity.

How does pharmacological alteration of HIF-1 α status affect oxygen-dependent radiosensitivity?

Before discussing how pharmacological alteration of HIF-1 α status by EDHB and R59949 treatments affects oxygen-dependent radiosensitivity of HeLa, LNCaP and MCF7 cells first we consider changes in HIF-1 α level and HIF-1 DNA binding. Various pharmacological interventions increase HIF-1 α protein level, mainly by stabilising the protein against proteasomal degradation. Several agents have been described as mimicking hypoxia (e.g. Co²⁺ treatment) but this may be misleading because other (side) effects of such drugs may influence subsequent cell proliferation or apoptosis differently. Here we used the relatively specific prolyl hydroxylase inhibitor EDHB to stabilise HIF-1 α because of its clear mechanism of action as a substrate analogue and its low toxicity at effective concentrations. HIF-1 α level was significantly increased in all EDHB-treated cells under both normoxic and SH conditions, most markedly in normoxic MCF7 cells (~7 fold; Tab 3.2, *upper panel*). This is consistent with other reports in which EDHB was used to stabilise HIF-1 α level (Warnecke et al., 2003; Floyd *et al.*, 2007; Kasiganesan *et al.*, 2007; Li *et al.*, 2008; Chu *et al.*, 2010).

HIF-1α level												
	Control				EDHB				R59949			
	normoxia	SH ¹			normoxia ¹	SH ²			normoxi	SH ²		
HeLa	1	x2.7	**	x4	***	x1.7	###	x0.3	*	x0.4	##	
LNCa	1	x2.2	**	x5	***	x2.0	##	x0.5	ns	x0.5	##	
MCF7	1	x2.5	***	x7	***	x2.0	###	x0.7	ns	x0.3	##	

HIF-1 DNA binding						
	Control		EDHB		R59949	
	normoxia	SH ¹	normoxia ¹	SH ²	normoxia ¹	SH ²
HeLa	1	x2.4	x6	x1.5	x1.1	x0.5
LNCaP	1	x1.2	x1.6	x1.6	x0.8	x0.9
MCF7	1	x1.4	x6	x3.0	x1.6	x0.9

Table 3.2. **Comparison of EDHB and R59949 effects on HIF-1 α protein level and HIF-1 DNA-binding in wild-type cells immediately after irradiation under normoxia or SH.** Fold changes (x) and significance are shown, except for DNA binding where n=2. Normoxic or SH (<0.2 % O₂ x 1 h) cultures were exposed to EDHB (0.5 mM for 2 h) or R59949 (0.3 mM for 3 h), then immediately cooled (<1min) to 4°C and lysed for preparation of whole or nuclear extracts, and analysed by ELISA for HIF-1 α level or HIF-1 DNA-binding activity respectively. Values are calculated from Figs. 3.16 and 3.17. Comparisons: ^{1,*} relative to normoxic control, ^{2,#} relative to SH control.

Although EDHB treatment raised HIF-1 α level more than hypoxia alone, the combined effect of both conditions together was less than additive. HIF-1 DNA binding activity was also increased in all EDHB-treated cells under both normoxia and SH (Table 3.2, lower panel). By contrast, in order to lower HIF-1 α level we chose the diacylglycerol kinase inhibitor R59949, which is considered to activate prolyl hydroxylase indirectly by reducing synthesis of phosphatidic acid suggested to act as a negative regulator (Temes *et al.*, 2005). Although there are few reports of the use of this drug to lower HIF-1 α level (Aragones *et al.*, 2001; Kyoung *et al.*, 2006; Gonsalves &

Kalra, 2010), and its mechanism of action remains to be confirmed, we found that R59949 decreased HIF-1 α level in all three cell lines tested after short exposure to a non-toxic concentration under both normoxic and SH conditions (Table 3.2, *upper panel*). R59949 treatment also decreased HIF-1 DNA binding in SH HeLa cells but had no measurable effect in MCF7 or LNCaP (Table. 3.2 *lower panel*). However this latter finding is hardly surprising because HIF-1 DNA binding was already low in control cells. Both EDHB and R59949 were used at concentrations considerably higher than the IC₅₀ values for their target enzymes, in order to obtain significant changes in HIF-1 α protein level after relatively short exposure times (2 h for EDHB and 3 h for R59949). Thus it is highly probable that they may also have caused non-specific effects that could perhaps include compensatory adaptations affecting radiosensitivity. Therefore by exposing HIF-1 α -deficient cells to EDHB and R59949 we attempted to distinguish whether the functional effect of these drugs (measured as 7-day proliferation) on oxygen-dependent radiosensitivity depended on HIF-1 α or reflected other actions.

EDHB increased HIF-1 α level and HIF-1 DNA binding activity under both normoxia and SH in all three cells lines (Table 3.2). Because EDHB had no effect on radiosensitivity in normoxic HIF-1 α knockdown cells (Table 3.3, *upper panel*) we conclude that its radioprotective effect in wild type cells (Table 3.3 *lower panel*) is mediated mainly through HIF-1 α . This is consistent with reports of increased radioresistance in normoxic cells treated with other HIF-1 α stabilising agents including DMOG and CoCl₂ (Ayrapetov *et al.*, 2011; Stoefer *et al.*, 2011). Interestingly, under severe hypoxia EDHB increased radioresistance of HIF-1 α knockdown HeLa and MCF7 cells without raising HIF-1 α levels (Fig. 3.21), indicating a protective effect independent of HIF-1 α . However this could not be seen in wild type cells which were

already radioresistant. In these cells the drug-induced radioprotection may have been masked by the protective effect of increased HIF-1 α . Overall, the effect of HIF-1 α -stabilising EDHB on oxygen-dependent radiosensitivity is fully consistent with a causal role for HIF-1 α in radioprotection.

R59949 decreased HIF-1 α level in all three cell lines under both normoxia and SH conditions but had no detectable effect on already low HIF-1 DNA binding activity, except SH HeLa where basal signal was somewhat higher (Table.3.2). Surprisingly the drug increased the radioresistance of knockdown cells both under hypoxia and normoxia, indicating a protective effect independent of HIF-1 α . One possible mechanism might be a compensatory increase in HIF-2 α protein level expressed in parallel to HIF-1 α in many cancer cell lines (Bracken *et al.*, 2006), secondary to HIF-1 α knockdown as shown in MCF7 cells (Carroll *et al.*, 2006). Although R59949 was also shown to inhibit hypoxic stabilisation of HIF-2 α (Temes *et al.*, 2005), its effect on HIF-2 α level in HIF-1 α knockdown cells has not been reported. Moreover HIF-2 α expression was associated with poor response to radiotherapy in patients with cervical cancer (Kim *et al.*, 2011). Although R59949 effect on HIF-2 α expression in wild-type cells was not investigated in the present study, the agent was reported to decrease both HIF-1 α and HIF-2 α levels with the same efficiency in renal cancer cells (Temes *et al.*, 2005). This indicates that the two HIF- α isoforms are not always regulated reciprocally in wild type cells. Here we observed that although R59949 reduced HIF-1 α level under hypoxia in all three cell lines tested it radiosensitised only MCF7 under the conditions used. In the other cell lines this may reflect an opposing protective effect of the drug which was more clearly evident in knockdown cells. If so, then this latter radioprotective effect varied between cell lines.

HIF-1α knockdown						
	Control		EDHB		R59949	
	normoxia ¹	SH ¹	normoxia	SH ³	normoxia ²	SH ³
HeLa	↑ x1.4 *	↓ x0.7 **	- x1	↑ x1.7	↑ x1.7 #	↑ x1.6
LNCaP	- x1 ns	↓ x0.8 *	↑ x1.4	- x1	(↓) x 0.8	↑ x1.4 †
MCF7	↑ x1.7	↓ x0.6 **	- x1	↑ x1.8 †	↑ x2.3	↑ x2.1

Wild type

	Control		EDHB		R59949	
	normoxia	SH ²	normoxia ²	SH ³	normoxia ²	SH ³
HeLa	1	↑ x2.7	↑ x1.6	(↑) x1.1	↑ x1.8	(↓) x0.9
LNCaP	1	↑ x1.7 #	(↑) x1.4	(↓) x0.7	- x1 ns	(↑) x1.3
MCF7	1	↑ x2.8	↑ x1.4	(↓) x0.8	(↑)x1 ns	↓ x0.7

Table 3.3. **Comparison of EDHB and R59949 effects on proliferation 7 days after irradiation under normoxia or SH in HIF-1 α knockdown and wild-type cells.** Radioprotection ($\uparrow\downarrow$), fold change (x) and significance are shown. Values are calculated from Figs. 3.20 and 3.23. Cells were transfected with HIF-1 α siRNA either 72 (HeLa, LNCaP) or 96 (MCF7) hours before irradiation. They were pretreated with EDHB (0.5 mM x 2 h) or R59949 (0.3 mM x 3 h) under normoxia or SH (< 0.2% O₂ x 1 h) prior to irradiation at 6.2 Gy, then subcultured. Cell proliferation was measured 7 days later using the resazurin reduction assay. Comparisons: ^{1,*} relative to wild type cells, ^{2,#} relative to normoxic control, ^{3,†} relative to SH control.

R59949 had no effect on the radiosensitivity of HeLa cells under hypoxia (in which high HIF-1 α and HIF-1 DNA binding activity were reduced), but caused clear protection (###, Table 3.3) under normoxia (where a low HIF-1 α level is reduced further). By contrast in MCF7 the sensitizing effect seen under hypoxia was lost under normoxia. Hypoxic radiosensitisation has also been reported in several studies that used other pharmacological agents to reduce HIF-1 α protein level and/or inhibit HIF-1 activity in different human cancer cell lines (Staab *et al.*, 2007; Koh *et al.*, 2008; Palayoor *et al.*, 2008; Schwartz *et al.*, 2009; Oommem & Prise, 2012; Yang *et al.*,

2013). In summary, R59949 effects are also consistent with a causal role for HIF-1 α in radioprotection but are less clear-cut because (a) in normoxic cells HIF-1 α is already low and lowered further by the drug so sensitization is difficult to detect, and (b) HeLa cells in particular showed a protective effect of the drug independent of HIF-1 α .

In conclusion therefore, these genetic and pharmacological experiments together show that HIF-1 α status regulates response to ionizing radiation in the three cell lines tested. This is consistent with the hypothesis that HIF-1 α plays a major causal role in oxygen-dependent radiosensitivity.

Chapter 4

HIF-1 α role in oxygen-dependent chemosensitivity

Introduction

Chemotherapy

Chemotherapy is a treatment that uses cytotoxic drugs to slow or stop the growth of tumour cells. It can be given before surgery or radiotherapy to shrink the size of the tumour or after those treatments to destroy any remaining tumour cells including metastases. Moreover it can also be applied in parallel with radiotherapy (chemoradiotherapy) to make treatment more efficient. Alternatively, chemotherapy can be used as a palliative treatment to reduce symptoms of cancer and improve quality of life when cure is not an option. The most common ways to apply chemotherapeutic drugs are intravenously or orally, but other routes such as intramuscular injection, administration directly into the tumour or a body cavity as well as topical cream application are also possible. Depending on the type of cancer, its development stage, localisation as well as patient age and general health condition, chemotherapy is normally given during regular sessions over a few months. Chemotherapy use is limited by its severe side effects that include fatigue, hair loss, vomiting, depression, infertility, anaemia and others. In addition response to chemotherapy can be impaired due to development of resistance during treatment.

Resistance to chemotherapy

Drug physiochemical properties can contribute to the effectiveness of chemotherapy but the tumour microenvironment plays a substantial role in controlling the distribution of anticancer drugs. Drugs are usually delivered to the tumour via blood so the abnormal vasculature of solid tumours may limit their distribution and cytotoxic

effects (Jang *et al.*, 2003). Another factor that may contribute to resistance involves increased efflux of the drugs from target cells via P-glycoprotein (Ouar *et al.*, 1999). Furthermore low oxygen concentration commonly found in solid tumours enhances their resistance to chemotherapy by several mechanisms. Hypoxia slows down cell proliferation or arrests the cell cycle, whereas most anticancer drugs are more effective against rapidly growing cells (Durand & Raleigh 1998). Moreover hypoxic cells are located further from blood vessels and due to disorganized and variable blood flow delivery of chemotherapeutic agents might be limited and lead to reduced chemosensitivity (Gillies *et al.*, 1999). Additionally, extracellular pH in hypoxic tumours is lower than in normoxic tissues due to accumulation of the glycolysis end product lactic acid. As a result, the intracellular uptake of weakly basic drugs such as doxorubicin, mitoxantrone or vincristine is inhibited, while in contrast weakly acidic drugs can concentrate intracellularly (Mahoney *et al.*, 2003, Gerwick *et al.*, 2006).

In addition to the physiochemical factors described above, hypoxia-induced resistance to conventional chemotherapeutic drugs has been shown in various tumour cell lines *in vitro*. For example, acquired resistance under hypoxia to doxorubicin-induced cytotoxicity was found in cell lines derived from human leukaemia and lymphoma (Xu *et al.*, 2005), pancreatic and cervical carcinoma (Chen *et al.*, 2009b), lung adenocarcinoma (Song *et al.*, 2006), prostatic carcinoma (Frederiksen *et al.*, 2007), breast metastatic carcinoma (Matthews *et al.*, 2001) and colon carcinoma (Sullivan *et al.*, 2008, Guin *et al.*, 2010). In addition hypoxia-mediated resistance to a growing number of cytotoxic drugs including cisplatin (Koch *et al.*, 2003; Liu *et al.*, 2008; Lau *et al.*, 2009; Lin *et al.*, 2011), etoposide (Brown *et al.*, 2006; Lee *et al.*, 2006), 5-fluorouracil (Marignol *et al.*, 2009; Ravizza *et al.*, 2009; Yoshida *et al.*, 2009),

gemcitabine (Yokoi & Fidler, 2004), paclitaxel (Merighi *et al.*, 2007; Lu *et al.*, 2008) and methotrexate (Li *et al.*, 2006) was also reported.

Attempts to overcome hypoxia-induced chemoresistance

Since effectiveness of chemotherapy in solid tumours is impaired by hypoxia, several strategies have been explored to overcome this resistance, including improving tumour blood flow, increasing tumour blood vessel permeability by damaging tumour endothelium, and reduction in the tumour interstitial fluid pressure (reviewed in Tredan *et al.*, 2007). Other approaches include use of recombinant anaerobic bacteria. These non-pathogenic spores-forming strains can colonise hypoxic necrotic regions of tumours and cause extensive local lysis of cells without affecting normal tissue. For example a genetically modified *Salmonella* strain was reported to selectively colonise tumours in Phase I clinical trials (Toso *et al.*, 2002). In addition a *Clostridium* strain genetically modified to convert nontoxic prodrug to its toxic form was reported to colonise hypoxic/necrotic areas of tumours in mice (Liu *et al.*, 2002). Therefore use of bacteria in cancer therapy is a novel strategy now being investigated in clinical trials (Wang *et al.* 2013). Non-toxic prodrugs which are activated to a toxic form under hypoxic conditions were also tested for their ability to improve chemotherapy but produced conflicting results. For example in phase III clinical trials tirapazamine in combination with chemotherapeutic drug was reported to either improve (von Pawel *et al.*, 2000) or have no effect (Williamson *et al.*, 2005; DiSilvestro *et al.*, 2014) on survival rates of patients with various types of cancer. Other prodrugs (e.g. AQ4N; Tredan *et al.*, 2009) which were reported to enhance activity of some chemotherapeutic drugs are still under

investigation. In addition to these approaches at the cell level hypoxia-induced drug resistance may also involve changes in protein expression that contribute to survival and proliferation of tumour cells. Since HIF-1 α is one of the major transcription factors affecting changes in gene expression under hypoxia, another approach to overcoming the resistance of hypoxic tumours to chemotherapy might be to target HIF-1 α .

HIF-1 and chemoresistance

As previously outlined in *Chapter 3* hypoxia which is commonly present in most solid tumours affects their response to therapies and contributes to poor prognosis following treatments. Moreover hypoxia-induced HIF-1 α expression is important for regulation of coordinated adaptive responses due to activation of various genes related to cell survival, metabolism, metastasis etc.. Therefore HIF-1 α has been proposed as a potential target in improving effectiveness of hypoxia-induced resistance to chemotherapy.

Despite elevated HIF-1 α under hypoxia, there is conflicting evidence in the literature about whether it plays a causal role in oxygen-dependent chemosensitivity of tumour cells. Some authors found pharmacological inhibition of HIF-1 α protein to overcome resistance to 5-fluorouracil (Muroso *et al.*, 2012). Others confirmed correlation of increased HIF-1 α level and enhanced resistance to etoposide (Brokers *et al.*, 2010). Inhibition of HIF-1 α by genetic approaches under hypoxia has also been found to increase sensitivity to cisplatin and doxorubicin (Song *et al.*, 2006; Sullivan *et al.*, 2008) or paclitaxel (Huang *et al.*, 2010). HIF-1 α deficient tumours showed increased sensitivity to chemotherapeutic agents carboplatin and etoposide (Unruh *et*

et al., 2003), 5-flourouracil, cisplatin (Sasebe *et al.*, 2006), methotrexate (Li *et al.*, 2006), etoposide (Brown *et al.*, 2006), vincristine (Liu *et al.*, 2008) and gemcitabine (Yang & Kang 2008). Possible mechanisms through which HIF-1 α overexpression might contribute to decreased drug sensitivity involve upregulation of P-glycoprotein, heme oxygenase-1 and manganese-superoxide dismutase (Sasebe *et al.*, 2006) suppression of drug-induced apoptosis by enhancing the Bcl-2/Bax ratio (Liu *et al.*, 2008) or induction of G₁ cell cycle arrest (Yoshida *et al.*, 2009; Huang *et al.*, 2010). By contrast, HIF-1 α -independent pathways were also suggested for hypoxia-induced chemoresistance (Dong *et al.*, 2001; Erler *et al.*, 2004; Piret *et al.*, 2006).

Although various HIF-1 α inhibitors have been developed (discussed in *Chapter 1*), they are not available yet for clinical use. However in clinical settings several groups associated expression of HIF-1 α in tumour tissue with poor prognosis or increased metastasis following concurrent chemoradiotherapy in patients with colorectal (Cao *et al.*, 2009), oesophageal (Ogawa *et al.*, 2011), gastric (Nakamura *et al.*, 2009), pancreatic (Kazuhiko *et al.*, 2011) or breast (Tiezzi *et al.*, 2013) cancer. By contrast HIF-1 α overexpression was also reported to have no impact on the prognosis of ovarian (Birner *et al.*, 2001) or gastric cancer (Urano *et al.* 2006) in patients following chemotherapy.

Thus there is evidence to suggest that HIF-1 contributes to hypoxia-induced resistance to chemotherapeutic drugs in tumour cells. However its role appears to be variable and may depend on chemotherapeutic drug or experimental model used. Therefore this study focused on testing the hypothesis that HIF-1 α plays a causal role in hypoxia-induced resistance to cytotoxic drugs conventionally used for treatment of solid tumours. If so then manipulating HIF-1 α status by pharmacological or genetic means

could improve the clinical effectiveness of chemotherapy in solid tumours. In order to generalise observed effects cell lines derived from three different common human tumour types were compared.

