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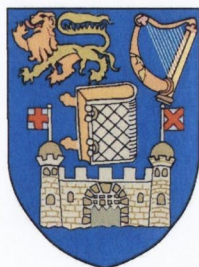
# **The role of HIF-1 alpha and hypoxia in the preferential response of prostate tumours to microtubule-targeting agents**

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**MB BCh BAO (Hons), IMRCSI**

**A thesis submitted for the degree of Doctor in Medicine  
(M.D.)**

**2008-2010**



**University of Dublin, Trinity College, Ireland**





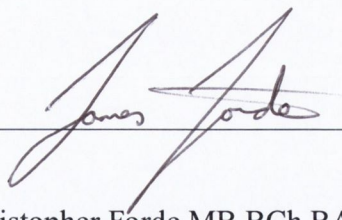
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# ABSTRACT

## Introduction

Tumour hypoxia is emerging as a common feature of prostate tumours associated with poor prognosis mainly due to their resistance to chemo-radiotherapy regimes. The efficacy of microtubule-targeting agents (MTAs) has recently been shown to be modulated by Hypoxia-Inducible-Factor-1 alpha (HIF-1 $\alpha$ ). HIF-1 $\alpha$  over expression has been reported to occur in almost 70% of all human tumours including prostate cancer. We proposed that administration of MTAs to patients with hypoxic prostate tumours may reduce the hypoxic response. Moreover, over expression of the hypoxia-responsive gene class III beta-tubulin (TUBB3) has been associated with docetaxel resistance in a number of cancer models. We proposed that TUBB3 down-regulation participates in docetaxel sensitivity and investigated the potential role of this gene in acquired docetaxel chemoresistance. We finally proposed to investigate cytotoxic effect of a novel MTA, PBOX-15, in hypoxic prostate tumours and its role in overcoming hypoxia associated radiation resistance.

## Methods

The cytotoxic effect of a panel of MTAs (docetaxel, paclitaxel, vincristine and PBOX-15) was determined in 22Rv1 and DU145 prostate cancer cells lines. Sensitivity was correlated with HIF-1 $\alpha$  gene and protein expression levels under aerobic and hypoxic conditions. Hypoxia-induced chemoresistance was next investigated in a pair of isogenic docetaxel-resistant PC3 cell lines. Basal



and hypoxia-induced TUBB3 gene expression levels were determined in all cell lines. Finally, the radio-sensitisation properties of PBOX-15 were investigated.

## **Results**

Prostate cancer cells were most sensitive to docetaxel under both aerobic and hypoxic conditions. Sensitivity correlated with reduced basal and hypoxia-induced HIF-1 $\alpha$  gene expression levels (down-regulated 5-fold and 16-fold; respectively in 22Rv1 and DU145 hypoxic untreated samples). Docetaxel appeared to reduce HIF-1 $\alpha$  protein expression in hypoxic cell lines. Acquired docetaxel resistance was not associated with TUBB3 over expression. PBOX-15 appears to radio-sensitise prostate cancer cell lines, overcoming hypoxia associated radiation resistance.

## **Conclusion**

We sought to evaluate the effect of MTAs including docetaxel on hypoxic prostate tumours as well as HIF-1 $\alpha$  gene and protein expression. We concluded that docetaxel may down-regulate HIF-1 $\alpha$  protein expression and the subsequent hypoxic response. HIF-1 $\alpha$  as a prognostic marker may help identify patients with disease likely to progress. Neoadjuvant treatment with docetaxel in localised tumours may help reduce the initial hypoxic response and future risk of biochemical failure. With regard to more advanced taxane refractory disease, further research into the mechanisms of taxane resistance may aid in the development of taxane analogues which are more effective in the treatment of the disease. Enhancing the efficacy of MTAs rather than replacing them with a novel agent may be of benefit in the treatment of those with prostate cancer.

## **ACKNOWLEDGEMENTS**

This thesis would not have been possible without the help of numerous people and I am sincerely indebted for all the advice, encouragement, and guidance I have received. It is a pleasure to thank the following people.

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# **PRESENTATIONS AND PUBLICATIONS**

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## **Poster Presentations**

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### **Poster Presentations (continued)**

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### **Manuscripts in preparation**

**Forde J.C., Perry A., Martin L., Hollywood D., Lawler M., Lynch T.H., Marignol L.** “Preferential sensitivity of prostate cancer cells to docetaxel correlates with down-regulation of HIF-1 $\alpha$  in hypoxia and lowered TUBB3 expression levels.”

**Forde J.C., Perry A., Martin L., Hollywood D., Lawler M., Lynch T.H., Marignol L.** “Microtubule-targeting compound PBOX-15 radio-sensitises cancer cells in vitro.”

**Forde J.C., Perry A., Martin L., Hollywood D., Lawler M., Lynch T.H., Marignol L.** “Enhancing the efficacy of taxanes in prostate cancer.”



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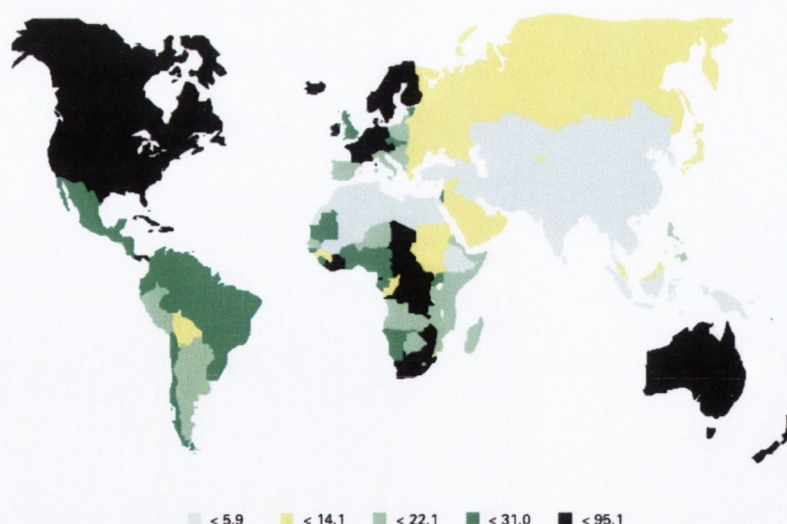
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# **CHAPTER 1**

## **Introduction**

## 1.1 Prostate cancer: Incidence, mortality and future challenges

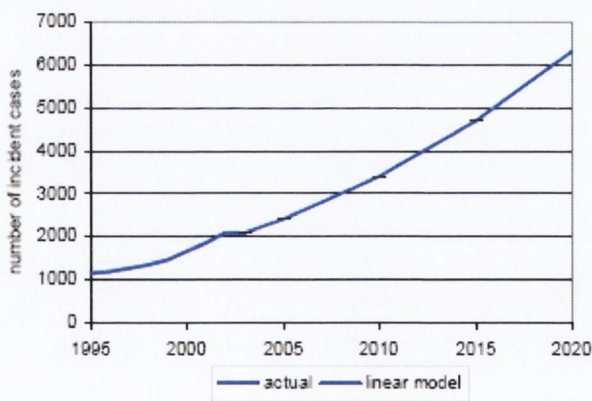
Prostate cancer is the most commonly diagnosed non-cutaneous cancer in men worldwide with more than 670,000 cases occurring every year (Ferlay, Autier et al. 2007). This accounts for one in nine of all newly diagnosed cancers in males. There has been a global rise in the incidence of prostate cancer over the past 20 years (Hsing, Tsao et al. 2000) with the incidence and prevalence rates varying widely around the world (Figure 1.1). By far the highest incidence rates are in North America and Northern and Western Europe with lower rates in China and other Asian countries (Quinn and Babb 2002). The incidence and prevalence of prostate cancer also vary with race and ethnicity. African-American men have the highest incidence rates while Orientals and other Asians have the lowest (Farkas, Marcella et al. 2000),(Gilligan, Wang et al. 2004).