Materials and Methods

Chemicals and reagents

Ethyl-3,4-dihydroxybenzoate (EDHB), 3-[2-[4-(bis(4-fluorophenyl)methylene)-1-piperidinyl]ethyl]-2,3-dichloro-2-thioxo-4(1H)-quinazolinone (R59949), deferoxamine mesylate salt, *cis*-diammineplatinum (II) dichloride (cisplatin), doxorubicin hydrochloride, 5-fluorouracil (5-FU), paclitaxel, dimethyl sulfoxide (DMSO) and Hepes buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cobalt(II) chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) was an AnalaR[®] grade reagent (BDH Laboratory Supplies, England). All other reagents were of the highest purity commercially available.

Stock solutions and storage

Stock solutions of EDHB (250 mM), R59949 (50 mM), cisplatin (100 mM), doxorubicin (10 mM), 5-FU (100 mM) and paclitaxel (10 mM) were prepared in sterile DMSO and aliquots were stored at -20°C. Prior to use stock solutions were thawed and diluted in sterile culture medium to the required final concentration. Deferoxamine (20 mM) and CoCl_2 (200 mM) were prepared fresh daily in sterile PBS then diluted in sterile culture medium to the required final concentrations.

Exposure to hypoxia

In order to induce hypoxia for oxygen-dependent chemosensitivity experiments we investigated two protocols that differed substantially in severity and duration of oxygen depletion. Subconfluent cultures were subjected to either 1 hour severe hypoxia

(<0.2% O₂ x 1 h) as described in *Chapter 3*, or to less severe hypoxia (0.51 ± 0.02% O₂ balanced with 5% CO₂, 94.5% N₂) for 24 hours. For the second option the cells were placed in the MIC-101 chambers and constantly flushed (0.5 L/min x 24 h) with water-saturated gas mixture at 37°C. TYGON® F-4040-A tubing with a low O₂ permeability coefficient ($\sim 10^{-11}$, [amount of gas (cm³) x tubing wall thickness (cm)]/[surface area of tubing (cm²) x time (seconds) x pressure drop across tubing wall (cmHg)]) was used to connect the gas flow regulator to the controlled-atmosphere chamber. Figure 4.1 shows schematically the arrangement used.

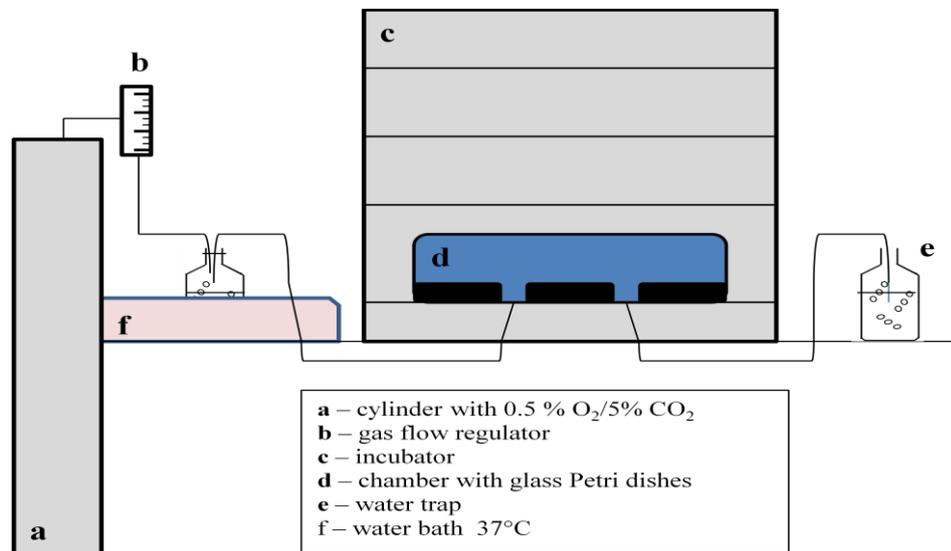


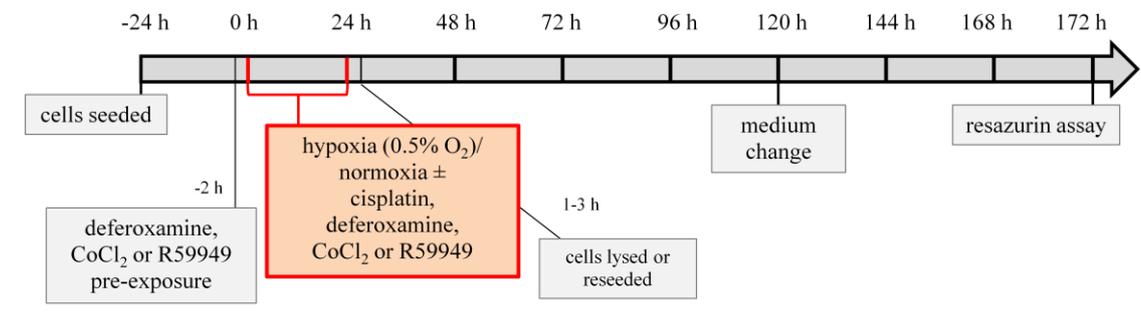
Figure 4.1 **Experimental arrangement for exposing cell cultures to prolonged hypoxia (24 h).** *Schematic, not to scale.*

Experimental design

Several key parameters were varied in order to establish an *in vitro* model of oxygen-dependent chemosensitivity in the three cell lines used. Wild type or transfected cells were seeded based on their doubling time and plating efficiency the day before

they were exposed to chemotherapeutic drug treatment, altering HIF-1 α protein level or hypoxia (0.5% O₂) as described before (*Chapter 3*). In the case of transfected cells, 12 hours before drug/hypoxia treatment transfected cells were reseeded into 6-well plates. Immediately after the 24 hour period of hypoxia cells were reoxygenated by opening the controlled atmosphere chamber, then either rapidly cooled (<1 min) and lysed, or harvested (<5 min), counted, reseeded at low density and allowed to grow for 7 days before measurement of cell proliferation. Figure 4.2 shows a summary of the standard experimental design used to investigate HIF-1 α protein role in oxygen-dependent chemosensitivity. Within this general format conditions were varied further as described in the *Results* section.

wild type cells



HIF-1 α knockdown cells

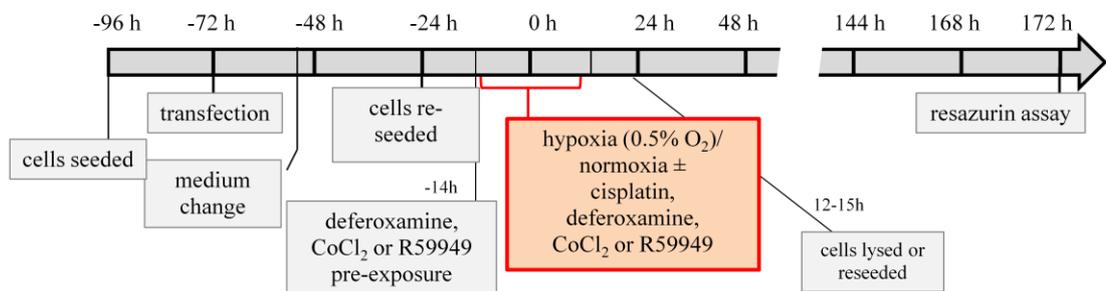


Figure 4.2. **General experimental design for study of HIF-1 α role in oxygen-dependent chemosensitivity.**

Results

Development of an *in vitro* model of oxygen-dependent chemosensitivity

In order to establish an *in vitro* model of oxygen-dependent chemosensitivity we used several structurally and mechanistically distinct chemotherapeutic drugs. However for logistical reasons only one, randomly picked drug was tested in pilot experiments using a limited number of cell lines. Initial conditions were chosen to match those in the oxygen-dependent radiosensitivity experiments (*Chapter 3*), to facilitate direct comparison. In the first of the experiments we tested whether exposure of HeLa cells to doxorubicin for 1 h (so that SH could be imposed concurrently) would show oxygen-dependent chemosensitivity. Drug concentration was varied across the range 3 nM - 3 μ M, for measurement of toxicity evident as reduced cell growth 7 days later. (Fig. 4.3, *panel A*). At the lowest concentration some evidence of adaptive and stimulation growth response was observed (hormesis). However in the doxorubicin concentration range over which significant toxicity was seen (0.3 – 3 μ M) no protection was conferred by simultaneous hypoxia (Fig. 4.3, *panel B*).

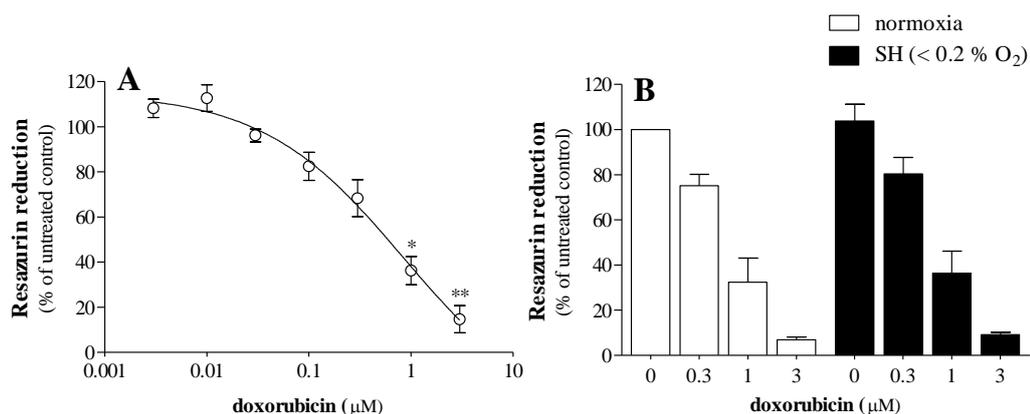


Figure 4.3. Cytotoxicity (A) and oxygen-dependent chemosensitivity (B) of acute doxorubicin exposure in HeLa cells. Cell cultures seeded in glass Petri dishes at a final density of 4×10^5 cell/dish were exposed to doxorubicin for 1 hour under either normoxia or SH (<0.2% O₂). Cell proliferation was measured 7 days later using the resazurin assay. Data shown are means \pm SE of 3 independent experiments. Comparisons *, doxorubicin-treated vs. control.

Most *in vitro* studies investigating oxygen-dependent chemosensitivity suggest that drug exposure for at least 8 h is needed in order to show increased resistance under conditions of reduced oxygen concentration. Therefore we adopted a model where drug exposure period was lengthened to 24 h. We selected cisplatin as a representative drug commonly used for treatment of human solid tumors including advanced cervical, bladder, testicular and ovarian carcinomas. Moreover cisplatin was reported to be less effective under hypoxia in testicular (Koch *et al.*, 2003) and gastric (Rohwer *et al.*, 2010) tumour cells. We also chose cisplatin because of its mechanism of action which resembles the damage caused by ionizing radiation (described in *Chapter 3*), in distorting the DNA structure in a way that if not repaired can lead to cell death. First concentration-dependent cytotoxicity of cisplatin was tested in each cell line under normoxia (Fig. 4.4).

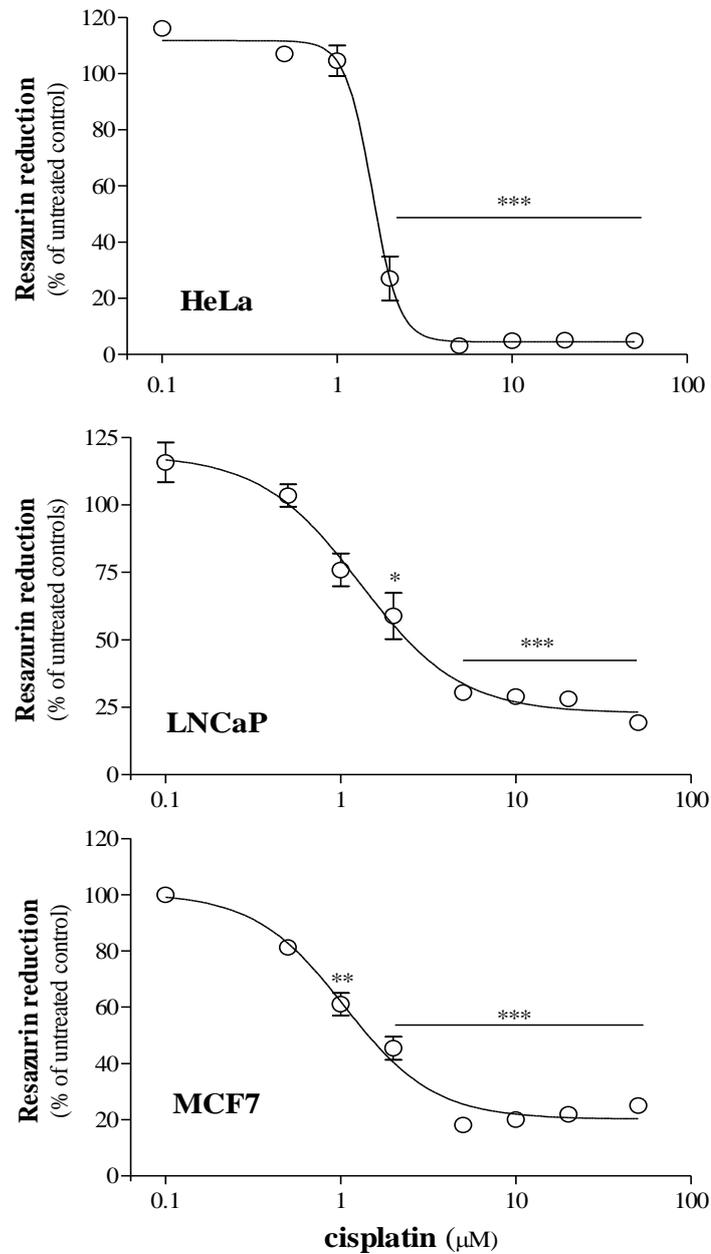


Figure 4.4. **Cytotoxicity of cisplatin under normoxia in three cancer cell lines.** Cell cultures seeded in glass Petri dishes at a final density of 4×10^5 cells/dish were incubated with 0.1-50 μ M cisplatin for 24 hours. Toxicity was measured 7 days later using the resazurin assay. Data shown are means \pm SE from 3 separate experiments. Where bars are not visible SE lies within the symbol shape. Comparison: *, drug-treated vs. untreated control.

After 24 h exposure to cisplatin the cells were cultured in drug-free culture medium for 7 days before using the resazurin reduction assay to measure toxicity in terms of reduced proliferation. Figure 4.4 shows the best-fit dose-response curves for each cell line, giving IC_{50} values for 24 h exposure to cisplatin of 1.7, 2.4 and 1.4 μM for HeLa, LNCaP and MCF7 cells respectively. As for doxorubicin, small but not significant hormesis was observed for 2 of the 3 cell lines (Fig. 4.4). However it is unlikely that severe hypoxia ($<0.2\% \text{ O}_2$) as used in the radiosensitivity model could be sustained for 24 h without causing major cell injury (see later, Fig. 4.6). Therefore the cisplatin concentration range (1-5 μM) found to cause strong but sub-maximal toxicity was then used to test whether exposure to SH for 1 h immediately before or after more prolonged drug exposure (24 h) would result in oxygen-dependent chemosensitivity. Figure 4.5 shows that only hypoxic pretreatment significantly protected HeLa cells exposed to 2 μM (1.7-fold increase in cell survival), or LNCaP cells exposed to 2 or 5 μM cisplatin (1.4 and 1.8-fold increase in survival). Cisplatin at 1 μM was not tested on HeLa cells because it did not produce any toxicity in contrast to the effect on LNCaP and MCF7 cells (Fig. 4.4).

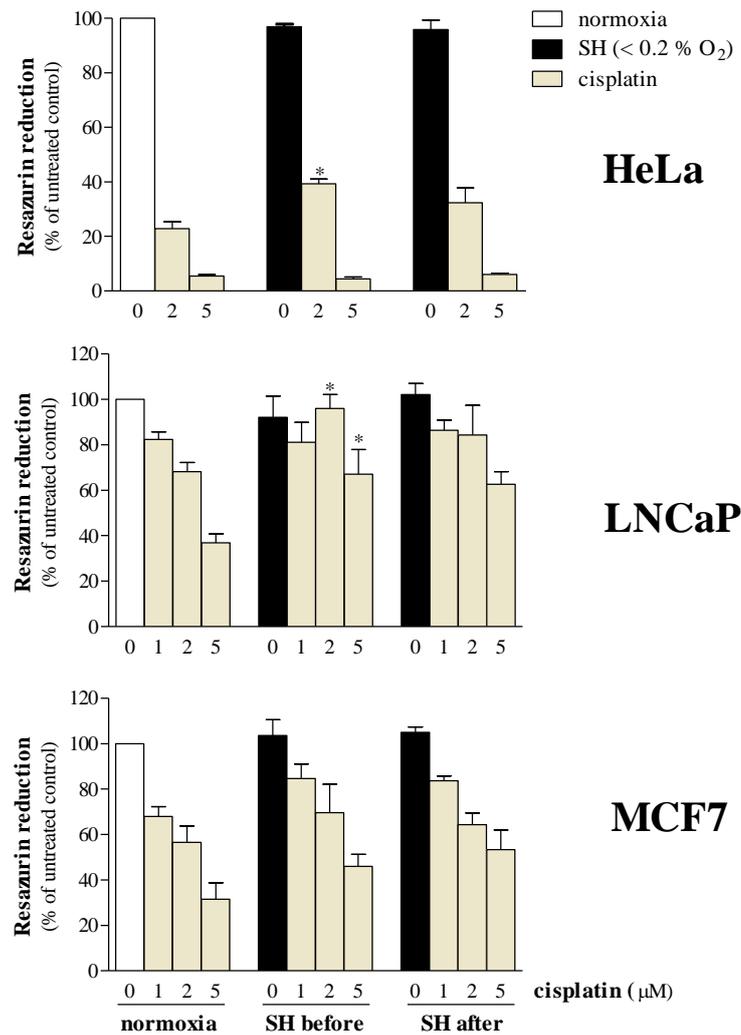


Figure 4.5. **Effect of 1 hour SH immediately before or after exposure to cisplatin on sensitivity of HeLa, LNCaP and MCF7 cells.** Cells cultures seeded in glass Petri dishes at a final density of 4×10^5 cells/dish were exposed to severe hypoxia (<0.2 O₂) for 1 h immediately before or after exposure to 1, 2 or 5 μM cisplatin for 24 h. Cell proliferation was measured 7 days later using the resazurin assay. Data shown are means \pm SE of 3 independent experiments. Comparisons *, SH treated vs. normoxic controls at equivalent cisplatin concentration.

Although some oxygen-dependent chemosensitivity was observed under these conditions it was not as marked as in the radiosensitivity model (~4-fold difference in survival between normoxic and SH cells).

Overall, oxygen-dependent chemosensitivity seen in cell cultures deprived of oxygen for 1 h concurrent with doxorubicin exposure did not show any evidence of SH-induced cell protection. Furthermore the protection afforded by 1 h SH before or after 24 h cisplatin exposure was neither substantial nor fully consistent between cell lines. Therefore since most *in vitro* models of oxygen-dependent chemosensitivity described in the literature involve a hypoxic period of 8 h or more, next we investigated how extending severe oxygen depletion (<0.2% O₂) affected HeLa cell viability. In order to do so the controlled-atmosphere chambers were clamped after the 38 min gassing period with oxygen-free nitrogen required to produce SH. At various times thereafter (2-24 h) the clamps were released, then reoxygenated cells were immediately subcultured and grown for 7 days before measuring proliferation in terms of resazurin reduction. Figure 4.6 shows that this proliferation decreased progressively as duration of SH insult was increased (from 10 % decrease in cell survival after 2 h hypoxia up to 60 % for 24 h).

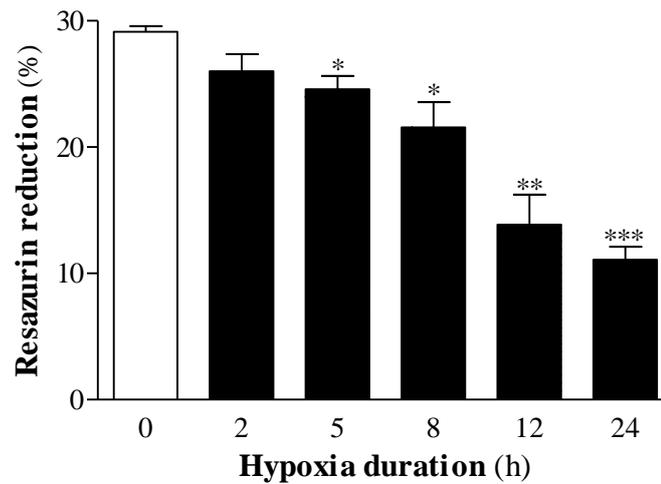


Figure 4.6. **Impaired cell proliferation after prolonged oxygen deficiency.** Cells cultures seeded in glass Petri dishes at a final density of 4×10^5 cells/dish were exposed to SH ($O_2 < 0.2\%$) for 2-24 hours, then subcultured and incubated for 7 days before measurement of cell proliferation using the resazurin assay. Data shown are means \pm SE of 3 independent experiments. Comparisons *, SH vs. normoxic control.

A complication of maintaining the bicarbonate-buffered cell culture in an atmosphere with $CO_2 < 5\%$ is that the pH of the medium will rise, although phenol red colour in the culture medium at the end of 24 h period under N_2 without CO_2 was not changed. Therefore we tested whether increasing the HEPES concentration to enhance CO_2 -independent pH buffering would reduce cell injury caused by prolonged SH. Culture medium originally containing 35 mM HEPES was further supplemented to a final concentration of 45 or 60 mM while maintaining the pH unchanged. Increased buffering stimulated cell growth under normoxia but did not reduce hypoxia-induced injury in a dose-dependent way (Fig. 4.7).

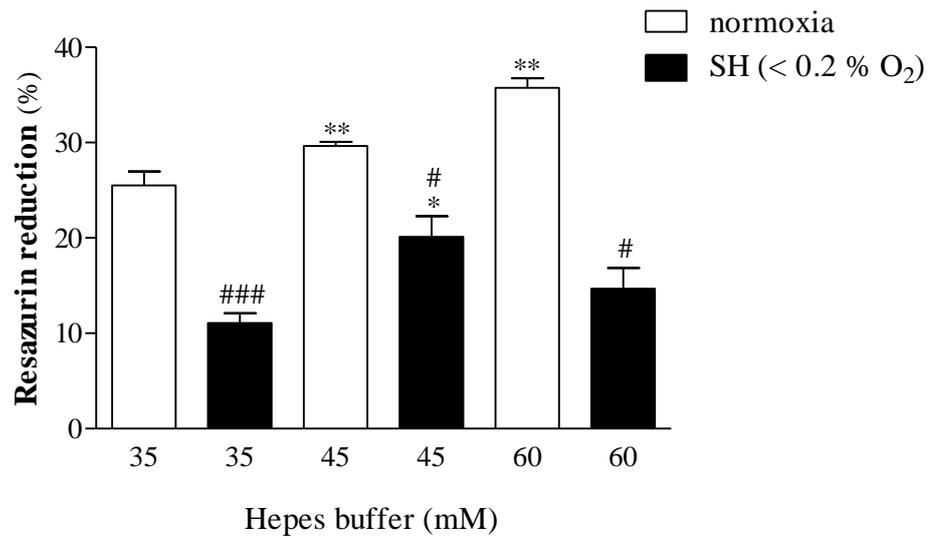


Figure 4.7. **Effect of increased CO₂-independent buffering (Hepes) on HeLa cell proliferation after 24 h SH without CO₂.** Cells seeded in glass Petri dishes at a final density of 4×10^5 cells/dish in medium with Hepes buffer as indicated were exposed to SH (O_2 -free N_2 , with no more than trace CO_2 present) for 24 hours, then subcultured and incubated for 7 days before measurement of cell proliferation using the resazurin assay. Data shown are means \pm SE of 3 independent experiments. Comparisons *, Hepes-supplemented vs. standard medium; #, hypoxia vs. normoxia.

We also tested whether Hepes supplementation of the culture medium affected the oxygen-dependent chemosensitivity of HeLa cells exposed to cisplatin for 24 h. However cisplatin toxicity was not significantly oxygen-dependent at either of the two higher Hepes concentrations (Fig. 4.8).

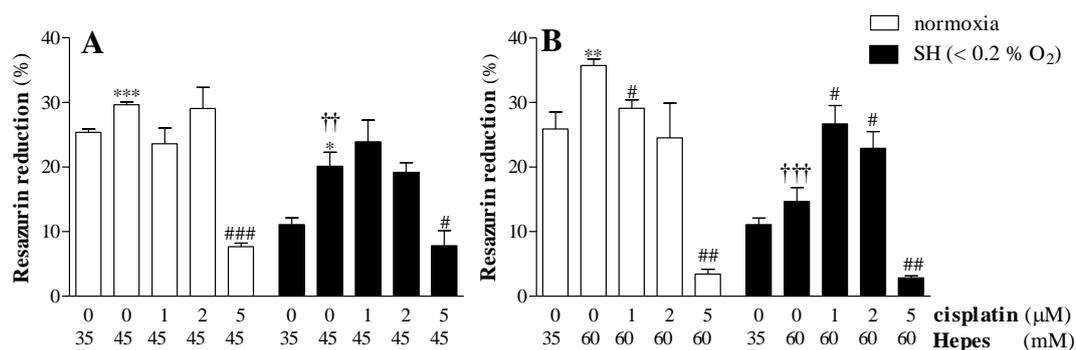


Figure 4.8. **Effect of 24 h oxygen depletion under increased Hepes buffer concentration conditions on chemosensitivity of HeLa cells to cisplatin.** Cells seeded in glass Petri dishes at a final density of 4×10^5 cells/dish in medium containing either 45 (A) or 60 (B) mM Hepes were exposed to hypoxia concurrently with 1, 2 or 5 μ M cisplatin for 24 hours, then subcultured and incubated for 7 days before measurement of cell proliferation using the resazurin assay. Data shown are means \pm SE of 3 independent experiments. Comparisons *, normoxia/hypoxia with raised Hepes concentration vs. normal Hepes concentration; #, cisplatin-treated vs. untreated control; †, hypoxia vs. normoxia at equivalent Hepes concentration.

The next protocol tested was to subject cells to 1 h hypoxia (O₂-free N₂) followed by 2 h normoxia (5% CO₂ in air) in 8 cycles over a 24 h concurrent exposure to cisplatin. During periods of normoxia Petri dishes containing cell cultures were removed from the controlled-atmosphere chambers but remained within the incubator under 5% CO₂, to ensure rapid re-equilibration of CO₂ levels in the culture medium. This treatment caused elevation of HIF-1 α level in LNCaP though not in HeLa cells (Fig. 4.9, panel A). However, repeated cycles of hypoxia (8 x 1 h, punctuated by 2 h periods of normoxia) produced no oxygen-dependent sensitivity to cisplatin in either cell line (Fig. 4.9, panel B and C).

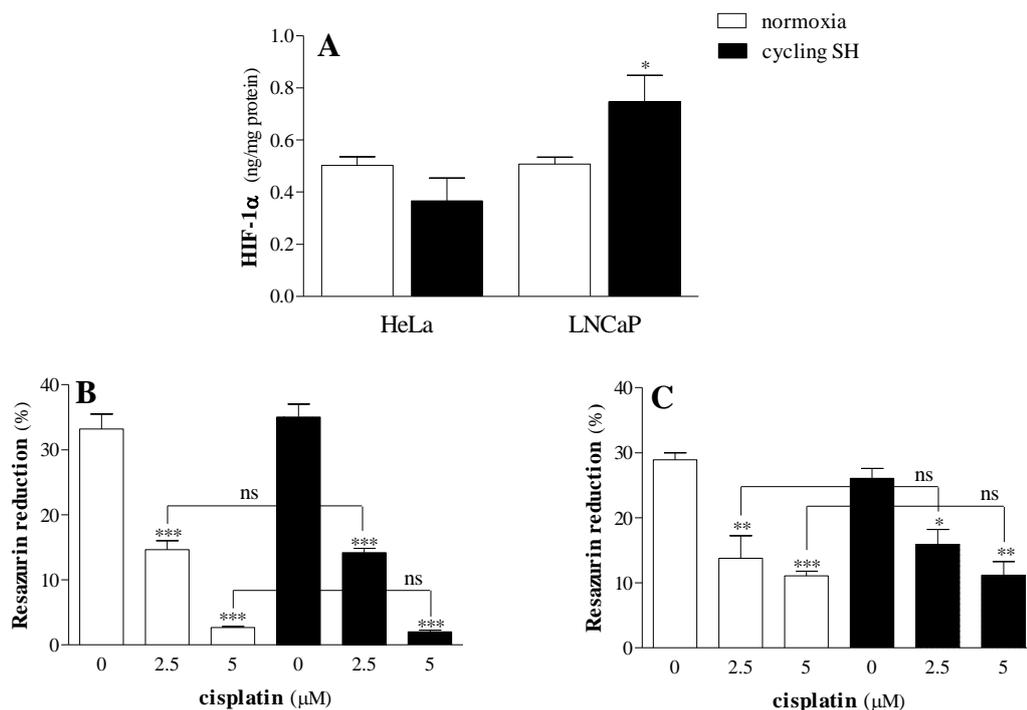


Figure 4.9. **Impact of repeated SH (8 x 1 h cycles at 2 h intervals) on HIF-1 α protein level (A) measured acutely and on (B) HeLa and (C) LNCaP cell proliferation measured after 7 days.** Cells seeded in glass Petri dishes at a final density of 1×10^6 (A) or 4×10^5 (B,C) cells/dish were subjected to 8 cycles of 1 h SH followed by 2 h reoxygenation, then either lysed or subcultured for 7 days. Cell proliferation was measured using the resazurin assay and HIF-1 α protein level was analysed by ELISA. Data shown are means \pm SE from 3 separate experiments. Comparison *, cisplatin treated vs. untreated controls. For both cell lines at both cisplatin concentrations $p > 0.05$ (ns) for SH vs. normoxia.

Since these variations in hypoxia duration and timing produced little or no oxygen-dependent sensitivity to cisplatin or doxorubicin in any of the cell lines tested, next we investigated whether it could be seen under less severe hypoxia (0.5 % O₂). HeLa and MCF7 cultures were exposed to cisplatin (0-200 μ M), 5-fluorouracil (0-500 μ M), doxorubicin (0-10 μ M) or paclitaxel (0-300 nM) for 24 h under either normoxia or MH

(0.5% O₂). Effect of drug exposure was measured either immediately or after 3 days. For the latter, drug-containing culture medium was replaced with drug-free one without reseeding the cells.

Resazurin reduction measured immediately after the 24 h drug exposure period showed a concentration-dependent toxicity of cisplatin and doxorubicin in both HeLa and MCF7 cells, an effect of paclitaxel in HeLa only and an effect of 5-fluorouracil in neither cell line, over the concentration range tested (Fig. 4.10). We also tested whether exposure of HeLa cells to cisplatin (150 μM) under conditions where the atmosphere-controlled chambers were constantly flushed with O₂-free N₂ for 24 h could increase cell survival. However, no oxygen-dependent chemosensitivity was seen under these conditions either (data not shown). By contrast, proliferation measured 3 days later showed concentration-dependent effects of all 4 drugs on HeLa but still no significant toxicity to either 5-fluorouracil or paclitaxel on MCF7 cells over the concentration ranges tested (Fig. 4.11).

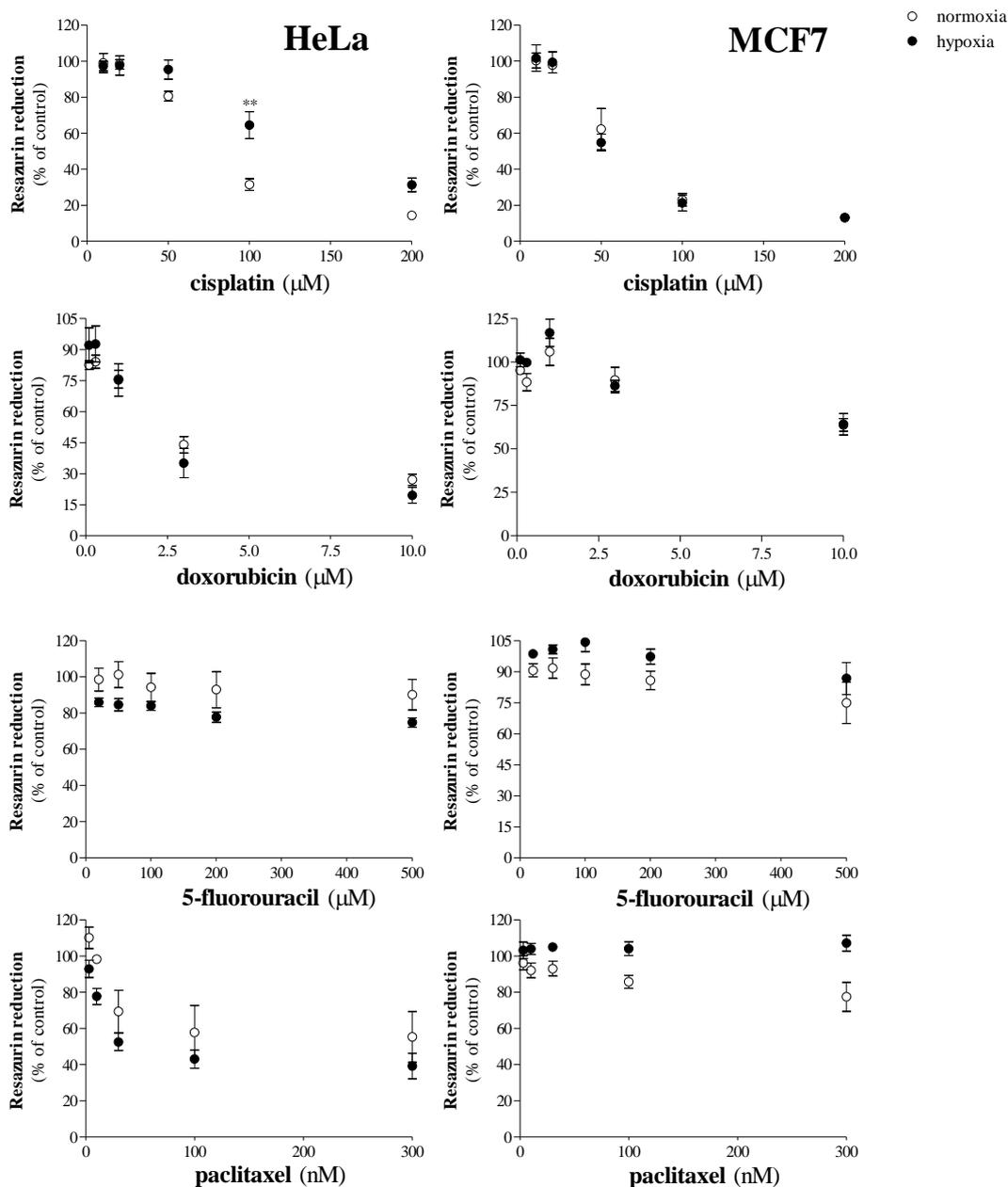


Figure 4.10. Acute sensitivity of HeLa and MCF7 cells exposed to cisplatin, doxorubicin, 5-FU and paclitaxel under normoxic and hypoxic conditions. Cells were incubated with chemotherapeutic drugs under either 21% O_2 (normoxia, white circles) or 0.5% O_2 (hypoxia, black circles) for 24 h, after which drug was removed and resazurin reduction measured. Data are normalized relative to normoxic or hypoxic controls, and are shown as means \pm SE from 3 separate experiments. Comparison *, hypoxia vs. normoxia at equivalent drug concentration.

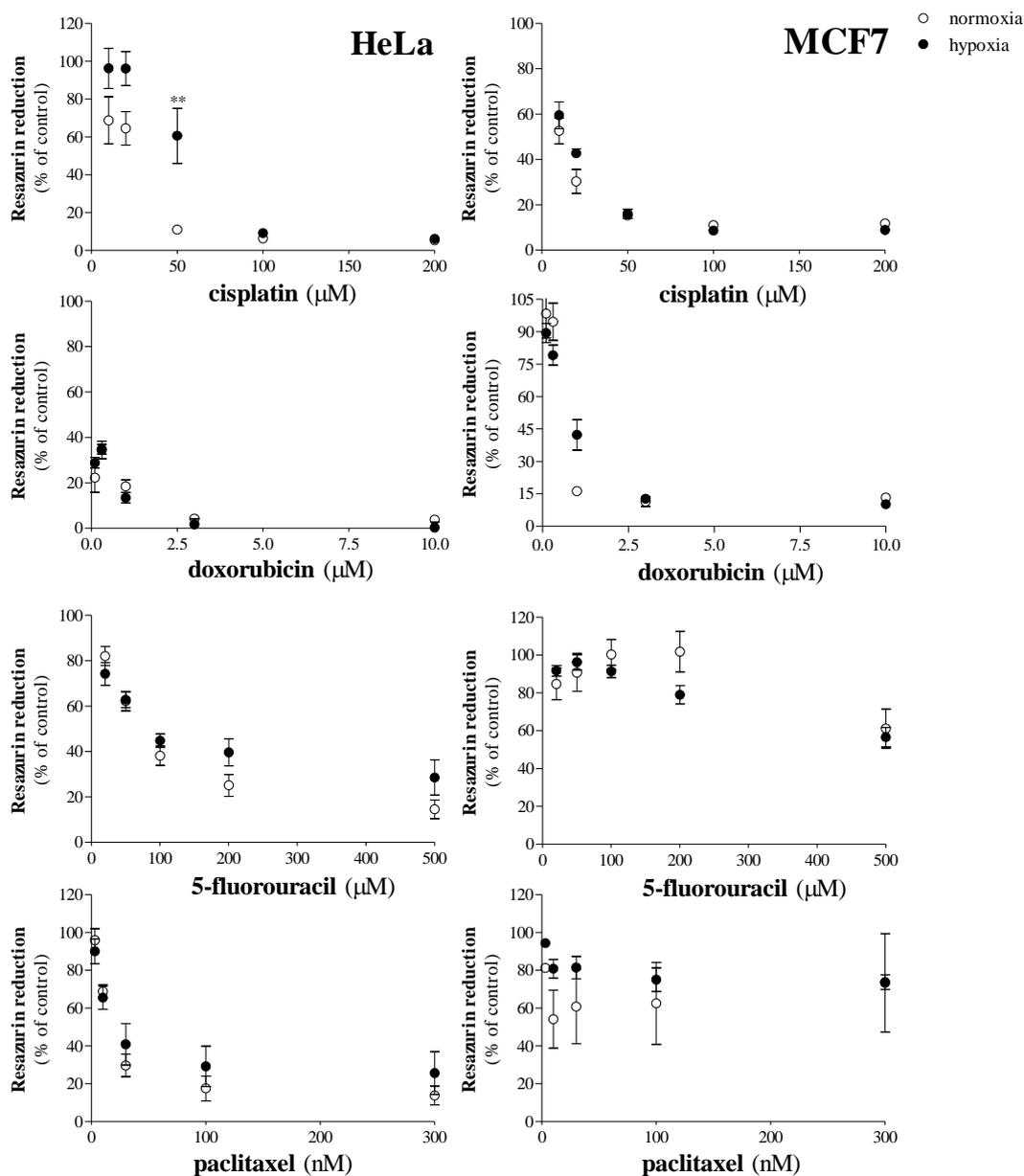


Figure 4.11. **Delayed sensitivity of HeLa and MCF7 cells exposed to cisplatin, doxorubicin, 5-FU and paclitaxel under normoxia or hypoxic conditions.** Cells exposed to chemotherapeutic drugs under either 21% O_2 (normoxia, white circles) or 0.5% O_2 (hypoxia, black circles) for 24 h were then further incubated in drug-free conditions for 3 days, after which resazurin reduction was measured. Data are normalized relative to normoxic or hypoxic controls, and are shown as means \pm SE from 3 separate experiments. Comparison: * hypoxic vs. normoxic at equivalent drug concentration.