**Figure 1.1:** The incidence of prostate cancer world-wide, age-standardized using the world standard population. Rate per 100,000 population. (Quinn and Babb 2002).



In Europe, 345,900 cases of prostate cancer were diagnosed in 2006 with 87,400 deaths attributed to the disease (Ferlay, Autier et al. 2007). In the UK, three-quarters of cases occur in men over 65 years with the largest number noted in those aged between 70 and 74 years (Cancer Research UK). Ireland suffers a similar burden from prostate cancer. One in eleven men living in Ireland will be diagnosed with prostate cancer during their lifetime. In 2006 alone, of the 2,399 men diagnosed with prostate cancer 547 died of the disease (NCRI 2006). By 2020, using current trends in prostate cancer incidence, rates will rise to 6,330 cases per year according to the National Cancer Registry of Ireland. This represents one of the largest increases in cancer incidence in Ireland to date (275% between 2000 and 2020) (Figure 1.2) (NCRI 2006). Increases in prostate cancer incidence are likely related to enhanced public awareness, improved diagnostic tools and the widespread determination of prostate-specific antigen (PSA) (Bono 2004).



**Figure 1.2** Projected number of cases of prostate cancer in Ireland 2005-2020.(NCRI 2006)

This increasing use of PSA in early detection and screening has contributed to an unfortunate trend of over-diagnosis and over-treatment in prostate cancer. We have seen a stage migration towards more localized, curable and less aggressive disease. A challenge we face in the future is to identify those patients with actively aggressive disease. This includes those patients with hypoxic prostate tumours. Hypoxia in prostate cancer is associated with treatment resistance and aggressive disease that is more likely to progress (Marignol, Coffey et al. 2008). Currently, chemotherapy in prostate cancer is reserved for those with advanced end stage metastatic disease. Chemotherapeutic agents such as taxanes have been reported to be of benefit in hypoxic tumours (Escuin, Kline et al. 2005). Taxanes may therefore be of benefit at an earlier stage in the management of prostate cancer.

## **1.2 Current management of prostate cancer**

### **1.2.1 Presentation and diagnosis**

During the early stages of prostate cancer, patients rarely have symptoms relating to the disease. Some may complain of lower urinary tract symptoms such as urgency, frequency, poor stream and nocturia; however, these symptoms are more commonly seen with benign prostatic disease such as benign prostatic hypertrophy (BPH). Those with advanced disease such as metastatic disease to bone may present with bone pain. Examination of patients involves a digital rectal exam (DRE) to assess the prostate gland for any abnormality. Often small discrete tumours are impalpable while larger tumours



may be felt as a hard nodule on examination of the prostate. In more advanced disease, DRE may show that the entire prostate is involved.

The majority of referrals to specialist urology clinics for patients suspected of having prostate cancer are as a result of an abnormal prostate specific antigen (PSA) test. Widespread use of PSA testing has led to a substantial increase in the number of patients diagnosed with prostate cancer. This glycoprotein is a serine protease of the kallikrein family and is produced almost exclusively by the prostate gland by both normal and malignant prostate cells with normal levels defined as between 0 - 4.0 ng/ml (Myrtle 1989). PSA was approved by the United States Food and Drug Administration in 1986 to monitor prostate cancer, and in 1994 was approved as a tool for detecting the disease in men aged 50 years and over (Fitzpatrick, Banu et al. 2009). Despite its widespread use, the role of PSA as a diagnostic marker is still controversial due to its low specificity and low sensitivity (Polascik, Oesterling et al. 1999). As well as being elevated in prostate cancer, it is also raised in benign conditions such as BPH and inflammatory conditions such as prostatitis. It can also be elevated after injury caused by surgical instrumentation and trauma. Other limitations include the fact that PSA levels are highly variable over time (Soletormos, Semjonow et al. 2005) and poorly distinguish indolent from aggressive cancers (Thompson, Pauler et al. 2004). However, a patient presenting with a raised PSA test and or an abnormal DRE warrants further



investigation with a biopsy of the prostate which can allow a histological diagnosis of prostate cancer to be made.

### **1.2.2 Treatment of localised and locally advanced disease**

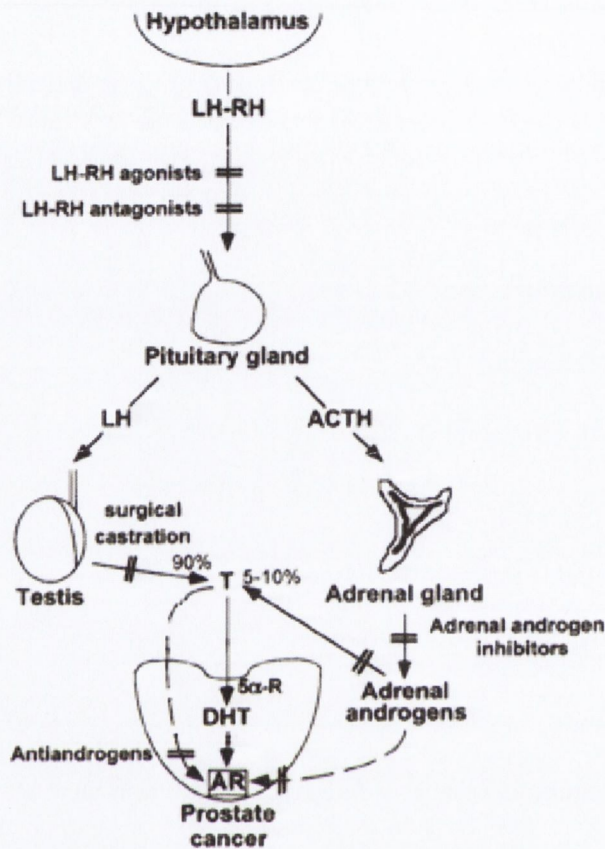
The clinical presentation of prostate cancer is classified as localised, locally advanced or metastatic. The management of localised disease, or disease confined to the prostate, includes observation (otherwise known as ‘watchful waiting’) and surgery in patients whose life expectancy exceeds ten years (Heidenreich 2007). Surgery for localised disease involves a radical prostatectomy, meaning excision of the entire prostate, the prostatic urethra along with the seminal vesicles. As surgery has been associated with a 30% risk of relapse and permanently decreased quality of life (Walsh 2000), radiation therapy is sometimes preferred. This can involve either external beam (Zimmermann, Geinitz et al. 2001) or brachytherapy (Cesaretti, Stone et al. 2007) but long-term results are dependent on stage of the disease (Lagerveld, Laguna et al. 2003). Treatment of locally advanced prostate cancer includes radiotherapy, watchful waiting, surgery as well as hormonal treatment which will be discussed in the next section.

### **1.2.3 Treatment of metastatic disease**

Up to a third of men with biochemical relapse (rising PSA profile) will progress to develop metastatic disease (Catton 2005) which is the cause of nearly all prostate cancer related death. A study following 794 patients with

metastatic prostate cancer showed 77% lived less than 5 years, 16% lived between 5 and 10 years, and only 7% over 10 years (Tangen, Faulkner et al. 2003). The standard treatment for metastatic prostate cancer is androgen deprivation therapy, either by surgical castration (orchidectomy) or more commonly medical castration (Figure 1.3). The overall aim of androgen deprivation treatment is to suppress testosterone, which stimulates prostate cancer growth. Bilateral orchidectomy has been shown to reduce testosterone levels by 90% within 24 hours of surgical castration (Maatman, Gupta et al. 1985). Medical castration involves the use of luteinising hormone-releasing hormone (LH-RH) agonists. LH-RH agonists cause an initial surge in luteinising hormone (LH) release and testosterone levels. These increased levels subsequently reduce LH-RH production via a negative feedback mechanism leading to reduced LH production. In the absence of LH, Leydig cell production of testosterone drops to castrate levels.