Hypoxic cells were significantly more resistant to chemotherapeutic drug-induced injury than normoxic controls only in the case of HeLa cells exposed to 100 μM cisplatin when measured immediately after the exposure period, or to 50 μM when assayed 3 days later. However the difference was more marked than seen previously using SH (<0.2% O_2 x 1 or 24 h). Therefore we tested whether oxygen-dependent sensitivity to cisplatin was evident when cell cultures were allowed to proliferate for the standard 7 day period used in radiosensitivity experiments (*Chapter 3*). MeWo cells were also used in place of LNCaP because the latter attached poorly so could be lost during medium changes. Normoxic and hypoxic cultures were exposed to two cisplatin concentrations (2.5 and 5 μM , previously found to cause sub-maximal toxicity under normoxia (Fig. 4.4)) for 24 h, then subcultured for 7 days after which resazurin reduction was measured. Under these conditions significant hypoxia-induced resistance to 2.5 μM cisplatin was observed in all 3 cell lines tested. It was most marked for HeLa and MeWo cells, with 4- and 3-fold increased cell survival respectively (Fig. 4.12). We also tested whether HIF-1 α level which was readily elevated by SH (<0.2% O_2 x 1h) was also raised by the MH (0.5% O_2 x 24 h) protocol. Figure 4.13 shows that prolonged exposure to 0.5 % O_2 increased HIF-1 α level in all cell lines tested, although not significantly in MeWo. However this change in HeLa and MCF7 was substantially lower than observed previously after SH treatment (Fig. 3.15). Therefore for all subsequent experiments evaluating HIF-1 α role in oxygen-dependent chemosensitivity we adopted a standard model of 24 h exposure of cells to 2.5 μM cisplatin in the presence of ambient or 0.5% O_2 , followed by subculture and measurement of proliferation 7 days later.

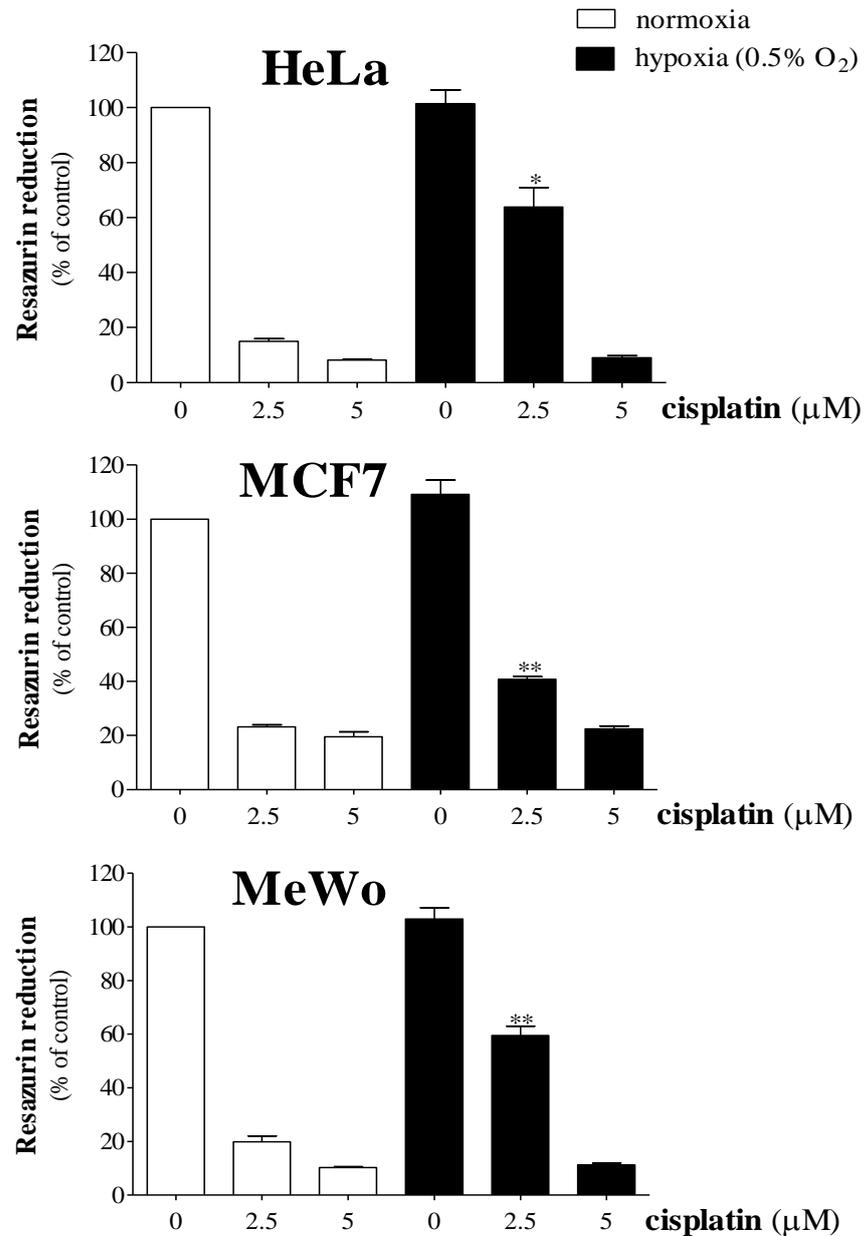


Figure 4.12. **Long term oxygen-dependent sensitivity of HeLa, MCF7 and MeWo cells to cisplatin.** Cells exposed to 2.5 or 5 μM cisplatin under either 21% O₂ (normoxia) or 0.5% O₂ (hypoxia) for 24 h were then subcultured in drug-free medium for 7 days after which resazurin reduction was measured. Data shown are means ± SE from 3 separate experiments. Comparison: * hypoxic vs. normoxic at equivalent cisplatin concentration.

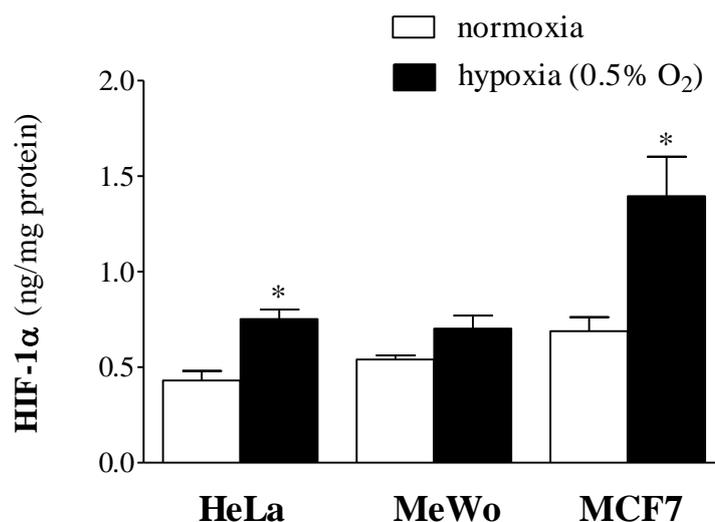


Figure 4.13. **Impact of prolonged hypoxia on HIF-1 α protein level in 3 cell lines.** Cell cultures seeded in glass Petri dishes at a final density of 1×10^6 cells/dish were subjected to 0.5% O₂ (hypoxia) for 24 h, then rapidly cooled, lysed and analysed for HIF-1 α protein level by ELISA. Data shown are means \pm SE from three separate experiments. Comparison:*, hypoxia vs. normoxia.

Sustained pharmacological alteration of HIF-1 α level

As described in *Chapter 3* the causal role of altered HIF-1 α status was tested using EDHB and R59949 (Fig. 3.4), to increase and decrease respectively the HIF-1 α level in cells. Because exposure to hypoxia in the oxygen-dependent chemosensitivity model was lengthened to 24 hours it was necessary to identify EDHB and R59949 treatment conditions that caused sustained effects on HIF-1 α level without marked toxicity. In the first of several pilot experiments these parameters were tested in parallel in two out of the three cell lines. Initially cell cultures were pre-treated with EDHB and R59949 at concentrations previously found to be effective (0.5 mM x 2 h and 0.3 mM x 3 h respectively) followed by exposure to the agents at 5-fold lower concentration over

the next 24 h. Figure 4.14 Panel A shows that prolonged exposure to EDHB failed to elevate HIF-1 α in either cell line tested. Treatment with R59949 lowered HIF-1 α levels, but also increased acute morphological damage to the cells (not shown) and toxicity as measured 7 days later. (Fig. 4.14, panel B).

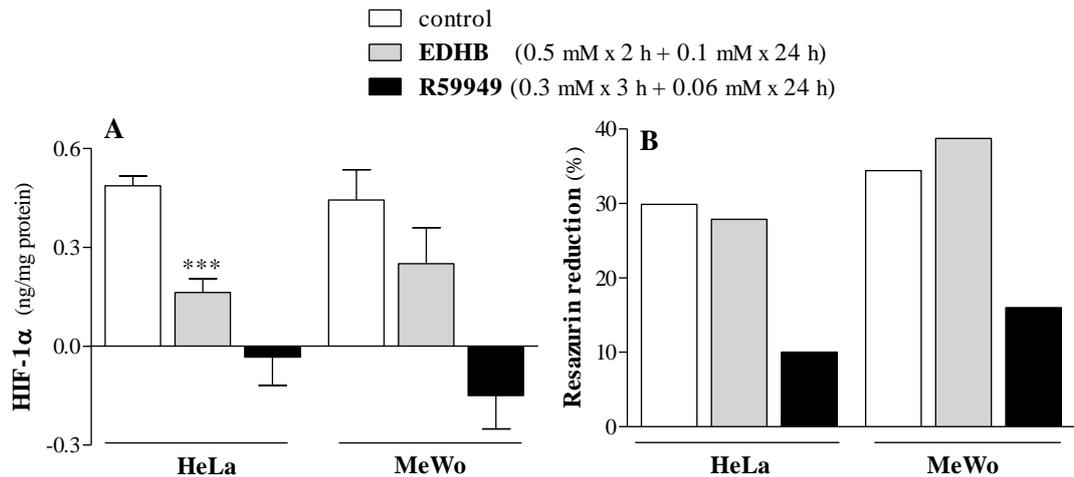


Figure 4.14. **Effect of prolonged EDHB and R59949 exposure on (A) HIF-1 α protein level and (B) subsequent cell proliferation.** Cell cultures at a final density of 1×10^6 (A) or 4×10^5 (B) cells/dish were treated with EDHB or R59949 as indicated, then either rapidly cooled, lysed and analysed for HIF-1 α protein level by ELISA (A) or subcultured for 7 days (B) and cell proliferation measured in terms of resazurin reduction. Data shown are from 3 experiments (A; mean \pm SE) or from a pilot experiment (B).

Therefore in the next series of experiments the concentrations of both agents were reduced 2.5-fold while maintaining the exposure duration, in an effort to reduce development of tolerance to EDHB and toxicity of R59949. Figure 4.15 panel A shows that in all three cell lines EDHB again failed to increase HIF-1 α protein level over 24 h, whereas this R59949 treatment decreased HIF-1 α level without increasing toxicity (Fig. 4.15, panel B).

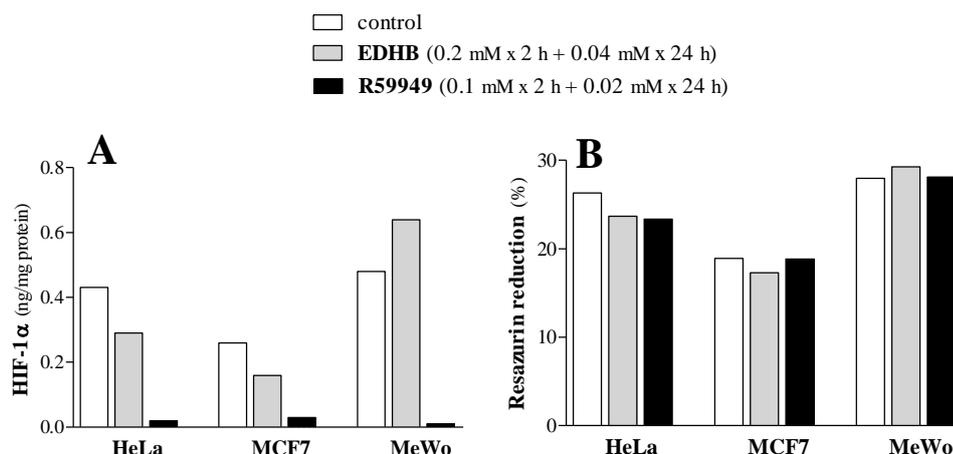
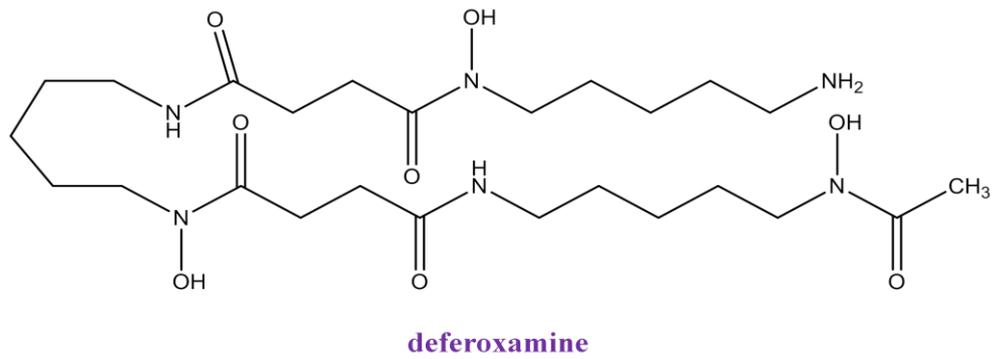


Figure 4.15 **Effect of prolonged exposure to EDHB and R59949 at lower concentration on HIF-1 α protein level (A) and cell proliferation (B).** Cell cultures at a final density of 1×10^6 (A) or 4×10^5 (B) cells/dish were treated with EDHB or R59949 as indicated, then either rapidly cooled, lysed and analysed for HIF-1 α protein level by ELISA (A) or subcultured for 7 days (B) and cell proliferation measured in terms of resazurin reduction. Data shown are from a pilot experiment.

Further permutations of EDHB concentrations were also tested (0.5 mM x 2 h followed by 0.25 mM or 0.5 mM x 24 h) but none elicited an increase in HIF-1 α protein level that was sustained over 24 h (data not shown). Therefore, two other agents known to stabilise HIF-1 α protein level were tested. Prolyl hydroxylases (PHDs) require an iron atom for catalytic activity so iron chelators such as deferoxamine inhibit them, thereby impairing the HIF-1 α hydroxylation that targets it for degradation (Yuan *et al.*, 2003). An IC_{50} range of 12-25 μ M has been reported for this indirect stabilisation of HIF-1 α (Kim *et al.*, 2010). Cobalt is a transition metal with an IC_{50} of 400 μ M (Yang *et al.*, 2004) that prevents HIF-1 α degradation both by replacing the iron in the iron-binding centre of the enzyme (Epstein *et al.*, 2001) and by impeding HIF-1 α interaction with

VHL even when it is already hydroxylated (Yuan *et al.*, 2003). These actions of deferoxamine and cobalt ions lead to stabilisation of HIF-1 α protein level (Fig. 4.16).

A



B

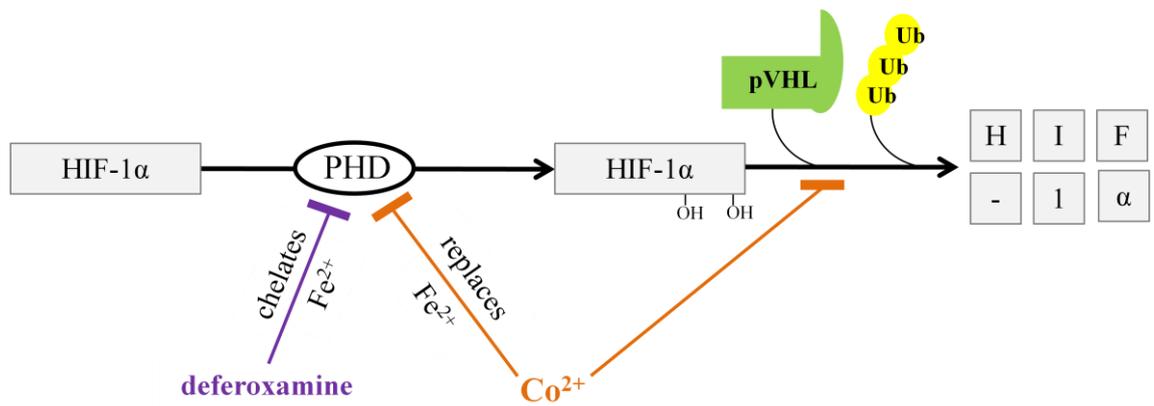


Figure 4.16. (A) Deferoxamine structure and (B) mechanism of action of deferoxamine and cobalt chloride.

A pilot experiment was carried out using HeLa cells to determine whether elevated HIF-1 α could be maintained after exposure to deferoxamine or CoCl₂ for 24 h. Figure 4.17 shows that both short (2 h) and prolonged (2 + 24 h) exposure to deferoxamine or CoCl₂ caused a substantial increase in HIF-1 α protein level in HeLa cells.

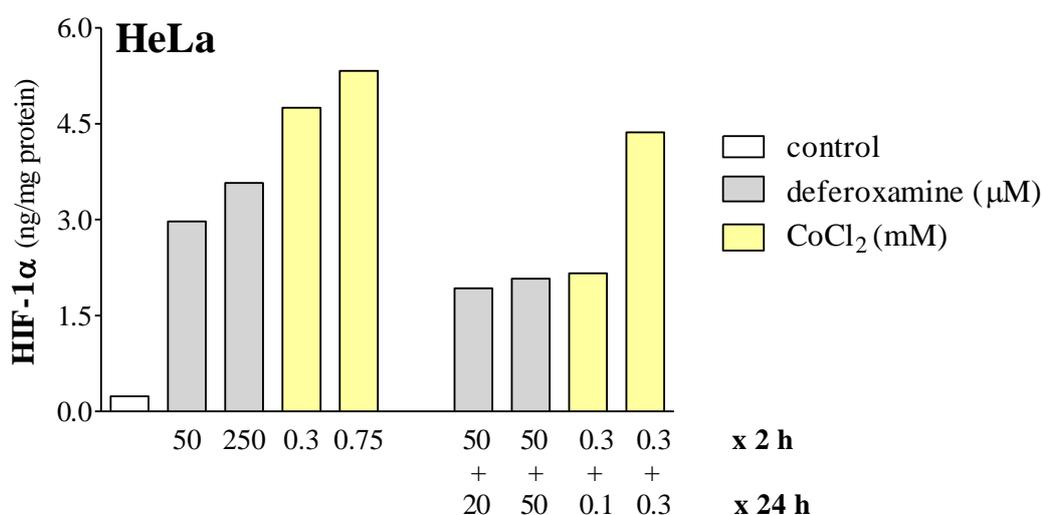


Figure 4.17. **Effect of deferoxamine or CoCl₂ exposure on HIF-1 α protein level in HeLa cells.** Cell cultures seeded in glass Petri dishes at a final density of 1×10^6 cells/dish were treated with deferoxamine or CoCl₂ at the concentrations shown either for 2 h or for 2+24 h, then rapidly cooled, lysed and analysed for HIF-1 α protein level by ELISA. Data shown are from a pilot experiment.

Therefore standard protocols of 50 μ M x 2 h then 20 μ M x 24 h for deferoxamine, 0.3 mM x 2 h then 0.1 mM x 24 h for CoCl₂ and 0.1 mM x 2 h then 0.02 mM x 24 h for R59949 were tested in all 3 cell lines (Fig. 4.18). All three treatments produced significant changes in HIF-1 α level after a 24 h exposure period. However they were not toxic (Fig. 4.19).

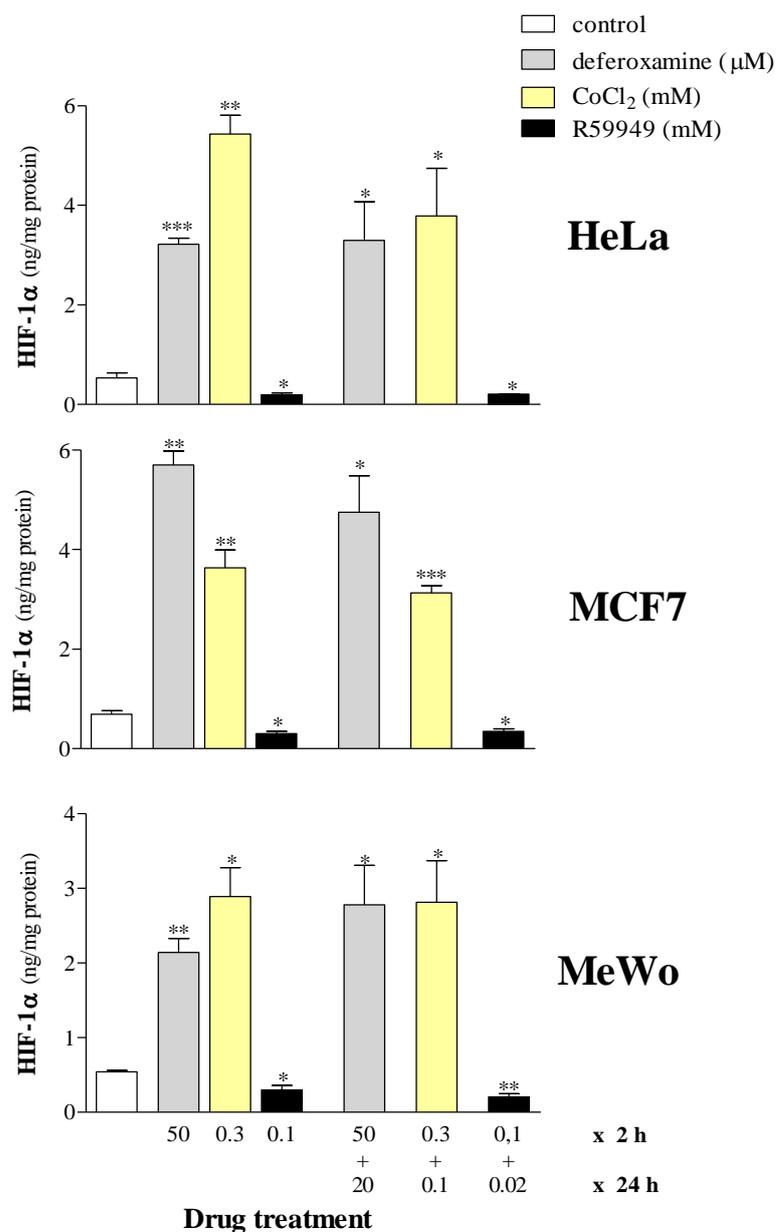


Figure 4.18. **Sustained alterations of HIF-1α protein level in 3 cell lines after deferoxamine, CoCl₂ or R59949 exposure.** Cell cultures seeded in glass Petri dishes at a final density of 1×10^6 cells/dish were treated with deferoxamine, CoCl₂ or R59949 at the concentrations indicated for 2 or 2+24 h, then rapidly cooled, lysed and analysed for HIF-1α protein level by ELISA. Data shown are means \pm SE from three separate experiments. Comparison: *, drug-treated vs. untreated controls. Note different scales on the vertical axes.

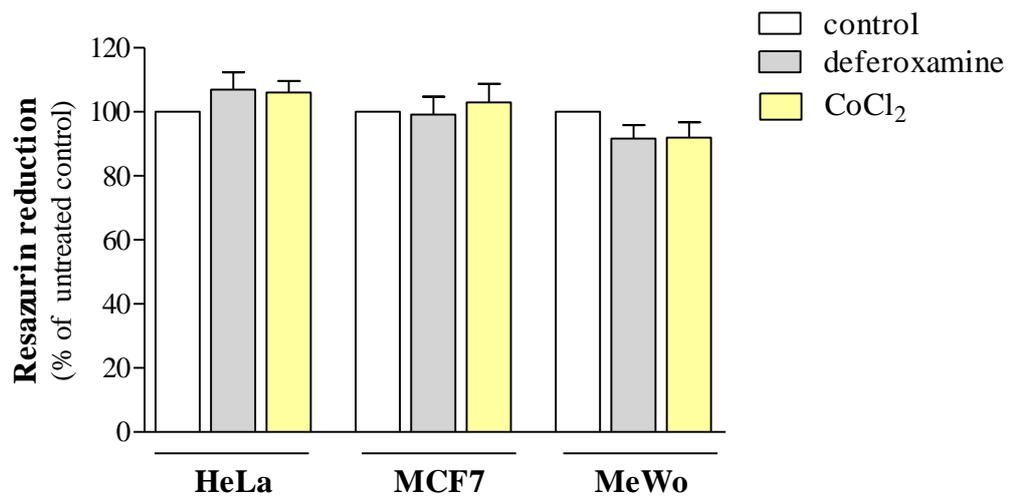


Figure 4.19. **Lack of toxicity of HIF-1 α stabilising deferoxamine and CoCl₂ treatments.** Cell cultures seeded into glass Petri dishes at a final density of 4×10^5 cells/dish were exposed to deferoxamine at $50 \mu\text{M} \times 2 \text{ h}$ followed by $20 \mu\text{M} \times 24 \text{ h}$, or CoCl₂ at $0.3 \text{ mM} \times 2 \text{ h}$ followed by $0.1 \text{ mM} \times 24 \text{ h}$, then harvested, counted and reseeded at low densities in drug-free medium. Resazurin reduction was measured 7 days later. Data shown are means \pm SE from 3 separate experiments ($p > 0.05$).

HIF-1 DNA-binding activity

In order to test whether HIF-1 α levels correlated with DNA binding activity we analysed nuclear extracts of cells exposed to hypoxia (0.5% O₂), deferoxamine, CoCl₂ or R59949 for 2 + 24 h. Figure 4.20 shows that under normoxia HIF-1 DNA-binding activity was low but following 24 h MH it was increased particularly in HeLa and MCF7 cells. This correlated with HIF-1 α protein level which was also raised significantly in those 2 cell lines when incubated under MH (Fig. 4.13). Deferoxamine and CoCl₂ treatments greatly increased HIF-1 α DNA binding activity in both normoxic and hypoxic cells (except hypoxic MeWo). Under hypoxia R59949 treatment caused marked decrease in HIF-1 DNA binding activity only in HeLa and MCF7 cells as compared to hypoxic controls (Fig. 4.20). However no effect was evident in normoxic cells, presumably because control levels of DNA binding were low already. Overall therefore, deferoxamine or CoCl₂-induced stabilisation and accumulation of HIF-1 α protein under normoxia also increased DNA-binding activity. Conversely, destabilisation of HIF-1 α using R59949 reduced DNA binding in hypoxic cells but not measurably in normoxic cells where DNA binding was close to the limit of detection of the assay used.

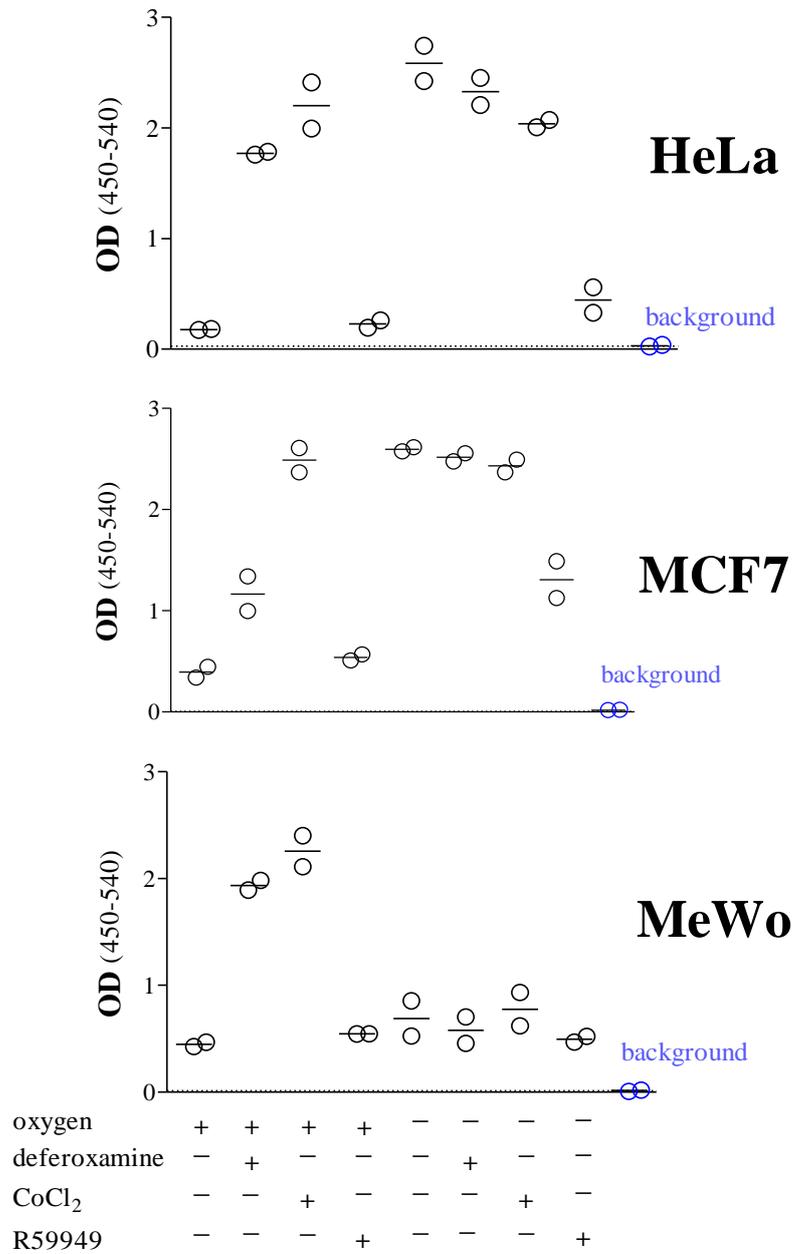


Figure 4.20. **HIF-1 DNA-binding activity.** Cells were treated with deferoxamine (50 μM x 2 h followed by 20 μM x 24 h), CoCl₂ (0.3 mM x 2 h followed by 0.1 mM x 24 h) or R59949 (0.1 mM x 2 h followed by 0.02 mM x 24 h) under either normoxia or hypoxia (0.5% O₂), then cooled to 4°C, lysed for preparation of nuclear extracts and analysed for HIF-1 DNA-binding activity. Data shown are individual values from 2 independent experiments.

HIF-1 α distribution between cytoplasmic and nuclear fractions

HIF-1 α protein is stabilised in the cytoplasmic fraction then translocates to the nucleus in order to dimerise with HIF-1 β subunit and bind to HREs of target genes. Since HIF-1 α protein level measured in whole cell extracts did not fully correlate with HIF-1 DNA binding in the case of hypoxic MeWo cells we also measured HIF-1 α distribution between cytoplasmic and nuclear fractions. Following 24 h MH HIF-1 DNA binding activity was increased and so the protein predominated in the nuclear fraction (95%). By contrast, although more than 70% of total HIF-1 α protein was present in the nuclear fraction of CoCl₂- and deferoxamine-treated MeWo cells (Table 4.1), the HIF-1 DNA binding activity was unaffected.

Cell type	Oxygenation status	Treatment	HIF-1 α (% of total)/Fraction	
			Cytoplasmic	Nuclear
HeLa	MH	-	43	57
MeWo	MH	-	5	95
MeWo	normoxia	CoCl ₂	6	94
MeWo	normoxia	deferoxamine	30	70
MeWo	MH	CoCl ₂	5	95
MeWo	MH	deferoxamine	26	74

Table 4.1. **Subcellular distribution of HIF-1 α protein between cytoplasmic and nuclear fractions in wild-type HeLa and MeWo cells.** MH (0.5 % O₂ x 24 h) cultures were immediately cooled to 4°C and lysed for preparation of nuclear and cytoplasmic fractions and measurement of HIF-1 α level by ELISA.

Role of HIF-1 α in oxygen-dependent sensitivity to cisplatin

Having established the model of oxygen-dependent sensitivity of HeLa, MCF and MeWo cells to cisplatin, we next investigated whether pharmacological manipulation of HIF-1 α protein level would affect cell response to chemotherapeutic drug. Briefly, the cells pre-exposed to deferoxamine, CoCl₂ or R59949 were further treated for 24 h with these agents under normoxia or hypoxia concurrently with cisplatin (2.5 μ M). Response of the cells to the treatment was measured 7 days later in terms of resazurin reduction.

Oxygen-dependent sensitivity to cisplatin was evident for all cell lines. Furthermore toxicity of deferoxamine, CoCl₂ or R59949 was not detected. However treatments likely to raise HIF-1 α level (deferoxamine, CoCl₂) did not confer significant protection on cells exposed to cisplatin in the presence of oxygen (Fig 4.21, *left column*). Under hypoxia, cisplatin induced cell injury was not significantly affected by HIF-1 α -modulating drug treatments except in the case of MeWo where deferoxamine and CoCl₂ sensitized cells whereas R59949 protected them (Fig 4.21, *right column*). Overall therefore, neither in normoxia nor hypoxia did altered HIF-1 α level affect cell response to cisplatin.

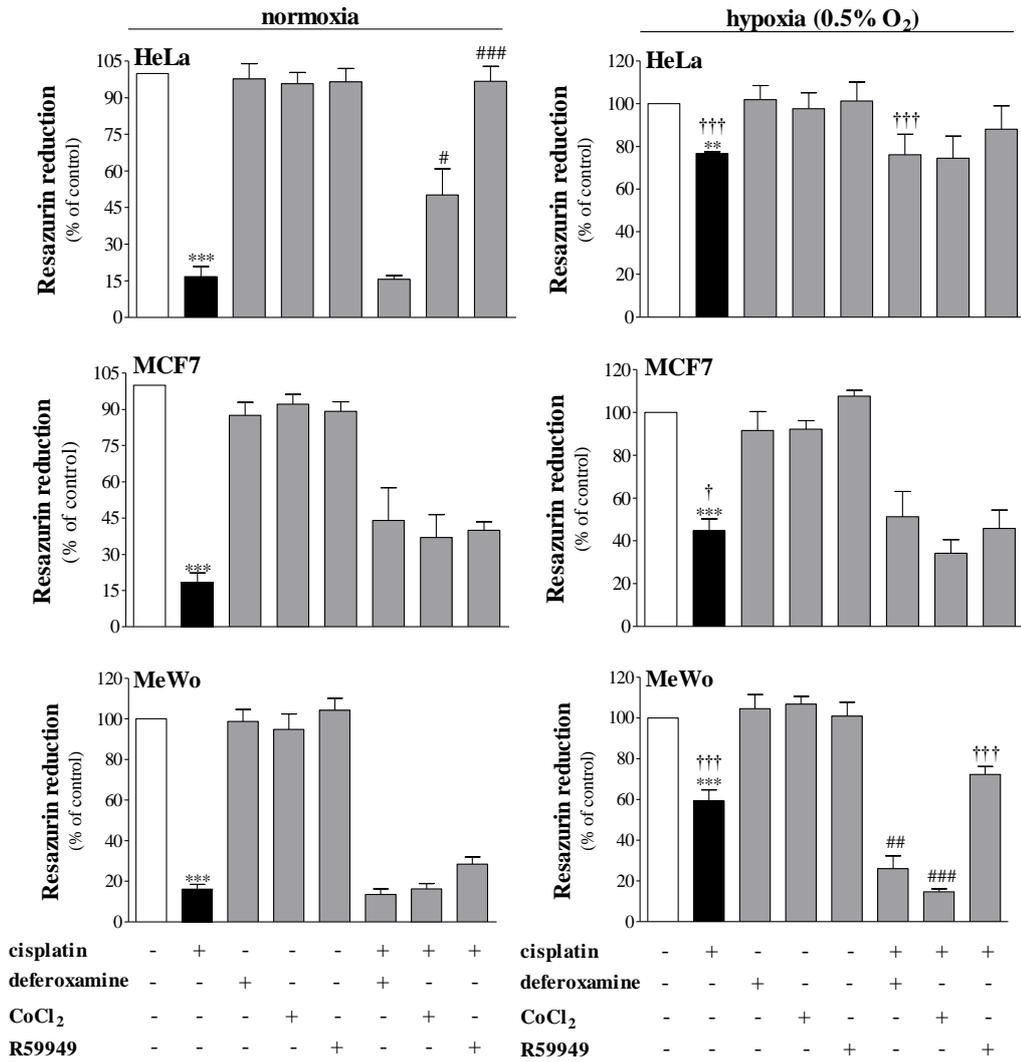


Figure 4.21. **Impact of altered HIF-1 α protein level on oxygen-dependent sensitivity of HeLa, MCF7 and MeWo cells to cisplatin.** Normoxic or hypoxic (0.5% O₂) cells in which HIF-1 α protein level was altered pharmacologically were exposed to cisplatin (2.5 μ M) for 24 h, then subcultured. After 7 days resazurin reduction was measured. Data are means \pm SE from 3 experiments. Comparisons: *, cisplatin-treated vs. untreated controls; #, cisplatin + deferoxamine/CoCl₂/R59949 treated vs. cisplatin alone; †, hypoxia vs. corresponding normoxia.