**Figure 1.3:** Strategies for hormonal therapy. LHRH, luteinizing hormone-releasing hormone; LH, luteinizing hormone; ACTH, adrenocorticotrophic hormone; T, testosterone; 5 $\alpha$ -R, 5 $\alpha$ -reductase; DHT, 5 $\alpha$ -dihydrotestosterone; AR, androgen receptor. Adapted from (Miyamoto, Messing et al. 2004).

The added use of direct acting anti-androgen blockade initially helps prevent ‘tumour flare’ which may worsen symptoms such as spinal cord compression. More recently, we have seen the development of LH-RH antagonists which bind immediately and competitively to the LH-RH receptors in the pituitary gland reducing LH concentrations by 84% within 24 hours of administration (Weckermann and Harzmann 2004).



Androgen deprivation results in reduction in PSA as well as palliation of symptoms such as severe bone pain and can provide disease control in over 80% for a median duration of 18 to 24 months (V.A.C.U.R.G 1967). Although subsequent second line hormone therapy (eg. antiandrogen withdrawal, glucocorticoid therapy) may be needed to diminish disease burden in some men, virtually all patients will eventually fail treatment, developing androgen independent or hormone-refractory prostate cancer (HRPC) within a median of 18 to 24 months (Petrylak, Tangen et al. 2004). Treatment for HRPC involves systemic cytotoxic chemotherapy.

#### **1.2.4 Radiotherapy in prostate cancer**

As we mentioned previously (section 1.2.2), radiotherapy is a commonly used as a treatment option in both localised and locally advanced disease. It can also be used in metastatic prostate cancer to alleviate symptoms such as bone pain. External beam radiotherapy (EBRT) is chosen by about one quarter of patients with prostate cancer as a primary treatment option (Potosky, Davis et al. 2004). This involves delivering up to 78 Gray (Gy) in 2 Gy fractions over a period of up to 8 weeks (Fowler 2005). In EBRT, high energy x-rays are produced by a linear accelerator directed at the prostate. The ionizing radiation damages the cancer cells by direct interaction with a critical target in the cell or indirectly through the generation of reactive oxygen species leading to apoptosis.

### **1.2.5 Treatment of hormone-refractory prostate cancer**

Chemotherapy is offered to appropriate patients with HRPC as well as patients with low volume disease who have failed radical local treatment and hormone therapy. It was previously considered to be relatively ineffective in HRPC (Eisenberger, Simon et al. 1985; Yagoda and Petrylak 1993). To date, three drugs (estramustine, mitoxantrone, and docetaxel) have been approved by the US Food and Drug Administration (FDA) for first-line chemotherapy in HRPC (Garmey, Sartor et al. 2008). Chemotherapy with mitoxantrone had been shown to palliate symptoms but did not extend overall survival (Gravis, Salem et al. 2007). The results of the TAX 327 and SWOG 99-16 trials for the first time showed an improvement in overall survival with docetaxel-based chemotherapy in patients with metastatic HRPC (de Wit 2008). Both trials compared docetaxel with mitoxantrone regimens and both showed an improvement in median survival of 2 months in those treated with docetaxel (Chang and Kibel 2009). A semi-synthetic taxane, docetaxel is currently the standard of care as a first line chemotherapeutic agent for the treatment of HRPC (Sinibaldi 2007). However many patients with HRPC will go on to require second- and even third-line treatment upon progression of the disease and options after this are limited. This has led to continued research into novel and more targeted chemotherapy regimes in prostate cancer.



### **1.3 Taxanes**

#### **1.3.1 Principles of action**

The principal mechanism of the taxane class of drugs is the disruption of microtubule function. They are known as microtubule targeting agents or MTAs. Microtubules are important for several cell functions including division, signaling and intracellular trafficking. They provide structural integrity as part of the cytoskeleton and play a role in the transport system for proteins, vesicles and organelles. They play a vital role in the formation of the mitotic spindle, dictating the segregation of chromosomes during mitosis. This makes them an attractive target for chemotherapeutic agents. In fact, taxanes are one of the most effective classes of chemotherapeutics and are used frequently in non-small cell lung, ovarian and breast cancers as well as prostate cancers (Chang and Kibel 2009). Taxanes work by binding to  $\beta$ -tubulin incorporated into microtubules. This prevents disassembly, stabilises the mitotic spindle and triggers apoptotic pathways. These apoptotic pathways include inactivation of Bcl-2 via phosphorylation, activation of JNK, Raf-1, and caspase dependent and caspase independent apoptotic mechanisms (Mathew and Dipaola 2007; Sinibaldi 2007). Docetaxel has previously been shown to have a higher affinity for  $\beta$ -tubulin than another taxane, paclitaxel, resulting in Bcl-2 phosphorylation at lower concentrations (Tannock, de Wit et al. 2004).



### **1.3.2 Taxane refractory prostate cancer**

Although docetaxel based therapies are the frontline treatments for HRPC, recent studies have focused on the increasing prevalence of taxane refractory prostate cancer. Mathew *et al.* defined it as evidence of disease progression during therapy or within 30 days of the last taxane dose (Mathew and Dipaola 2007). It is estimated that between 40 to 50% of HRPCs do not show substantive and sustained PSA decreases following treatment with taxanes with the median duration of response limited to between 6 to 9 months (Petrylak, Tangen et al. 2004; Tannock, de Wit et al. 2004). Taxane resistance can be classed as primary or secondary. In primary resistance, there is progression of disease without evidence of response to taxane treatment, whereas in secondary resistance there is an initial clinical response followed by disease progression (Yonemori, Katsumata et al. 2005).

### **1.3.3 Mechanisms of taxane resistance**

Several mechanisms of taxane resistance and their pathways have been studied previously. These include the direct interaction of taxanes with tubulin. Several different isotypes of tubulin have been described and a switch from class I to class III tubulin predominance has been observed to result in decreased impairment of microtubule dynamic instability by paclitaxel (Kamath, Wilson et al. 2005). Also the presence of a point mutation in tubulin at the taxane binding site causing taxane resistance has been proposed. This has been investigated in lung cancer patients where a strong correlation was seen

between tubulin point mutations and taxane resistance (Monzo, Rosell et al. 1999). Another possible mechanism is the over expression of the MDR-1 gene which encodes an efflux pump that is able to efflux taxanes out of the cell, thus hampering their retention and thus their efficacy (Horwitz, Lothstein et al. 1986). The influence of p53, which is activated when damage occurs to a mitotic spindle, may also be responsible for taxane resistance. Presence of mutant p53 could predict taxane sensitivity (Ioffe, White et al. 2004). Other studies have looked at whether the expression Bcl-2 can be modulated with certain agents affecting taxane efficacy (Morris, Cordon-Cardo et al. 2005). The research into the mechanisms of taxane resistance is ongoing and it is likely that several of these mechanisms operate simultaneously.