Effect of HIF-1 α knockdown on oxygen-dependent chemosensitivity

To further analyse whether stabilisation of HIF-1 α protein and subsequent regulation of gene expression contribute to increased resistance of cells to cisplatin we carried out experiments where effect of HIF-1 α protein knockdown on oxygen-dependent chemosensitivity was investigated.

It was previously established that transfection with targeted siRNA significantly reduced HIF-1 α protein level in all 3 cell lines (*Chapter 2*). For the purpose of these experiments we also tested whether exposure of transfected cells to HIF-1 α stabilizing agents deferoxamine and CoCl₂ would affect HIF-1 α protein level. Both compounds increased HIF-1 α levels (as compared to HIF-1 α knockdown cells) but not beyond the basal levels in wild-type cells (data not shown). Because HIF-1 α levels in transfected cells reached the sensitivity limit of the ELISA, the effect of R59949 was not tested. Briefly, cells transfected with targeted siRNA were pre-exposed to deferoxamine, CoCl₂ or R59949 and further treated for 24 h with these agents under normoxia or hypoxia concurrently with cisplatin (2.5 μ M). In order to capture the period where HIF-1 α gene expression was maximally reduced, the 24 h treatment of cells with cisplatin, agents that alter HIF-1 α level or hypoxia started 12 hours before the time point at which HIF-1 α expression was minimal. After treatment cells were subcultured and resazurin reduction was measured 7 days later.

Knockdown cells of all three cell lines were sensitive to cisplatin under normoxia, though not as markedly as wild-type controls. However the clear difference in chemosensitivity of normoxic and hypoxic cells that was evident in wild type cells,

was lost in knockdown cells of two out of three lines due to increased protection of normoxic cisplatin-treated cells (HeLa and MCF7 cells). Hypoxia-induced resistance to cisplatin was also lost in HIF-1 α knockdown MeWo cells (Fig. 4.22, lower panel).

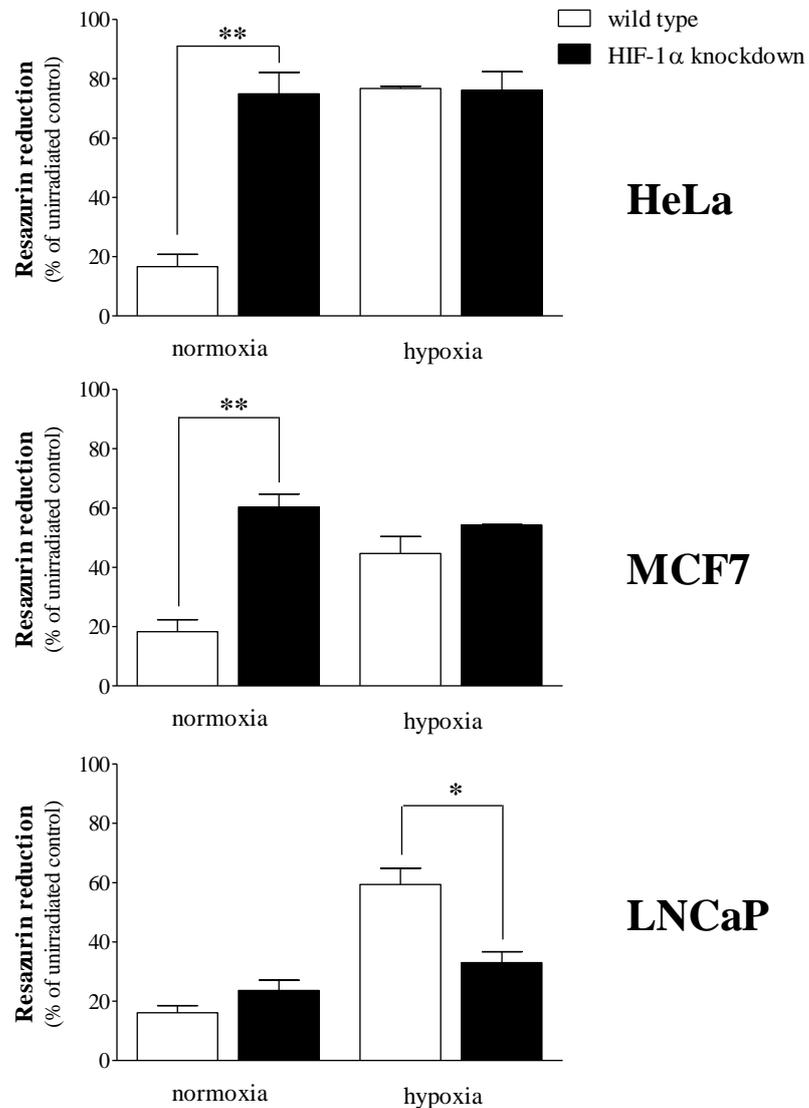


Figure 4.22. Oxygen-dependent sensitivity to cisplatin in HIF-1 α knockdown cells. Normoxic or hypoxic (0.5% O₂) cells were transfected with siRNA either 60 (HeLa, MeWo) or 84 (MCF7) h before being exposed to cisplatin (2.5 μ M) for 24 h, then harvested and subcultured. Cell proliferation was measured 7 days later in terms of resazurin reduction. Data shown are means \pm SE from 3 separate experiments. Comparisons: *, knockdown vs. wild type.

Deferoxamine, CoCl_2 or R59949 treatment reduced survival of knockdown HeLa and MCF7 cells under normoxia. Although stabilization of HIF-1 α protein level caused by deferoxamine and CoCl_2 was small in knockdown cells, both agents sensitised HeLa knockdown cells to cisplatin under normoxia and hypoxia, while having no effect in MCF7. By contrast, although both agents protected MeWo cells under hypoxia, only CoCl_2 did so under normoxia. R59949 partly protected knockdown MeWo cells against cisplatin toxicity under both normoxia and hypoxia but had no such effect in the other cell lines (Fig 4.23).

In summary, oxygen-dependent sensitivity to cisplatin was evident in wild-type cells of all three cell lines but was abolished in HIF-1 α knockdown cells. HIF-1 α level was significantly altered in wild-type cells by HIF-1 α -modulating drug treatments but not in knockdown cells. The two HIF-1 α -stabilising agents had mixed and inconsistent effects on cisplatin induced cell injury in both normoxic and hypoxic wild type and HIF-1 α knockdown cells. By contrast HIF-1 α -destabilizing R59949 increased resistance to cisplatin under most conditions tested unless cisplatin toxicity was only moderate. Overall therefore, neither in wild type nor HIF-1 α knockdown cells did pharmacological manipulation of HIF-1 α level affect cell response to cisplatin injury under either normoxia or hypoxia in a way consistent with a substantial causal role for HIF-1 α in oxygen-dependent chemosensitivity.

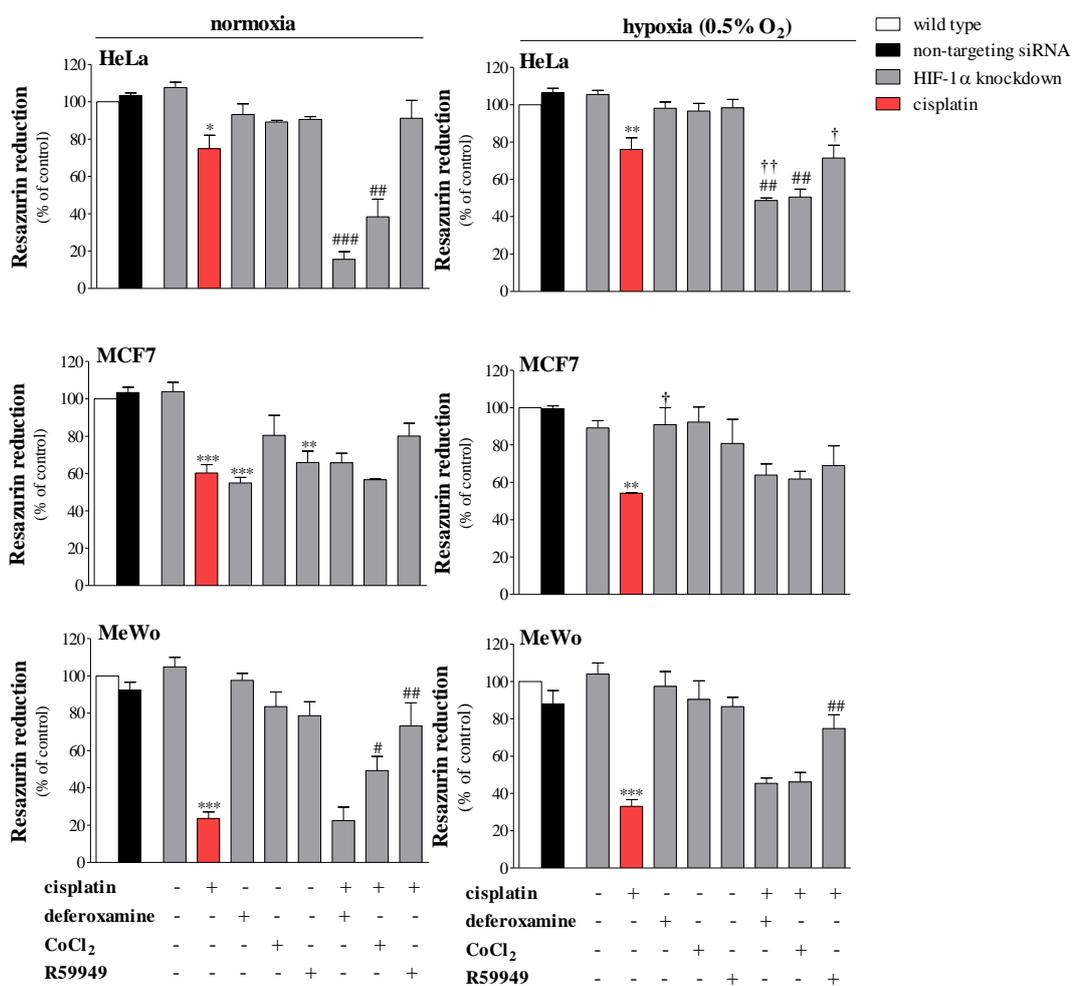


Figure 4.23. **Effect of deferoxamine, CoCl₂ or R59949 on oxygen-dependent sensitivity to cisplatin in HIF-1α knockdown HeLa, MCF7 and MeWo cells.** Cultures were transfected with HIF-1α siRNA either 60 (HeLa, MeWo) or 84 (MCF7) h before treatment. Normoxic or hypoxic (0.5% O₂) cells were exposed to deferoxamine, CoCl₂ or R59949 concurrently with cisplatin (2.5 μM) for 24 h then subcultured. Cell proliferation was measured 7 days later using the resazurin reduction assay. Data are means ± SE from 3 separate experiments. Comparisons: *, cisplatin/deferoxamine/CoCl₂/R59949-treated vs. untreated controls; #, cisplatin+drug treated vs. cisplatin alone †, hypoxia vs. corresponding normoxia.

Discussion

Hypoxia-induced drug resistance is a major barrier to effective cancer chemotherapy. Here we developed an *in vitro* model of oxygen-dependent chemosensitivity in order to investigate the role of HIF-1 α . Under these conditions we observed oxygen-dependent chemosensitivity across only a narrow range of cisplatin concentrations, but 7 days after drug exposure for 24 h it was significant in all 3 cell lines tested. Because EDHB failed to sustain increased HIF-1 α level over the 24 h period of concurrent treatment with chemotherapeutic drug, instead we used either CoCl₂ or deferoxamine to stabilise HIF-1 α , and R59949 to decrease it. Expected changes in HIF-1 α protein level were observed in wild type and HIF-1 α knockdown cells incubated either under normoxia or MH (0.5 % O₂ x 24 h) and co-exposed to CoCl₂, deferoxamine or R59949, as measured by ELISA in samples taken acutely (within less than 1 minute of reoxygenation). HIF-1 α DNA-binding activity was also increased in MH- and CoCl₂- or deferoxamine-treated cells, and lowered by R59959 under hypoxic conditions. However, in neither wild type nor HIF-1 α knockdown cells did pharmacological agents alter cell response to cisplatin-induced injury as expected if HIF-1 α played a causal role in oxygen-dependent chemosensitivity. Moreover increased resistance to cisplatin observed in HIF-1 α knockdown cells complicated interpretation of effects of HIF-1 α -modulating drugs.

To our knowledge this is the first study to focus primarily on determining the role of HIF-1 α in oxygen-dependent sensitivity of tumour cells to a widely-used chemotherapeutic drug. We found that suppression of HIF-1 α expression with targeted siRNA conferred protection on normoxic tumour cells against injury by cisplatin. This

relation has not been reported before and suggests that basal levels of HIF-1 α present under normoxia contribute to cisplatin toxicity. Furthermore, it contrasts with previous reports that elevated HIF-1 α levels in hypoxic tumour cells correlate with increased chemoresistance. However we also found that pharmacologically altered HIF-1 α levels in tumour cells do not predict their oxygen-dependent chemosensitivity. This suggests that factors other than HIF-1 α contribute to relative drug resistance, at least in our model of prolonged moderate hypoxia. Comparison of different tumour cell lines under the same hypoxia conditions showed that changes in HIF-1 α level and HIF-1 DNA binding have different time courses. We also found that hypoxia duration and severity affect HIF-1 α level and HIF-1 DNA binding differently. Variations in impact of altered HIF-1 α status on cisplatin toxicity between different tumour cell lines suggest that interventions directed at HIF-1 α as an adjunct to chemotherapy are likely to be more effective in some tumour types than in others. Collectively these observations may inform the design of new therapeutic strategies that target the HIF-1 pathway to improve the clinical effectiveness of cisplatin chemotherapy.

Development of an *in vitro* model of oxygen-dependent chemosensitivity

Reduced oxygen tension decreases the *in vitro* toxicity of many conventional chemotherapeutic agents in cell lines derived from human solid tumours (reviewed in *Introduction*). However the wide variety of cell lines and hypoxic treatments used makes it difficult to identify clearly the relationship between oxygen tension and diminished response to cytotoxic drugs. Therefore when discussing the effect of chemotherapeutic drugs under hypoxic conditions *in vitro*, duration and severity of

oxygen depletion, parallel or sequential exposure to hypoxia and chemotherapeutic drug as well as the time after which cell proliferation was assayed should be considered.

Here several different protocols were investigated using 1 h SH (<0.2% O₂), to facilitate comparison with radiosensitivity experiments. However, neither 1 h SH concurrent with drug exposure nor 1 h SH following 24 h drug exposure increased cell resistance to doxorubicin or cisplatin respectively (Table 4.2). By contrast, SH insult for 1 h immediately before 24 h drug exposure increased cell survival. However, although significant, this oxygen-dependent chemosensitivity was considerably less than the oxygen-dependent radiosensitivity observed before (*Chapter 3*). Moreover it was confined to only one of three cell lines tested while SH-induced radioprotection was universal. As far as we are aware the effect of such short (1 h) and severe (<0.2% O₂) hypoxia on survival of cancer cells exposed to chemotherapeutic drugs has not been reported before. It is also possible that this limited oxygen-dependent chemosensitivity might occur in other cell types or with different chemotherapeutic drugs.

Repeated cycles of 1 h severe hypoxia (SH) alternating with normoxia over 24 h concurrent with cisplatin exposure did not affect chemosensitivity of the cell lines tested either. Possibly the 2 h reoxygenation periods between 1 h SH insults were long enough to erode any hypoxia-induced resistance to the drug that might have developed. Severe hypoxia (<0.2% O₂) sustained for 24 h while cells were exposed to cisplatin also failed to elicit oxygen-dependent sensitivity but caused a significant decrease in cell proliferation (~60%). Furthermore, supplementation of the medium with HEPES to minimize pH change in the absence of 5% CO₂ stimulated cell growth under normoxia, obscuring any oxygen-dependent chemosensitivity. Overall, these different protocols

showed that short severe hypoxia (<0.2% O₂ x 1 h) before, during or after exposure to chemotherapeutic drug is usually insufficient to elicit oxygen-dependent chemosensitivity *in vitro*, at least in the cancer cell lines tested. By contrast, oxygen deficiency is chronic *in vivo* and results from fluctuating blood flow through abnormal vessels. In turn the delivery of chemotherapeutic drugs to hypoxic regions of the tumour is curtailed.

Hypoxia (O ₂ x duration)	Drug x exposure time	Cell line	Oxygen-dependent chemosensitivity		
			Day 0	Day 3	Day 7
<0.2% x 1 h	doxorubicin x 1 h	HeLa	-	-	No
<0.2% x 1 h before drug	cisplatin x 24 h	HeLa	-	-	Yes
		LNCaP	-	-	Yes
		MCF7	-	-	No
<0.2% x 1 h after drug	cisplatin x 24 h	HeLa	-	-	No
		LNCaP	-	-	No
		MCF7	-	-	No
<0.2% x 24 h	cisplatin x 24 h	HeLa	-	-	No
<0.2% x 1 h) x 8 (over 24 h)	cisplatin x 24 h	HeLa	-	-	No
		LNCaP	-	-	No
0.5% x 24 h	cisplatin x 24 h	HeLa	Yes	Yes	Yes
		MCF7	No	No	Yes
		MeWo	-	-	Yes
	doxorubicin x24 h	HeLa	No	No	-
		MCF7	No	No	-
	5-FU x 24 h	HeLa	No	No	-
		MCF7	No	No	-
	paclitaxel x 24 h	HeLa	No	No	-
		MCF7	No	No	-

Table 4.2 **Development of an *in vitro* model of oxygen-dependent chemosensitivity.** Drug effect was measured in terms of resazurin reduction immediately after exposure (Day 0), and/or three or seven days later.

Most *in vitro* models of hypoxia-induced chemoresistance reported in the literature involve parallel exposure to hypoxia and chemotherapeutic drug, but for much longer than 1 h and with less severe oxygen deprivation. Treatment durations range from 12 h (Yokoi & Fidler, 2004), 24-48 h (Comerford *et al.*, 2002; Xu *et al.*, 2005; Merighi *et al.*, 2007; Hao *et al.*, 2008; Lu *et al.*, 2008; Chen *et al.*, 2009b; Lau *et al.*, 2009; Lin *et al.*, 2011), 72 h (Koch *et al.*, 2003; Yoshida *et al.*, 2009) or 96 h (Marignol *et al.*, 2009) to as long as 144 h (Rharass *et al.*, 2008). A further variation involves pre-incubating tumour cells under hypoxia before exposure to a drug is continued under conditions of reduced oxygen concentration (Roberts *et al.*, 2009). Hypoxia-enhanced resistance of tumour cells to chemotherapeutic drugs is also reported for sequential exposures. The most commonly used *in vitro* model involves prolonged (12-24 h) incubation under hypoxia (<0.2-1% O₂) followed by short (1-4 h) exposures to drug under standard normoxic conditions (Sakata *et al.*, 1991; Matthews *et al.*, 2001; Schnitzer *et al.*, 2006; Song *et al.*, 2006; Frederiksen *et al.*, 2007; Sullivan *et al.*, 2008; Guin *et al.*, 2011). Conversely, drug treatment preceding a drug-free period of hypoxia has also been reported to show oxygen-dependent chemosensitivity (Lee *et al.*, 2006). These different combinations of prolonged incubation under less severe hypoxic conditions appear to induce resistance to chemotherapeutic drugs more effectively than the 1 h x <0.2% O₂ protocol tested here. Therefore it was necessary to change the hypoxic treatment to one that was longer but less severe, to minimise the impact on proliferation of control cells not exposed to cisplatin.