## **1.4 Hypoxia in prostate cancer**

### **1.4.1 Evidence of hypoxia in prostate tumours**

We previously introduced of the concept of tumour hypoxia and its association with aggressive disease (section 1.1). There is a growing body of experimental evidence that recognizes hypoxia as a major factor that influences tumour proliferation and the malignant progression of prostate tumours (Marignol, Coffey et al. 2008). Hypoxia is defined as a loss of oxygen in tissues and is widespread in solid tumours due to the tumour's ability to outgrow the existing vasculature. The microenvironment within a tumour is characterised by fluctuations in hypoxia and energy deprivation leading to genetic adaptations that increase invasiveness of tumours as well as their metastatic behaviour.



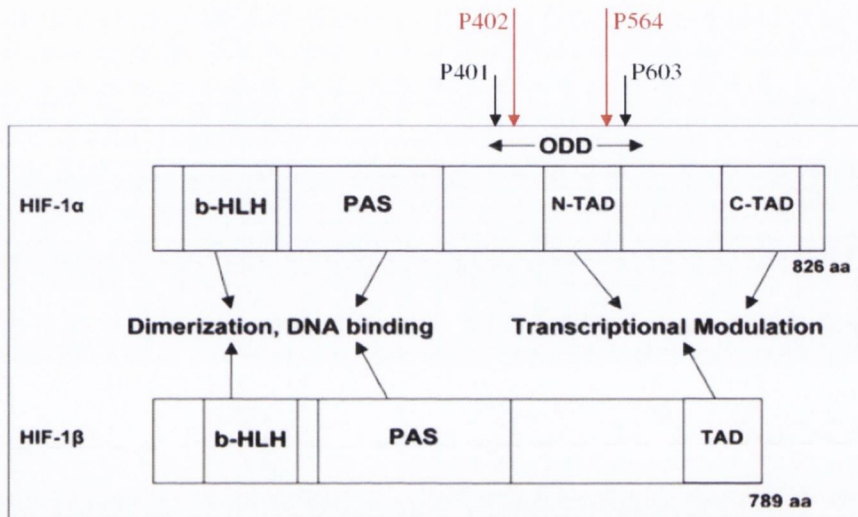
Evidence of tumour hypoxia in prostate tumours has been documented through detection of molecular markers of hypoxia by immuno-molecular imaging and physical measurements (Rasey, Koh et al. 1996; Bostwick and Iczkowski 1998; O'Donoghue, Zanzonico et al. 2005; Pallares, Rojo et al. 2006). The presence of hypoxic regions in prostate tumours has also been confirmed with positron emission tomography of [18F] fluoro-misonidazole (Rasey, Koh et al. 1996). It has been demonstrated that there are significantly lower oxygen measurements in the pathologically involved portion of the prostate (average median pO<sub>2</sub> 9.9mmHg) in comparison to periprostatic normal muscle (average median pO<sub>2</sub>, 28.6 mm Hg;  $p < 0.0001$ ) (Movsas, Chapman et al. 2000). The same authors also found that increasing levels of hypoxia within the prostate gland correlated significantly with increasing clinical stage, patient age and Gleason score.

#### **1.4.2 The role of hypoxia-inducible factor-1 alpha in tumour progression**

A number of studies have reported a role for hypoxia-inducible factor-1 alpha or HIF-1 $\alpha$  in the cytotoxicity of chemotherapeutic drugs disrupting the microtubule system, such as docetaxel, paclitaxel and vincristine (Escuin, Kline et al. 2005; Zeng, Kizaka-Kondoh et al. 2007). The HIF-1 complex is a proangiogenic transcription factor which plays a critical role in tumour progression and metastasis as well as overall tumour survival. HIF-1 is responsible for activating a number of genes in hypoxia which will ultimately result in cellular adaption to low oxygen conditions (Dery, Michaud et al. 2005). Initially discovered in 1992 as a regulator of hypoxia induced



erythropoietin expression (Semenza and Wang 1992), further studies have found the HIF-1 complex to be made up of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits. (Figure 1.4).



**Figure 1.4:** The hypoxia inducible factor-1-complex (HIF-1) is a dimer composed of two proteins involved in hypoxic gene transactivation, HIF-1 $\alpha$  and HIF-1 $\beta$ . Dimerization between these two factors is mediated by the helix-loop-helix (HLH) and aryl hydrocarbon nuclear translocation (ARNT) domains. DNA binding involves the basic (b-) region of the b-HLH domains. HIF-1 modulates transcription through its transactivation domains (TAD). Between amino acids 401 and 603, HIF-1 $\alpha$  contains an oxygen-dependent degradation domain (ODD) which controls its degradation under normoxia. The critical proline residues that mediate VHL binding when hydroxylated are Pro402 and Pro564, which are both located in the oxygen-dependent domain (ODD).

These heterodimers are basic helix-loop transcription factors that regulate the transcription of over 60 target genes allowing cellular adaptation to hypoxic environments (Wang, Jiang et al. 1995; Vaupel 2004). HIF-1 $\beta$  is also known as aryl hydrocarbon nuclear translocation or ARNT (Wang and Semenza 1995). It is strictly a constitutively expressed nuclear protein independent of oxygen tension (Maxwell, Pugh et al. 2001). In contrast, HIF-1 $\alpha$  is a cytoplasmic protein responsive to oxygen levels. In the presence of oxygen,

HIF-1 $\alpha$  is continuously degraded by the ubiquitin-proteasome system. This degradation process takes place only when certain conserved prolyl residues of HIF-1 $\alpha$  are hydroxylated, a modification requiring O<sub>2</sub>-dependent enzyme activity (Semenza 2001). Only HIF-1 $\alpha$  containing modified prolyl sites are then recognized by the von Hippel-Lindau protein, which is the recognition component of an E3 ubiquitin ligase that finally targets HIF-1 $\alpha$  for proteasomal degradation (Vaupel 2004). This rapid degradation makes HIF-1 $\alpha$  an extremely labile protein with a half life of less than five minutes (Dery, Michaud et al. 2005). However in hypoxia, HIF-1 $\alpha$  degradation is inhibited by the inability of the von Hippel-Lindau protein to bind to HIF-1 $\alpha$  (Figure 1.5 A) (Vaupel 2004). The stabilised HIF-1 $\alpha$  is translocated to the nucleus, where it heterodimerises with HIF-1 $\beta$  subunits producing an active HIF-1 complex. This activated HIF-1 interacts with specific hypoxic response elements (HRE's) which are found in the promoter regions of a variety of target genes (e.g. p300/CBP, STAT3). This leads to the activation and transcription of these genes to produce a variety of proteins that promote cell survival under hypoxic conditions (Semenza 2002) (Figure 1.5 B).

Overall, HIF-1 $\alpha$  over expression has been reported to occur in almost 70% of all human tumours including prostate cancer and their metastases compared with their adjacent normal tissue (Zhong, De Marzo et al. 1999). This same study also found overexpression of HIF-1 $\alpha$  in the pre-neoplastic tissue prostate intraepithelial neoplasia (PIN) suggesting that HIF-1 $\alpha$  overexpression



may occur early in carcinogenesis. Studies have also confirmed that HIF-1 $\alpha$  levels are up-regulated in high-grade PIN lesions relative to corresponding benign prostate specimens (Zhong, Semenza et al. 2004). This evidence would make HIF-1 $\alpha$  an interesting target for novel chemotherapeutic agents in prostate cancer.

#### **1.4.3 Hypoxia-inducible factor-1 alpha and tumour angiogenesis**

The process of new blood vessel formation or angiogenesis provides a principle mechanism for maintaining adequate blood flow in expanding cell populations including tumor tissue (Vaupel 2004). In adults, angiogenesis occurs in response to tissue hypoxia/ischemia and plays an important role in determining the progression of cancer. A critical molecular pathway involving the activation HIF-1 in hypoxia results in the production of mediators of angiogenesis including vascular endothelial growth factor (VEGF) (Semenza 2003) . VEGF is one of the most potent stimulators of angiogenesis which is essential for proliferation and migration of vascular endothelial cells. Almost all cells in the human body express VEGF in response to hypoxia and VEGF activity is regulated at a variety of levels including gene transcription, translation and protein excretion (Semenza 2001). There has been substantial published evidence, both experimental and clinical, that HIF-1 is paramount to this angiogenic process (Goonewardene, Sowter et al. 2002; Maxwell and Ratcliffe 2002).

In addition other vascular related gene products produced via the HIF-1 $\alpha$  pathway also mediate angiogenesis via the regulation of blood flow and vascular tone. This also links HIF-1 activity to potentially influencing tumor blood flow (Vaupel 2004). Further evidence also links HIF-1 $\alpha$  expression of VEGF in hypoxia mediated apoptosis. Hypoxic states which result in an amplification of normal HIF-1-dependent responses via loss of p53 function contributes to the angiogenic switch during tumorigenesis, therefore facilitating tumor angiogenesis (Ravi, Mookerjee et al. 2000). The selective targeting of HIF-1 $\alpha$  by chemotherapeutic agents may help disrupt tumour angiogenesis and subsequent tumour progression.

#### **1.4.4 Taxanes as hypoxic cell sensitisers**

Hypoxia-induced treatment resistance is well established in response to both radiation therapy and chemotherapy. The presence of tumour hypoxia appears to impair the effectiveness of radiotherapy with radio-sensitivity found to be progressively limited as tumour pO<sub>2</sub> levels fall. Hypoxic cells are up to three times more resistant to the effect of sparsely ionising radiation than well oxygenated cells (Hall 2006).

Changes in phenotype following hypoxic shock may also participate to reduce cytotoxic properties of commonly used chemotherapeutic drugs as well as compromise the outcome of androgen ablation therapies (Park, Kim et al. 2006). Tumour hypoxia is progressively associated with reduced oxidative



defence, gene instability, apoptosis resistance and may be associated with transition to androgen-independence in prostate cancer (Marignol, Coffey et al. 2008). While the presence of oxygen is essential for fixation of radiation-induced DNA damage, many conventional anticancer drugs also require oxygen for maximal activity (Teicher 1994). Moreover, hypoxia-induced cell cycle disturbances and inhibition of DNA double strand break repair processes under conditions of hypoxia may also reduce the ability of phase-specific chemotherapeutic agents for tumour targeting as well as reducing their toxicity (Figure 1.5 C) (Bristow and Hill 2008). It has been proposed that combination treatment with chemotherapeutic agents such as taxanes may radio-sensitise cancer cells. This has recently been studied in head and neck cancers using paclitaxel with some encouraging results (Herscher and Cook 1999).

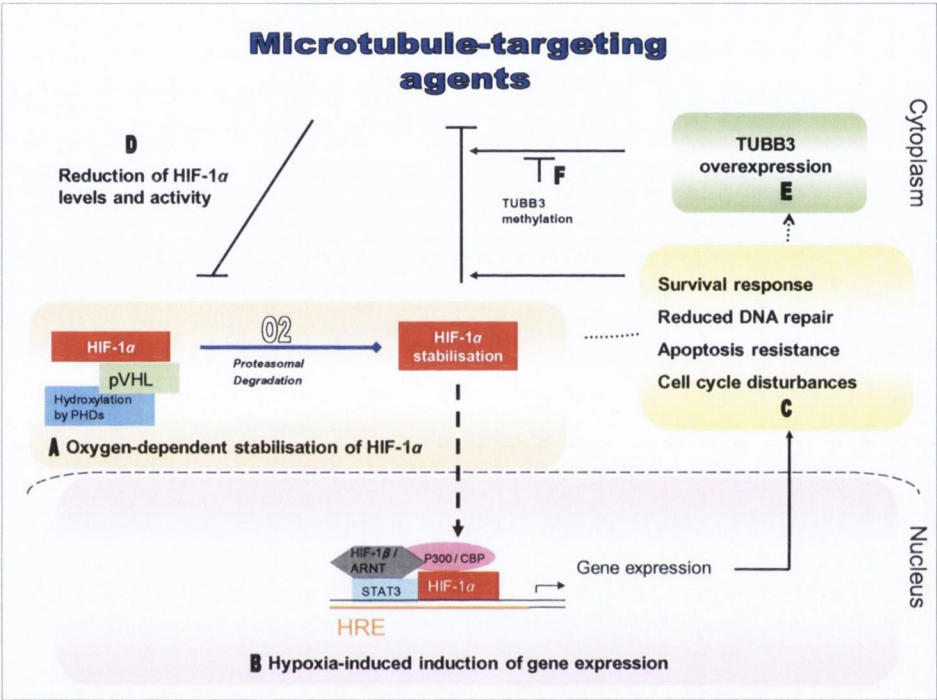
The HIF-1 $\alpha$  protein is also involved in physiological processes involving large-scale microtubule reorganization. While HIF-1 $\alpha$  overexpression was associated with increased drug-resistance (Liu, Ning et al. 2007), clinically relevant microtubule-targeting agents including taxanes were in fact shown to down-regulate HIF-1 $\alpha$  protein levels and activity (Escuin, Kline et al. 2005), modulating the sensitivity of tumour cells to these agents (Figure 1.5 D) (Zeng, Kizaka-Kondoh et al. 2007). Hypoxia-induced chemoresistance therefore appears to be both cell type and drug-specific (Skvortsova, Skvortsov et al. 2004; Thews, Gassner et al. 2007). Since taxanes are the only single chemotherapeutic agent to date with proven efficacy in prolonging overall

survival in prostate cancer patients (Tester, Ackler et al. 2006); they may thus have a role in down-regulating HIF-1 $\alpha$  and the subsequent hypoxic response.

#### **1.4.5 Resistance to microtubule targeting agents**

Changes in expression of a number of genes have been shown to be implicated in resistance to microtubule targeting agents. Over expression of the Class III beta-tubulin (TUBB3) gene has been found in breast (Paradiso, Mangia et al. 2005), lung (Seve, Isaac et al. 2005) and pancreatic cancer (Lee, Cao et al. 2007) and has been proposed as a major mechanism of drug resistance to microtubule targeting agents (Kamath, Wilson et al. 2005) (Figure 1.5 E). It has been previously demonstrated that lung cancer cells could be sensitized to paclitaxel by down-regulation of TUBB3 (Kavallaris, Burkhart et al. 1999) and more recently it has been found that hypoxia via HIF-1 $\alpha$  is able to induce the expression of TUBB3 (Raspaglio, Filippetti et al. 2008). Moreover, increased expression of TUBB3 upon hypoxia was abolished in taxane-resistant cells through methylation of the 3' enhancer (Lee, Cao et al. 2007). These results therefore suggest that TUBB3 methylation may prevent TUBB3 over expression in hypoxic cells and inhibit taxane resistance (Figure 1.5 F). TUBB3 methylation status may thus represent a determining factor governing response to these compounds.





**Figure 1.5:** Modulation of microtubule-targeting agent's activity in hypoxia. **A.** Oxygen deprivation inhibits proteasomal degradation of Hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ). **B.** Stabilised HIF-1 $\alpha$  F is translocated to the nucleus where it binds to hypoxia response elements (HRE) along with other transcription factors to induce gene expression. **C.** Hypoxia-induced gene expression participates in the induction of survival response, apoptosis resistance and along with hypoxia-induced cell cycle disturbances and DNA repair inhibition, participates in chemoresistance. **D.** Microtubule-targeting agents reduce HIF-1 $\alpha$  protein levels and activity and may prevent HIF-1 $\alpha$  mediated responses. **E.** Class III beta-tubulin (TUBB3) over expression in hypoxia contributes to treatment resistance. **F.** TUBB3 promoter methylation may prevent onset of resistance.