Another factor which differentiates *in vitro* models of oxygen-dependent chemosensitivity is the time after which cytotoxicity of the chemotherapeutic drug is assessed. Most *in vitro* studies use acute measurements of impact while only a few

focus on longer term effects on cell proliferation which may have greater biological relevance. Here we tested effects on cell growth immediately, three days and seven days after concurrent hypoxia and drug exposure. Our results show that oxygen-dependent sensitivity as measured acutely or after 3 days was evident in only one of the three cell lines tested, when exposed to a single drug (cisplatin; Table 4.2). This contrasts with several reports of acute hypoxia-induced resistance in the literature (Koch *et al.*, 2003; Hao *et al.*, 2008; Chen *et al.*, 2009b; Marignol *et al.*, 2009). However direct comparison is difficult as reported hypoxia/drug exposure conditions differ from those used here (24 h exposure to chemotherapeutic drug under normoxia or hypoxia (0.5% O₂) followed by measurement of cell viability in terms of resazurin reduction capacity). In general however, acute oxygen-dependent chemosensitivity is only evident after longer exposure (>24 h) and less severe oxygen deficiency (>0.5% O₂) (Koch *et al.*, 2003), using apoptosis (Yokoi & Fidler, 2004; Cosse *et al.*, 2007) or DNA damage (Matthew *et al.*, 2009) as acute endpoints. By contrast when cells were allowed to proliferate for 7 days we found a clear oxygen-dependent sensitivity to cisplatin for all cell lines tested (Table 4.2, *red*), albeit over a narrow range of drug concentration. Several other studies using different cell lines and drugs have also shown increased oxygen-dependent chemosensitivity when cytotoxicity was assayed 8-21 days after hypoxia and drug treatment (Liu *et al.*, 2008; Ravizza *et al.*, 2009; Forde *et al.*, 2012). Here cisplatin was the only drug tested because it also showed oxygen-dependent toxicity acutely and after three days. However although oxygen-dependent resistance to three other drugs was not evident after three days, the possibility cannot be excluded that it might have become evident later.

In summary, hypoxic insults of varying duration and severity applied to several cancer cell lines and chemotherapeutic drugs failed to show clear oxygen-dependent chemosensitivity *in vitro*. In our hands oxygen-dependent chemosensitivity was evident *in vitro* only under a limited set of conditions. Others have also reported that even under the same hypoxic conditions oxygen-dependent chemosensitivity can be cell type specific (Cosse *et al.*, 2007; Merighi *et al.*, 2007) or drug-dependent (Flamant *et al.*, 2010; Forde *et al.*, 2010). Therefore effects of hypoxia-induced chemoresistance which are investigated using so many different *in vitro* models of oxygen-dependent chemosensitivity cannot easily be compared directly. Rather the particular conditions used (duration and severity of hypoxia, as well as time after which drug cytotoxicity was assayed) must be taken into account.

HIF-1 α level and HIF-1 DNA binding

Hypoxia is an intrinsic factor in the development and growth of most malignant tumours. Although HIF-1 α is considered a key regulator of tissue response to oxygen deficiency its expression can be cell-type specific (Schilling *et al.*, 2012). Moreover, as shown here, hypoxia-induced HIF-1 α stabilisation as well as DNA-binding activity can vary according to the hypoxia regime used. Severe but short hypoxia (<0.2% O₂ x 1 h; SH) caused a greater increase in HIF-1 α level than longer but less severe oxygen deprivation (0.5% O₂ x 24 h; MH) across the cancer cell lines tested. By contrast, SH increased HIF-1 DNA-binding activity only slightly whereas MH produced a much stronger, albeit cell type specific, effect (Table 4.3).

	HIF-1α level				HIF-1 DNA binding	
	SH ¹		MH ¹		SH ¹	MH ¹
HeLa	x2.7	**	x1.7	*	x2.4	x14.5
LNCaP	x2.2	**	n.d.		x1.2	n.d.
MCF7	x2.5	***	x2.0	*	x1.4	x6.6
MeWo	n.d.		x1.3	ns	n.d.	x1.5

Table 4.3. **Comparison of HIF-1 α levels and HIF-1 DNA-binding activity in HeLa, LNCaP, MCF7 and MeWo cells after SH or MH treatments, relative to normoxia.** Normoxic, SH (<0.2% O₂ x 1 h) or MH (0.5 % O₂ x 24 h) cultures were cooled to 4°C and lysed either for measurement of HIF-1 α level by ELISA or for preparation of nuclear extracts and analysis of HIF-1 DNA-binding activity. Comparisons: ¹,* relative to normoxic control. Abbreviations: SH, severe hypoxia; MH, moderate hypoxia; n.d., not determined; n.s., not significant.

Oxygen-dependent cell proliferation and sensitivity to cisplatin

As discussed in *Chapter 3*, hypoxia itself can influence cell proliferation capacity. In general the proliferation rate of tumour cells under hypoxic conditions decreases in inverse relation to severity and duration of oxygen depletion. Here we found that exposure of three human cancer cell lines to moderate hypoxia (0.5% O₂ x 24 h) had no effect on their subsequent proliferation (Fig. 4.12). This lack of effect of hypoxia alone made it easier to discern the long-term effects of chemotherapeutic drug and to study the role of HIF-1 α in oxygen-dependent chemosensitivity.

Decreased oxygenation also contributes to resistance of solid tumours to many chemotherapeutic drugs, therefore limiting the effectiveness of chemotherapy. Here we tested whether resistance to cisplatin, a conventional anticancer drug, is induced by hypoxia in three human tumour cell lines. Cisplatin toxicity was indeed reduced under hypoxia (Fig.4.12). Moreover the extent of hypoxia-induced resistance was cell type

specific, with the greatest effect observed for HeLa and the least for MCF7 cells. This confirms and extends the findings of others who showed hypoxia-induced cisplatin resistance in testicular germ (Koch *et al.*, 2003), gastric (Liu *et al.*, 2008), pancreatic (Chen *et al.*, 2009b), liver (Lau *et al.*, 2009) and colon (Lin *et al.*, 2011) tumour cells. By contrast *increased* sensitivity to cisplatin under hypoxic conditions was also reported for Chinese hamster ovarian (Skov *et al.*, 1998) and breast, lung and lymphoma (Strese *et al.*, 2013) cancer cell lines.

Does HIF-1 α knockdown affect oxygen-dependent sensitivity to cisplatin?

The most direct way to determine whether HIF-1 α contributes to oxygen-dependent chemosensitivity is to test the functional consequences of down regulating its expression. Here we found that transfection with specific siRNA transiently but markedly reduced expression of HIF-1 α in HeLa, MCF7 and MeWo cells (Fig. 2.8). The maximum decrease in expression of HIF-1 α was either 72 h (HeLa, MeWo) or 96 h (MCF7) after transfection. Because MH and drug treatment duration was 24 h, transfection was timed to bracket by 12 h on either side the point at which HIF-1 α knockdown was greatest. Under these conditions cell proliferation capacity as measured 7 days after either normoxia or MH treatment was unaffected by HIF-1 α knockdown (Fig. 4.22). However, knockdown abolished oxygen-dependent chemosensitivity in all cell lines, consistent with a major causal role for HIF-1 α . This was due to reduced toxicity of cisplatin in normoxic HeLa and MCF7 cells, and increased sensitivity in hypoxic MeWo.

Several groups have reported that reduced HIF-1 α expression enhances the chemosensitivity of various tumour cell lines under hypoxia (Brown *et al.*, 2006; Song *et al.*, 2006; Sullivan *et al.*, 2008; Min *et al.*, 2012). In the present study we found that transient HIF-1 α knockdown increased sensitivity to cisplatin-induced cell injury under MH conditions, but only in MeWo cells. Why HIF-1 α knockdown did not reverse the MH-induced resistance to cisplatin in HeLa and MCF7 cells remains unclear. It has been reported by others that in some cell lines hypoxia-induced drug resistance can only be partly reversed when HIF-1 α expression is knocked down (Schnitzer *et al.*, 2006, Ravizza *et al.*, 2009). Moreover a more detailed study in which HIF-1 α was suppressed using various genetic approaches (shRNAi, siRNA or plasmid transfection) showed that hypoxia-induced drug resistance is fully HIF-1 α -independent in some cancer cell types (Adamski *et al.*, 2013). Other possible mediators of relative drug resistance under hypoxia include transcription factors like AP-1 or other signaling pathway mediators such as PI3K, Pim-1 and NF κ B (Yokoi & Fidler, 2004; Piret *et al.*, 2006; Chen *et al.*, 2009b; Rohwer *et al.*, 2010; Adamski *et al.*, 2013).

It has been reported that HIF-1 α suppression increases the chemosensitivity of various cancer cell lines under normoxia (Sasabe *et al.*, 2006; Daskalow *et al.*, 2010). By contrast, here we found either unchanged (MeWo) or increased resistance to cisplatin (HeLa and MCF7) in HIF-1 α knockdown cells relative to wild type controls under normoxia. The underlying reason(s) remain unclear but may involve low basal levels of the protein in wild type cells, altered expression of other proteins in response to HIF-1 α knockdown (e.g. HIF-2 α) as well as different experimental models and conditions. In summary therefore, HIF-1 α knockdown sensitised MH MeWo cells to cisplatin and protected HeLa and MCF7 under normoxia. Consequently the difference

between normoxic and hypoxic chemosensitivity was abolished in all three cell lines under the conditions used.

How does pharmacological alteration of HIF-1 α status affect oxygen-dependent sensitivity to cisplatin?

Before discussing how pharmacological alteration of HIF-1 α status affects oxygen-dependent chemosensitivity of HeLa, MCF7 and MeWo cells we first consider changes in HIF-1 α level and HIF-1 DNA binding. Since hypoxia differentially regulates HIF-1 α level and DNA-binding activity across these tumour cell lines (Table 4.3) we also used either CoCl₂ or deferoxamine to test HIF-1 α role in oxygen-dependent chemosensitivity, as well as R59949 to examine the effect of HIF-1 α destabilisation. All treatments produced significant alterations in HIF-1 α level that were highly consistent across the tumour cell lines tested (Table 4.4, *upper panel*). Moreover these effects are in full agreement with those reported by others. Both cobalt chloride and deferoxamine readily increase HIF-1 α level, and are commonly used to study HIF-1 α -mediated effects not only in tumour cells (Yuan *et al.*, 2003, Vengellur & LaPres, 2004; Guo *et al.*, 2006; Triantafyllou *et al.*, 2006) but in endothelial cells too (Kim *et al.*, 2006).

Under normoxic conditions both CoCl₂ and deferoxamine raised HIF-1 DNA binding in parallel with protein level in all cell lines tested. Under MH conditions DNA binding activity was already high so could not be raised further by either agent in HeLa and MCF7 cells. By contrast, in MeWo cells neither hypoxia nor CoCl₂ or deferoxamine raised DNA binding activity very much (Table 4.4, *lower panel*). The reason for this limited response is unclear. Subcellular localization of HIF-1 α between

cytoplasmic and nuclear fractions was not markedly different to that in the other cell lines, albeit based on single measurements (Table. 4.1). Since at least 70 % of total HIF-1 α was already present in the nuclear fraction, other factors are probably involved such as post-translational modification of HIF1 α or its partner proteins in DNA binding and transcriptional activity. HIF-1 α regulation in MeWo cells may be distinct, because for example it was reported that HIF-1 α protein in the nucleus of hypoxic melanoma cells is stabilised by the apoptosis inhibitor bcl-2 (Trisciuglio *et al.*, 2010).

HIF-1α level								
	Control (N)		CoCl₂		Deferoxamine		R59949	
HeLa	1		x7	*	x6	*	x0.4	*
MCF7	1		x5	***	x7	*	x0.5	*
MeWo	1		x6	*	x5	*	x0.4	**

HIF-1 DNA binding								
	Control		CoCl₂		Deferoxamine		R59949	
	N	MH ¹	N ¹	MH ²	N ¹	MH ²	N ¹	MH ²
HeLa	1	x14	x12	x0.9	x10	x0.8	x1.2	x0.2
MCF7	1	x7	x6	x0.9	x3	x0.9	x1.4	x0.5
MeWo	1	x1.5	x5	x0.9	x4	x1.1	x1.2	x0.7

Table 4.4. **Comparison of CoCl₂, deferoxamine or R59949 effects on HIF-1 α protein level and HIF-1 DNA-binding in wild-type HeLa, MCF7 and MeWo cells under normoxia and MH.** Fold changes (x) and significance are shown, except for DNA binding where n=2. Normoxic (N) or MH (0.5 % O₂ x 24 h) cultures were exposed to CoCl₂ (0.3 mM for 2h followed by 0.1 mM for 24 h), deferoxamine (50 μ M for 2 h followed by 20 μ M for 24 h) or R59949 (0.1 mM for 2 h followed by 0.02 mM x 24 h), then immediately (<1min) cooled to 4°C and lysed for preparation of whole or nuclear extracts, and analysed by ELISA for HIF-1 α level or HIF-1 DNA-binding activity respectively. Values are calculated from Figs. 4.18 and 4.20. Comparisons: ^{*1} relative to normoxic control, ² relative to MH control.

As discussed earlier, in order to lower HIF-1 α level we chose the diacylglycerol kinase inhibitor R59994. However although R59949 significantly reduced HIF-1 α below basal levels in all cell lines tested (Table. 4.4, *upper panel*), it had no measurable effect on HIF-1 DNA binding activity (Table. 4.4, *lower panel*) which was already near the limit of detection in control cells under normoxic conditions. By contrast, under hypoxia HIF-1 DNA-binding activity was elevated, so the effect of R59949 was more readily apparent and caused a marked decrease in DNA-binding in both HeLa and MCF7 cells. The lack of R59949 effect in MeWo cells may have been due to the less marked increase in HIF-1 DNA binding under hypoxia than was seen in the other two cell lines.

Overall, CoCl₂, deferoxamine and R59949 caused expected changes in HIF-1 α level and HIF-1 DNA binding activity under both normoxia and MH in all three cell lines (except MH MeWo; Table. 4.4). Cell cultures were exposed to all agents 2 h before and in parallel with other treatments (hypoxia, cisplatin) in order to sustain significant changes in HIF-1 α protein level. Thus they may also have caused non-specific effects independent of HIF-1 α including compensatory adaptations affecting chemosensitivity. Therefore by exposing HIF-1 α -deficient cells to CoCl₂, deferoxamine and R59949 we attempted to distinguish whether the functional effect of these drugs (measured as proliferation after 7 days) on oxygen-dependent sensitivity to cisplatin depended on HIF-1 α or reflected other actions (Table. 4.5, *upper panel*).

HIF-1 α knockdown

	Cisplatin		Deferoxamine		CoCl ₂		R59949	
	normoxia ¹	MH ¹	normoxia ²	MH ³	normoxia ²	MH ³	normoxia ²	MH ³
HeLa	↑ x4.5 **	- x1 ns	↓ x0.2 ###	↓ x0.6 ††	↓ x0.5 ##	↓ x0.6 ††	- x1.2ns	- x0.94 ns
MCF7	↑ x3.3 **	- x1.2ns	- x1.1 ns	- x1.2ns	- x0.94ns	- x1.1ns	- x1.3ns	- x1.3ns
MeWo	(↑) x1.5ns	↓x0.6 *	- x0.95 ns	- x1.4ns	↑ x2.1 #	- x1.4ns	↑ x3.1 ##	↑ x2.3††

Wild type

	Cisplatin		Deferoxamine		CoCl ₂		R59949	
	normoxia	MH ²	normoxia ²	MH ³	normoxia ²	MH ³	normoxia ²	MH ³
HeLa	1	↑ x4.6###	- x0.9 ns	- x1 ns	↑ x3 #	- x1 ns	↑ x5.8 ###	- x1.1 ns
MCF7	1	↑ x2.4#	(↑) x2.4 ns	- x1.1ns	(↑) x2 ns	- x0.8 ns	(↑) x2.2 ns	- x1ns
MeWo	1	↑ x3.7###	- x0.9 ns	↓ x0.4 ††	- x1 ns	↓ x0.25 †††	- x1.7 ns	- x1.2 ns

Table 4.5. Comparison of CoCl₂, deferoxamine or R59949 effects on proliferation 7 days after cisplatin treatment under normoxia or hypoxia in HIF-1 α knockdown and wild type HeLa, MCF7 and MeWo cells. Sensitivity to cisplatin ($\uparrow\downarrow$), fold change (x) and significance are shown. Values are calculated from Figs. 4.20-4.21. Cultures were transfected with HIF-1 α siRNA either 60 (HeLa, MeWo) or 84 (MCF7) h before treatment. Normoxic or MH (0.5 % O₂ x 24 h) cultures were exposed to deferoxamine, CoCl₂ or R59949 concurrently with cisplatin (2.5 μ M) for 24 h, then subcultured. Cell proliferation was measured 7 days later using the resazurin reduction assay. Comparisons: ^{1,*} relative to wild type cells, ^{2,#} relative to normoxic control, ^{3,†} relative to MH control.

All HIF-1 α -modulating drugs altered the sensitivity of HIF-1 α knockdown cells to cisplatin under both normoxia and MH, consistent with effects independent of HIF-1 α . CoCl₂ and deferoxamine sensitised HIF-1 α knockdown HeLa cells both under normoxia and MH, possibly reflecting their small toxicity evident under normoxia. Why both CoCl₂ and R59949 increased resistance to cisplatin in knockdown MeWo cells remains unclear but indicates protective effects independent of HIF-1 α .

Although oxygen-dependent sensitivity to cisplatin was evident in all wild type cells the effects of HIF-1 α -modulating drugs were mixed (Tab.4.5, *lower panel*). Except for CoCl₂-treated HeLa, both HIF-1 α -stabilising drugs failed to enhance cisplatin resistance in wild type cells under normoxia. Under MH although HIF-1 α level and HIF-1 DNA-binding activity were increased the sensitivity to cisplatin remained unaffected in both CoCl₂- and deferoxamine-treated HeLa and MCF7 cells. This is consistent with the lack of further effect of the HIF-1 α -stabilising drugs in cells where HIF-1 α level and HIF-1 DNA binding were already high due to hypoxia. By contrast, a distinct survival pattern was found in MH MeWo cells as hypoxia-induced cisplatin resistance was abolished by CoCl₂ or deferoxamine treatments. This unexpected effect might partly reflect the unchanged DNA binding activity found in MH MeWo cells after CoCl₂ or deferoxamine treatment. Interestingly cisplatin has been reported to reduce HIF-1 α level in human ovarian cancer cells by inhibiting synthesis and stimulating degradation (Duyndam *et al.*, 2007). Whether the same applies to other cancer cell types remains to be tested. However this could partly explain the limited increase in cisplatin resistance we observed in both normoxic and MH HeLa and MCF7 as well as normoxic MeWo cells treated with HIF-1 α -stabilising agents. Although R59949 either reduced or sustained HIF-1 α at low level in all cell lines, it either completely or partly protected

against cisplatin injury in HeLa and MCF7, or MeWo cells respectively. These effects suggest an HIF-1 α -independent effect of R59949 on survival of cisplatin-treated tumour cells, but the mechanism remains to be identified.