#### **1.4    PBOX-15**

To overcome taxane resistance, a novel approach may be required. Currently in our laboratory we are working with a novel series of potential anti-cancer compounds, the pyrrolo-1,5-benzoxazepine (PBOX) compounds. These have been shown induce apoptosis in a variety of human chemotherapy-resistant cancer cell lines and in primary ex vivo material derived from cancer patients (Mulligan, Greene et al. 2006). One member of this compound series, PBOX-15, was found to cause microtubule depolymerisation in a human breast carcinoma cell line, MCF-7 (Mulligan, Greene et al. 2006). Activation of JNK is essential during PBOX-induced apoptosis (Mc Gee, Campiani et al. 2002) and Bcl-2 phosphorylation is a critical step in the apoptotic pathway of PBOX-6 (Mc Gee, Greene et al. 2004), another member of the PBOX family of compounds with similar mechanism of action to that of PBOX-15. PBOX-15 has also been found to be much more potent than some other anti-microtubule agents (Ling, Jiang et al. 2002). The effects of the PBOX family of compounds have recently been compared to paclitaxel with PBOX found as having a distinct effect on the microtubule network (Mulligan, Greene et al. 2006). Paclitaxel is a known microtubule polymerizer that with increasing concentration causes an increase in microtubule mass and a distinctive bundling of the microtubules. PBOX-15 was found to cause dissolution of the microtubule network via microtubule depolymerization. This study suggested that PBOX-15 binds to an as-yet-uncharacterised novel site on tubulin. With its



different mechanism of action on tubulin, PBOX-15 may be of use in overcoming resistance associated with hypoxia as well as taxane resistance.

## **1.6 Clinical potential**

As we mentioned previously (section 1.1), the use of PSA in early detection/screening is not without controversy and has contributed to a stage migration towards more localized, and curable, prostate cancers. In the modern PSA era, we must aim to differentiate men who have disease destined to progress and cause morbidity/mortality from those who will not require immediate, or possibly even delayed, therapeutic intervention. This has led to the concept of risk stratification in prostate cancer to help optimise treatment for low and high risk groups with the aim of stratifying patient care (Thompson 2006). Recently, we have also seen the use of prognostic nomograms which have been useful for predicting outcomes in patients with prostate cancer (Ross 2001). Ongoing research is looking at new potential prognostic markers by molecular, biochemical, imaging and other means identifying patients who are highest risk. This includes those at risk of progressing to taxane refractory disease.

Advances in imaging have shown potential to identify patients with hypoxic prostate cancer tumours prior to treatment. Blood oxygen level-dependent (BOLD) MRI showing a high sensitivity for defining intra-prostatic tumour hypoxia may have future potential as a non-invasive technique for

mapping hypoxic tumours (Hoskin, Carnell et al. 2007). Recent studies detailing non-invasive imaging of hypoxia with PET (Yapp, Woo et al. 2007) may also have a prognostic and/or diagnostic role in the clinical management of the disease. Other studies have also analysed a range of potential biomarkers other than PSA in prostate cancer (Parekh, Ankerst et al. 2007). Due to the expression of HIF-1 $\alpha$  in the early stages of prostate carcinogenesis and its correlation with diagnostic and prognostic indicators for early relapse and metastatic disease, this protein is attractive as a future prognostic biomarker of aggressive hypoxic prostate cancers (Kimbrow and Simons 2006).

In this project, we propose to determine whether hypoxic prostate tumours may specifically benefit from treatment with microtubule-targeting agents by studying their effects in a number of cell line models. In addition to the chemotherapeutic agents currently used in the clinical setting (docetaxel, paclitaxel and vincristine), we propose to investigate the novel chemotherapeutic agent, PBOX-15. We aim to determine the activity of these agents under hypoxic conditions in prostate cancer cell lines *in vitro* and correlate hypoxia-induced response to HIF-1 $\alpha$  gene and protein expression. We also plan to investigate if PBOX-15 has radio-sensitisation properties overcoming radiation resistance associated with hypoxia. We then finally hope to determine the role of TUBB3 expression on chemotherapeutic outcome.



# **CHAPTER 2**

## **Aims and Objectives**

## 2.1 Aim 1: Evaluation of docetaxel as a hypoxic cell sensitiser

### Hypothesis 1

Microtubule-targeting agents preserve their tumour cell killing activity under conditions of hypoxia.

#### *Objective 1*

We initially proposed to evaluate the cytotoxic activity of microtubule-targeting agents (docetaxel, paclitaxel and vincristine) under conditions of hypoxia using clonogenic assays in prostate cancer cell lines *in vitro*. We hoped to identify which drugs preserve their cytotoxicity under hypoxic conditions.

**Significance:** Patients with hypoxic tumours may benefit from treatment with microtubule-targeting agents.

### Hypothesis 2

HIF-1 $\alpha$  levels and activity may be down-regulated by microtubule-targeting agents, limiting HIF-1 $\alpha$  mediated surviving response.

#### *Objective 2*

We next evaluated the role of HIF-1 $\alpha$  in the cytotoxic activity of these drugs. We proposed to monitor HIF-1 $\alpha$  protein (Western blot) and gene (Taqman®) expression over time in treated hypoxic prostate cancer cell lines *in vitro*. We hoped to correlated persistence of cytotoxic activity in hypoxia with down-regulation of HIF-1 $\alpha$ .



**Significance:** Administration of microtubule-targeting agents to patients with hypoxic tumours may reduce the hypoxic response.

**Hypothesis 3**

TUBB3 expression may influence the sensitivity of hypoxic cells to microtubule-targeting agents.

***Objective 3***

We proposed to determine TUBB3 gene expression pattern in response to hypoxia using a Taqman® gene expression assay. We also proposed to determine if TUBB3 over expression is responsible for docetaxel resistance in a docetaxel resistant prostate cancer cell line.

**Significance:** TUBB3 expression may represent a mechanism for docetaxel resistance in prostate cancer.

**Aim 2: Evaluation of the potential of PBOX-15 for the management of hypoxic prostate tumours**

**Hypothesis 4**

Administration of a novel microtubule-targeting agent, PBOX-15, may be of benefit in the treatment of hypoxic prostate tumours.

***Objective 4***

We proposed to evaluate the cytotoxic activity of a novel microtubule-targeting agent, PBOX-15, in prostate cancer cell lines in both aerobic and hypoxic conditions. We planned to correlate this cytotoxic activity with both HIF-1 $\alpha$  gene and protein expression.

**Significance:** PBOX-15 may be of benefit in the treatment of hypoxic prostate tumours.

**Hypothesis 5**

PBOX-15 may radio-sensitise prostate cancer cells.

***Objective 5***

We proposed to evaluate the radio-sensitising effect of PBOX-15 in aerobic and hypoxic prostate cancer cell lines.

**Significance:** PBOX-15 may help overcome radiation resistance associated with hypoxia.



# **CHAPTER 3**

## **Materials and Methods**

### **3.1 Cell culture**

#### **3.1.1 Cell lines**

In this study, 3 human prostate cell lines were used: DU145, 22Rv1 and PC3. DU145 and 22Rv1 human prostate cancer cell lines were obtained from the American Type Culture Collection (Teddington, Middlesex, UK). DU145 was established from a metastatic prostate cancer brain lesion (Stone, Mickey et al. 1978). 22Rv1 is a human prostate carcinoma epithelial cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft (Sramkoski, Pretlow et al. 1999). PC3 was established from a metastatic prostate cancer bone lesion. PC3 cell lines used included an age-matched docetaxel-sensitive (PC3) and docetaxel-resistant (PC3-D12) lines that were kindly provided by Prof. William Watson, University College Dublin, Ireland.