To summarise, wild type cells showed a clear and consistent oxygen-dependent sensitivity to cisplatin which was abolished by HIF-1 α knockdown. However HIF-1 α knockdown itself caused mixed effects on cisplatin toxicity that depended on cell line and may reflect compensatory changes in expression of other proteins. Overall, the effects of HIF-1 α -modulating drugs were broadly similar in both wild type and knockdown cells, suggesting that they are partly or largely independent of HIF-1 α . It is possible that the *in vitro* model of oxygen-dependent chemosensitivity developed and used here was not sufficiently robust for pharmacological investigation of HIF-1 α causality, given the inherent variability of drug effects reflecting HIF-1 α -dependent and -independent mechanisms. These latter might include for example (a) hypoxia induced changes in HIF-1 α /HIF-2 α balance, (b) compensatory increase of HIF-2 α level in HIF-1 α knockdown cells and/or (c) direct cisplatin effects on HIF-1 α level.

Therefore taking these genetic and pharmacological experiments together it remains unclear whether oxygen-dependent sensitivity to cisplatin in these cell lines depends on HIF-1 α or reflects compensatory changes in the proteome of HIF-1 α knockdown cells as well as non-specific effects of HIF-1 α -modulating drugs.

Chapter 5

General Discussion

The objective of this study was to test whether and how HIF-1 α contributes to oxygen-dependent radio- and/or chemoresistance in tumour cells. If so then this could provide a promising therapeutic target for lessening hypoxia-induced radio- and chemoresistance of human solid tumours and thereby improve the clinical effectiveness of therapy. In our *in vitro* model, cell lines derived from four different human solid tumours were subjected to hypoxia that was either short and severe (<0.2% O₂ x 1h) or longer and more moderate (0.5% O₂ x 24 h) then irradiated near the clinical range or exposed to chemotherapeutic drug(s). The impact of treatment(s) was determined using the resazurin reduction assay, a method validated against other proliferation assays as highly sensitive and reproducible (Anoopkumar-Dukie *et al.*, 2005). We focused on the longer term effects on cell proliferation (7 days after treatment/intervention) rather than an acute response because the former is likely to be more relevant to tumour progression *in vivo*. HIF-1 α protein level in whole cell lysates and HIF-1DNA binding in nuclear extracts were measured by ELISA. Hypoxia increased HIF-1 α level and DNA-binding activity but a low basal level of the protein was also constitutively present in all four cancer cell lines under normoxia. Using both genetic and pharmacological approaches we have shown that HIF-1 α plays a causal role in oxygen-dependent radiosensitivity. However although oxygen-dependent chemosensitivity was abolished when HIF-1 α expression was transiently knock down using specific targeted siRNA, consistent with a causal role for HIF-1 α , results with the parallel pharmacological experiments were not conclusive.

In vitro models allow study of tumour biology under controlled conditions. However most do not include interaction of different cell types, or fluctuating delivery of O₂, nutrients or chemotherapeutic drugs and removal of metabolic end products that may influence the *in vivo* response of solid tumours to radio- and chemotherapy.

A key difference between cells in culture and *in vivo* relates to the availability of O₂. The O₂ level in normal tissue culture is usually that of ambient air (21%, ~ 150 mmHg), whereas *in vivo* partial pressure of the gas decreases considerably, reaching a minimum in the mitochondria where O₂ is reduced by cytochrome oxidase in the inner membrane. Moreover the O₂ level of even the same cell type within normal or tumour tissues is very variable. For example oxygenation of skeletal muscle was reported to vary between 2.8-4.2% (Kiaer & Kristensen, 1988) while that of normal cervix was 0.28-14% (Vaupel *et al.*, 2001). By contrast all cells in culture are exposed to approximately the same unphysiologically high O₂ level which imposes an oxidative stress. This reflects an imbalance between increased ROS generation and reduced levels of antioxidants present in cell culture media (Halliwell, 2003). Increased oxidative stress in turn may cause cellular adaptation by activation of various signaling pathways that promote proliferation (Liu *et al.*, 2001). Alternatively it can alter the pattern of gene expression and energy metabolism to trigger apoptosis (Gorman *et al.*, 1997) or cell senescence (Colavitti & Finkel, 2005). By contrast because *in vivo* most cells are exposed to lower O₂ levels ROS generation is reduced. Moreover extracellular fluids have greater availability of antioxidants. This difference in oxidative environment therefore constrains the extrapolation of *in vitro* observations to likely *in vivo* cell behaviour. Furthermore there is evidence that ROS are involved in HIF-1 regulation at

both cell (Jung *et al.*, 2008; Niecknig *et al.*, 2012) and organism (Pialoux *et al.*, 2009) levels.

Although O₂ level in *in vivo* models of cell hypoxia can be set and manipulated over a range, this simplifies greatly the usual pattern of hypoxia in solid tumours which is normally variable, both spatially and temporally. This is a consequence of (a) graded reduction in oxygen level with distance of individual cells from the blood vessels, and (b) fluctuating O₂ delivery which results from irregular vascularisation and transient block or release of blood flow. Further consequences of abnormal vasculature include (a) insufficient delivery of nutrients or chemotherapeutic drugs, and (b) reduced removal of metabolic end products leading to acidosis of the tumour microenvironment. These latter changes can be avoided in cell culture where pH is maintained under hypoxia and consistent, defined drug exposure can be maintained.

In the present study we used two *in vitro* models of hypoxia to test HIF-1 α role in oxygen-dependent radio- and chemosensitivity. The short severe regimen (<0.2% O₂ x 1 h) resembles acute but temporary hypoxia that may occur in solid tumours with interruption and resumption of blood flow. On the other hand the moderate but longer regimen (0.5% x 24 h) more closely resembles the chronic hypoxia of cells distant from blood vessels within tumour masses. One of the major cell responses to reduced oxygen level is stabilisation of HIF-1 α which then acts as a transcription factor to upregulate expression of many target genes involved in promoting proliferation, angiogenesis and metastasis. However it is becoming evident that HIF-1 α is also present and functional at normal oxygen concentrations in various tumour cell lines (Zhong *et al.*, 1998; Schilling *et al.*, 2012). Due to sensitivity of the ELISA assay used here we could detect

constitutive HIF-1 α protein in all four cancer cell lines under normoxia. This suggests that HIF-1 is not simply a on/off switch that responds to oxygen concentration but rather it is an important regulator of gene expression to maintain cell homeostasis across a broad range of oxygen concentration.

Cell cultures in most *in vitro* experimental models comprise a single cell type grown as a monolayer. Alternatively cells may be grown in three dimensional tumour-like spheroids (Dubessy *et al.*, 2000). By contrast solid tumours are composed of different cell types within a more complex microenvironment. For example presence of tumour stem cells with self-renewal and differentiation capacity contribute to tumour maintenance, recurrence and heterogeneity (Dontu *et al.*, 2003; Hassanein *et al.*, 2011). Moreover at different stages of tumour growth, infiltration can occur by different host cell types including immune cells, vascular and lymphatic endothelial cells, as well as by cancer-associated fibroblastic cells. Crosstalk between these various host cell types and tumour cells as well as quantitative or qualitative changes in the extracellular matrix of the tumour have been implicated in tumour progression and metastasis (Sounni & Noel, 2013). In addition, presence of other cell types within a tumour can promote increased radio- and chemoresistance (Bao *et al.*, 2006; Liu *et al.*, 2006, Blazek *et al.*, 2007). Whether HIF-1 α expression in cell types other than tumour cells influences radio- or chemoresistance is still under investigation. However it was reported that HIFs are required for cancer stem cell survival and participation in tumour progression (Li *et al.*, 2009) and also for regulating cancer stem cell phenotype (Heddleston *et al.*, 2010). Moreover suppression of HIF-1 α level in tumour-associated macrophages, which are found close to or within the tumour mass, slows tumour progression (Doedens *et al.*, 2010; Werno *et al.*, 2010). However in the present study we focused on tumour cell

response in order to understand the role at this level specifically. Based on our observations we conclude that HIF-1 α plays a causal role in oxygen-dependent radioresistance whereas in our *in vitro* model of oxygen-dependent sensitivity to cisplatin its role is less clear-cut and perhaps more limited.

Genetic approaches which significantly reduce expression of target proteins are commonly used in *in vitro* studies. Target genes can be knocked down in any cell type using siRNA but the effect is temporary and incomplete. Here we used targeted siRNA which transiently decreased HIF-1 α expression to test how this intervention influenced oxygen-dependent radio- and chemosensitivity of the cell cultures. Although siRNA used was specific for HIF-1 α so should not directly affect the expression of any other genes, these under HIF-1 regulation are also likely to be suppressed indirectly. In addition, compensatory changes in expression of other genes are also possible. These secondary effects might in turn influence cell response to ionizing radiation or cytotoxic drug. For example it was reported that knockdown of HIF-1 α increased expression of HIF-2 α in glioma cells thereby promoting their angiogenic phenotype (Anelli *et al.*, 2008). Knockdown of one HIF- α isoform also caused a reciprocal increase in expression of the other that promoted survival in hepatocellular tumour spheroids (Menrad *et al.*, 2010). Similar reciprocal regulation of HIF- α subunits was reported for breast (Carroll & Ashcroft, 2006) and renal (Raval *et al.*, 2005) cancer cells. Increased HIF-1 α protein levels in HIF-2 α knockdown cells were found to reflect enhanced protein translation (Schulz *et al.*, 2012).

Here we treated cell cultures with EDHB, CoCl₂, deferoxamine and R59949 to pharmacologically alter HIF-1 α level and DNA binding in the *in vitro* model of oxygen-

dependent radio- and chemosensitivity. EDHB and R59949 are less commonly used than CoCl_2 and deferoxamine to investigate HIF-1 α role in tumour cells. Their mechanism of action is relatively clear in the case of EDHB, CoCl_2 and deferoxamine but less so for R59949. Nevertheless all of these drugs target HIF-1 α only indirectly, though their actions on PHDs (or upstream of PHD in the case of R59949). Moreover in order to alter HIF-1 α significantly during a short exposure time we used relatively high concentrations of both EDHB and R59949. Therefore they are likely to have had non-specific effects in addition to regulating PHD activity. For example EDHB has been shown to promote iron deficiency in cultured cells by activation of iron regulatory protein 1, independently of HIF-1 α (Wang *et al.*, 2002). EDHB was also reported to induce haem oxygenase-1 expression via a HIF-1 α -independent mechanism in some cancer cell lines (Li *et al.*, 2008). Such HIF-1 α independent effects could contribute to changes in cellular metabolism and affect cell proliferation (Hill *et al.*, 2005; Ohara *et al.*, 2013). Both CoCl_2 and deferoxamine directly inhibit PHD activity by iron replacement or chelation respectively (Schofield & Ratcliffe, 2004). However other haem-containing enzyme(s) important in various cellular functions are also affected by CoCl_2 and deferoxamine, such as haem oxygenase-1 (Gong *et al.*, 2001), PI3K (Chachami *et al.*, 2004) and COX-2 (Woo *et al.*, 2006). Since PHD genes contain HRE sequences they are themselves induced under hypoxia by HIF-1 α (D'Angelo *et al.*, 2003; Metzen *et al.*, 2005). Thus inhibition of PHDs by EDHB, CoCl_2 or deferoxamine may result in compensatory upregulation (negative feedback) of the enzyme due to hypoxia-induced HIF-1 α stabilisation. Apart from regulation of HIF-1 α protein, PHDs are key enzymes in collagen biosynthesis (Myllyharju, 2003), which contributes to extracellular matrix deposition in cancer progression. Inhibition of collagen PHDs by

EDHB has recently been shown to decrease tumour fibrosis and metastasis in a mouse model of breast cancer (Glikes *et al.*, 2013). Hypoxia also decreases PHD-dependent hydroxylation of I κ B kinase- β , thereby releasing repression of NF κ B that may contribute to tumour development and progression (Cummins *et al.*, 2006). R59949 which is proposed to act as an indirect activator of PHDs (Temes *et al.*, 2005) could also influence the stability of protein other than HIF-1 α . It acts by inhibiting type I DGKs (α , β , γ) which in turn reduces synthesis of phosphatidic acid, thereby disinhibiting PHDs (Morita *et al.*, 2009) and further destabilizing HIF-1 α . Inhibition of type I isoform DGK α by R59949 has also been implicated in caspase-mediated apoptosis in some cancer cells (Dominguez *et al.*, 2013). By contrast R59949 does not affect type II DGKs, one isoform of which was reported to regulate growth of some cancer cell types (Whitworth *et al.*, 2012).

Since HIF-1 α level is prognostic of tumour response to therapy, if the relationship is causal then interventions that target HIF-1 α function may improve clinical effectiveness of radio- and chemotherapy. In the present study we show that knockdown of HIF-1 α expression with targeted siRNA reduced hypoxia-induced radioresistance in all cancer cell lines tested. Pharmacological destabilization of HIF-1 α by R59949 had a similar although less marked effect. Similarly EDHB-induced HIF-1 α stabilisation promoted radioprotection of hypoxic cells but not much beyond the effect of hypoxia alone. Hence we conclude that HIF-1 α plays a causal role in oxygen-dependent radioresistance of tumour cells, so genetic and/or pharmacological interventions targeting its stability and activity should be considered as potential candidates for improving the clinical effectiveness of radiotherapy. By contrast in our *in*

vitro model of oxygen-dependent sensitivity to cisplatin the causal role of HIF-1 α was less clear and could reflect the experimental conditions used. Therefore we cannot yet conclude whether targeting HIF-1 α function might also overcome hypoxic limitation of chemotherapy. Furthermore, since we used only one chemotherapeutic drug and specific conditions of hypoxia the effects observed cannot be extrapolated easily to *in vivo* settings.

It has already been reported that overexpression of HIF-1 α in tumours provides a prognostic marker of likely clinical outcome after radiotherapy, including in cervical, oropharyngeal and nasopharyngeal cancers ((Dellas *et al.*, 2008; Aebersold *et al.*, 2001; Xueguan *et al.*, 2008). Moreover HIF-1 α expression was positively correlated with initial response to concurrent chemoradiotherapy in patients with either oesophageal and head and neck cancer (Ogawa *et al.*, 2011; Koukourakis *et al.*, 2002). Expression of both HIF-1 α and its target VEGF were positively associated with advanced stage and poor prognosis in patients after surgical removal of colorectal cancer (Cao *et al.*, 2009). In contrast, HIF-1 α expression was not correlated with survival in patients with squamous cell carcinoma of the uterine cervix and glottis larynx (Mayer *et al.*, 2004; Douglas *et al.*, 2013). Nevertheless if HIF-1 α status influences response to treatment then development of therapies targeting HIF-1 α function may improve clinical responsiveness of tumours resistant to radio- and chemotherapy due to hypoxia. To date several drugs that destabilise HIF-1 α or inhibit its transcription, translation, DNA binding or transcriptional activity have been associated with improved response of tumour cells or/and tumours to radio- or chemotherapy (reviewed in *Chapter 1*). Although many of these also generated promising results in preclinical or early clinical trials, most failed in further clinical development (Galluzzi *et al.*, 2013). The reasons

include general cytotoxicity in the case of echinomycin (Onnis *et al.*, 2009) and 2-methoxyestradiol (Harrison *et al.*, 2011; Bruce *et al.*, 2012), hepatotoxicity in animal models (Supko *et al.*, 1995) or poor bioavailability during phase II trials in the case of geldanamycin and its derivatives (Ronnen *et al.*, 2006; Heath *et al.*, 2008; Solit *et al.*, 2008). Other preclinical limitations may also exist because not all clinical trials results have been published. For example PX-478 which was a very promising HIF-1 α inhibitor reported to enhance hypoxic radiosensitivity *in vitro* (Palayoor *et al.*, 2008), demonstrated antitumour activity and inhibited tumour progression *in vivo* (Welsh *et al.*, 2004; Jacoby *et al.*, 2010) and in phase I clinical trial (Tibes *et al.*, 2010), but was subsequently discontinued for unknown reasons. Another recent example is EZN-2968, an antisense oligodeoxynucleotide specific for HIF-1 α , which was administered to patients with refractory solid tumours. Although HIF-1 α mRNA and protein expression were both found to be lower (Jeong *et al.*, 2014) this clinical trial was also suspended.

Therefore since HIF-1 α was discovered in the 1990s, many inhibitors have been described and developed. However so far no drugs directed at the HIF-1 pathway have been licensed for clinical use. Several are still in clinical trials, but lack of specificity as well as the complexity of the HIF-1 pathway remain as significant obstacles to their introduction as a successful new therapeutic strategy. Nevertheless results from ongoing clinical trials should become available within the next few years, while further strategies now being developed (for example isoform-specific HIF- α inhibitors, inhibitors against HIF-1 α downstream targets, screening for natural-product based HIF-1 α inhibitors etc.) may eventually allow oxygen-dependent resistance to radio- and chemotherapies to be overcome by targeting the HIF pathway.

Future directions

For logistic reasons the mechanisms underlying the role of HIF-1 α in oxygen-dependent radiosensitivity could not be investigated in the present study. However it would be worth investigating the role of HIF-1 α in DNA repair and cell cycle progression under acute hypoxia conditions. It would also be interesting to test whether the effects of mitochondrial function and nitric oxide synthase activity on oxygen-dependent radiosensitivity observed previously (Anoopkumar-Dukie *et al.*, 2009) are mediated by HIF-1 α .

It is clear from this study that HIF-1 α level and DNA binding are reduced when R59949 is applied. However at the high concentration used HIF-1 α -independent actions of the drug may have obscured its potential radiosensitisation effects. Thus it would be worth extending the study to include other agents such as ZnCl₂, PX-478 or chetomin that also destabilise HIF-1 α . These were found to be effective in either reduction of HIF-1 α level (ZnCl₂, Nardinocchi *et al.*, 2010) or in radiosensitisation of hypoxic tumour cells (PX-478, Palayoor *et al.*, 2008; chetomin, Staab *et al.*, 2008). However to best of our knowledge no one has tested them in an *in vitro* model of acute severe hypoxia. Interestingly, cisplatin used here in an *in vitro* model of oxygen-dependent chemosensitivity has been reported to suppress HIF-1 α level in ovarian cancer cells (Duyndam *et al.*, 2007). Thus it would be worth testing whether the same applies to the cancer cell types used here.

Recently the role of HIF-2 α has been studied more intensively. Interestingly, a reciprocal increase in HIF-2 α protein level was reported in some HIF-1 α knockdown tumour cells (Carroll *et al.*, 2006). Therefore it should also be tested whether the HIF-2 α

status of tumour cells changes under conditions used here and influences their oxygen-dependent radio- and chemosensitivity.

Finally it is also clear from this study that a causal role of HIF-1 α in oxygen-dependent radio- and chemosensitivity is not universal between cell lines. Thus other cell lines representing various human solid tumours should be tested. Ideally oxygen-dependent radiosensitivity experiments should also be tested in the *in vitro* model of MH, to allow comparison with radiosensitivity under SH. This would in turn improve the extrapolation of observations to the level of solid tumours *in vivo*.

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