#### **3.1.2 Cell line continuity**

DU145, 22Rv1 and PC3 cell lines were cultured in RPMI 1640 plus glutamax supplemented with 10% fetal calf serum (FCS), 50 $\mu$ /ml penicillin and 5 $\mu$ /ml streptomycin (all Invitrogen, Paisley, UK). Cell cultures were grown in T75 flasks (Nunc, Roskilde, Denmark) in an incubator (Thermo Fischer Scientific, OH, USA) at 37°C and 5% CO<sub>2</sub>. Cultures were handled within a Class II laminar flow cabinet (Medical Air Technology, Oldham, UK). Cell lines were passaged weekly as described below (section 3.1.3) and fed once a week by replacing the culture media with fresh media. Mycoplasma testing was



regularly performed every 2-3 months on all cell lines (Appendix B) using the Mycoplasma PCR ELISA kit (Roche, West Sussex, UK). Cells consistently tested negative for mycoplasma contamination.

### **3.1.3 Passaging cell lines**

Media was removed from T75 Flasks containing the appropriate cell lines. Next 5ml of trypsin (Invitrogen, Paisley, UK) was added to the T75 flask and incubated for 5 minutes at 37°C. Next, the trypsin was neutralized with 5mls of fresh media added to bring the volume to 10mls. Cells were transferred to a 20ml sterile tube (Sterilin, Caerphilly, UK) and centrifuged at 1500rpm for 5 minutes in a Centra GP8R centrifuge (Thermo IEC, MA, USA). All but 0.2 ml of the resulting supernatant was discarded and the pelleted cells were re-suspended in the remaining media. Then 1-3mls of fresh media (depending on the cell line) was added. Cells were then transferred to a new T75 flask containing 20mls of fresh media. The 22Rv1 cell line was routinely passaged at a 1:6 ratio, where as DU145 and PC3 lines were split at a 1:10 ratio.

### **3.1.4 Counting cells**

After cells were trypsinized and thoroughly re-suspended in 1ml appropriate growth media, 10 $\mu$ l of cells were added to 90 $\mu$ l of Trypan blue solution 0.4% (Sigma-Aldrich, Poole, UK). After mixing, approximately 10 $\mu$ l of cell/Trypan blue solution was loaded onto each side of an improved Neubauer hemacytometer (Hawksley, Lancing, UK) with a 22x40mm coverslip

(Chance Proper, Warley, UK). Cells in the central 25 squares of each chamber were counted under fluorescence using a Dialux 20EB microscope (Leitz, Germany). The number of cells per chamber was averaged over 2 chambers and the cell concentration was calculated as:

$$\text{Cells/ml} = \text{cells/chamber} \times 10^5$$

### **3.1.5 Hypoxia and irradiation procedures**

Hypoxia was achieved in a Concept 400 hypoxia chamber (Ruskin Technologies, Leeds, UK) set at 0.5% O<sub>2</sub> and 5.0% CO<sub>2</sub>.

Ionising radiation was delivered using an RS225 cell irradiator (Gulmay Medical, Camberley, UK) at a dose rate of 3.25Gy/min (200V, 15mA).

### **3.1.6 Drug preparation**

Docetaxel and paclitaxel (Sigma-Aldrich, Poole, UK) were diluted in 100% ethanol to a stock concentration of 1mM. Vincristine and 5-Fluorouracil (Sigma-Aldrich, Poole, UK), were diluted in DMSO to a stock concentration of 1mM. PBOX-15 was kindly provided by the Institute of Molecular Medicine, Trinity College Dublin, and was also diluted in ethanol to a concentration of 1mM. The stock solutions were stored at -20C.



### 3.2 Clonogenic assays

Cells were trypsinized and counted as outlined previously (section 3.1.4). The cells (1000-5000 cells/well) were then plated in 6-well plates (Sarstedt, Wexford, Ireland). The plates were agitated after addition of the cells to ensure uniform seeding. Cells were left to seed overnight at 37°C. The next day, the cells were treated according to protocol (see next paragraph). One week later, the plates were removed from incubator and media removed. A staining solution made up of crystal violet (Sigma-Aldrich, Poole, UK) and 70% methanol was added to each well. Plates were then rinsed and left to air dry. Colonies were counted using the Colcount™ (Oxford Optronix, Oxford, UK). The plating efficiency was calculated in the untreated controls and the surviving fraction in treated cells as follows.

- Plating efficiency (%) = [number of clones / number of cells plated ] \* 100
- Surviving fraction (%) = [number of clones / (number of cells plated x plating efficiency) ] \* 100

For hypoxia experiments, the cells were treated with either docetaxel (1nM), paclitaxel (1nM), vincristine (1nM), 5-Fluorouracil (100nM) or PBOX-15 (250nM) under aerobic or hypoxic conditions. Forty-eight hours later, the drug was removed from all plates and 2 ml of fresh media was re added to each well and the plates returned to the incubator until colony formation.

For radiation experiments, PBOX-15 stock solution was made up to the following concentrations: 1nM, 10nM, 100nM, and 250 nM. Each concentration was then added to the relevant 6-well plates. Another plate was left untreated. Plates were then placed in the hypoxic chamber at 0.5% O<sub>2</sub> for 24 hours. At the end of drug treatment, the plates were taped with paraffin tape in the hypoxic chamber to minimise exposure to aerobic conditions before being irradiated with 2Gy. The media was then removed from the plates and fresh media added. Plates were then left in an incubator until colonies had formed.

### **3.3 MTT assay protocol**

5x10<sup>4</sup> cells/well were plated onto 96-well plates (Sarstedt, Wexford, Ireland) prior to treatment with a range of concentrations of docetaxel, paclitaxel, vincristine (0.1, 1, 10, 100, 250 nM); 5-Fluorouracil and PBOX-15 (0.01, 0.05, 0.1 and 1nM) for 48 hours under aerobic or hypoxic conditions. At the end of drug treatment, media containing the drug was removed from each well and replaced with 100µL of fresh media. 15µL of dye (CellTiter 96<sup>®</sup> Non-Radioactive Cell Proliferation Assay, Promega, Southampton, UK) was then added to each well and the plates left in the incubator for a further 4 hours. 100µL of solubilisation solution (Promega, Southampton, UK) was then added to each well. After a further 1 hour in the incubator the plates were read on a SpectraFlour Plus fluorometer (Tecan, Dorset, UK) at a wavelength of 570 nm. The absorbance at 570 nm was directly proportional to cell number allowing



calculation of the concentration of cytotoxic agent necessary to kill half of the cell population or IC50 values.

### **3.4 HIF-1 $\alpha$ gene expression**

#### **3.4.1 Purification of mRNA**

DU145 and 22Rv1 cell lines were cultured until approximately 90% confluent as previously described (section 3.1.2). For each T75 flask, 20mls of appropriate culture media (RPMI 1640 plus glutamax supplemented with 10% fetal calf serum, 50 $\mu$ /ml penicillin and 5 $\mu$ /ml streptomycin) was measured and placed into ventilated T75 flasks and placed into the hypoxic chamber (0.5% O<sub>2</sub>) for 24 hours prior to relevant experiment. The same was done with sterile PBS (Invitrogen, Paisley, UK) solution.

The first experiment involved investigating the levels of HIF-1 $\alpha$  mRNA expression in both cells lines at the specific hypoxia time points of 0, 4, 8, 16 and 24 hours. Following this the time point of 4 hours of hypoxic exposure was used to assess the impact of drug treatment on HIF-1 $\alpha$  mRNA expression in prostate cancer cell lines. The following concentrations of drugs were used; docetaxel at 1nM, 5-FU at 100nM and PBOX-15 at 250nM.

Cells were treated for 4 hours in the relevant T75 with a control T75 left untreated in hypoxia. The flasks were individually scraped using a 23cm cell scraper (Nunc, Roskilde, Denmark) and then transferred into a 15ml sterile tube

(Sarstedt, Wexford, Ireland). The above steps were carried out within the hypoxia chamber to minimize cell exposure to normoxia and potential degradation of the HIF-1 $\alpha$  gene. The same experiment was repeated in aerobic conditions.

Purification of total RNA from cultured prostate cancer cells was done using the RNeasy Mini Kit (Qiagen Ltd, Crawley, UK) as per protocol. Before starting the process, 10 $\mu$ l of  $\beta$ -mercaptoethanol ( $\beta$ -ME) per 1ml of Buffer RLT was added and the specified amount of 100% Ethanol was added to Buffer RPE.

Cells were disrupted by adding 350 $\mu$ L of Buffer RLT solution (with 10 $\mu$ l of  $\beta$ -mercaptoethanol per 1ml of Buffer RLT already added). The resulting lysate was pipetted directly into a QIAshredder spin column placed in a 2ml collection tube and centrifuged for 2 minutes at 13,500 rpm. 350 $\mu$ L of 70% ethanol was then added to the homogenized lysate, and mixed well by pipetting. Then up to 700 $\mu$ l of the sample, including any precipitate that may have formed was transferred to an RNeasy spin column placed in a 2ml collection tube and centrifuged for 15 seconds at 10,000 rpm. The flow-through was then discarded. 700 $\mu$ l of Buffer RW1 was then added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. The flow through was again discarded. 500  $\mu$ l Buffer RPE was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm



to wash the spin column membrane. Flow through was discarded. Another 500  $\mu$ l of Buffer RPE was added to the RNeasy spin column and centrifuged for 2 min at 10,000 rpm to wash the spin column membrane. The long centrifugation dried the spin column membrane, ensuring that no ethanol was carried over during RNA elution. The RNeasy spin column was then placed in a new 2 ml collection tube and centrifuged at 13,500 rpm for 1 minute. This step was performed to eliminate any possible carryover of Buffer RPE. The RNeasy spin column was then placed in a new 1.5 ml collection tube. 25  $\mu$ l RNase-free water was added directly to the spin column membrane and centrifuged for 1 minute at 10,000 rpm to elute the RNA. This step was repeated again to increase the RNA yield to 50  $\mu$ l. RNA samples were then quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). Samples were then immediately stored at minus 80° Celsius.

### **3.4.2 Conversion of RNA to cDNA**

Conversion of the RNA to cDNA was performed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Warrington, UK) as per protocol. Using up to 2  $\mu$ g of total RNA per 20  $\mu$ L reaction, the RT reaction mix was prepared using the kit components before preparing the reaction plate. To prepare the RT reaction mix (per 20  $\mu$ L reaction) the kit components were allowed to thaw on ice.

The following table outlines the volume of components needed to prepare the required number of reactions.

| Component                      | Volume/Reaction ( $\mu\text{L}$ ) |
|--------------------------------|-----------------------------------|
| 2X RT Buffer                   | 10.0 $\mu\text{L}$                |
| 20X RT Enzyme Mix              | 1.0 $\mu\text{L}$                 |
| Nuclease-free H <sub>2</sub> O | to 20 $\mu\text{L}$               |
| RNA Sample                     | to 9 $\mu\text{L}$                |
| Total per Reaction             | 20.0 $\mu\text{L}$                |

To prepare the cDNA RT reactions 20  $\mu\text{L}$  was aliquoted of RT reaction mix into each well. Tubes were sealed and the plate briefly centrifuged to eliminate any air bubbles. Tubes were placed on ice until ready to load onto the thermal cycler. To perform reverse transcription, the thermal cycler was programmed to the following conditions:

|                                    | Step 1 | Step 2 | Step 3   |
|------------------------------------|--------|--------|----------|
| Temperature ( $^{\circ}\text{C}$ ) | 37     | 95     | 4        |
| Time (minutes)                     | 60     | 5      | $\infty$ |

Tubes were stored at 2 to 8 $^{\circ}\text{C}$  short-term (up to 24 hours before use) and -15 to -25 $^{\circ}\text{C}$  long-term.



### 3.4.3 Gene expression protocol

Gene expression was performed using the TaqMan® Gene Expression Assays kit (Applied Biosystems, Warrington, UK). Stored cDNA was thawed along with kit reagents on ice and gently vortexed. MicroAmp® Optical 96-Well Reaction Plates (Applied Biosystems, Warrington, UK) were used. Two gene expression assays were used: HIF-1 $\alpha$  as the target gene and PGK-1 (Phospho Glycerate Kinase 1) as an endogenous control (both from Applied Biosystems, Warrington, UK). The number of reactions needed for each assay was calculated. Each reaction was repeated in triplicate. A master mix solution for each assay containing per reaction: 5  $\mu$ l of TaqMan® Gene Expression Master Mix, 0.5  $\mu$ l of the gene expression assay and 2  $\mu$ l of RNase-free water was made. 7.5  $\mu$ l of this master mix solution was then added to each well. Finally, 2.5  $\mu$ l of the relevant cDNA sample was then added to each well. 96-Well Reaction Plates were inserted into the 7500 Real Time PCR System (Applied Biosystems, Warrington, UK). Thermal cycling was programmed as follows and repeated 40 times:

|                 | Step 1 | Step 2 | Step 3 | Step 4 |
|-----------------|--------|--------|--------|--------|
| Temperature(°C) | 50     | 95     | 95     | 60     |
| Time            | 2 min  | 10 min | 15 sec | 1 min  |

Results were then analysed using the 7500 software v2.0.1 (Applied Biosystems, Warrington, UK).

### **3.5 Western blot for HIF-1 $\alpha$**

#### **3.5.1 Cell exposure to hypoxia**

The DU145 and 22Rv1 cell lines used in these experiments were cultured until approximately 90% confluent as previously described (section 3.1.2). The T75 flask was placed in to the hypoxic chamber (0.5% O<sub>2</sub> and 5.0% CO<sub>2</sub>) and the original culture media was discarded using a 25ml pipette (Drummond Scientific, PA, US). Next, 20mls of fresh hypoxic media (see preparation in section 3.4.1) was replaced into each T75 flask. The relevant drug (docetaxel, 1nM; 5-Fluorouracil, 100nM; PBOX-15, 250nM) was added to each flask with a control untreated T75 flask left untreated. Cells were then incubated for the pre-determined exposure time. In this study cells were exposed to 0 and 6 hours time points in hypoxia. Flasks that were exposed to 0 hrs hypoxia had their media replaced with 20mls of fresh non-hypoxic media prior to protein extraction.

#### **3.5.2 Preparation of whole cell lysate**

Protein extraction from cells was prepared using whole cell lysate (Appendix A – Table 1). The whole cell lysate and method for protein extraction was modified from a previous published technique (Palayoor, Tofilon et al. 2003). The lysis buffer was pre-prepared (minus PMSF and DDT), into 1ml aliquots and stored at -20°C until required. PMSF and DDT were also prepared separately into 5 $\mu$ l and 2 $\mu$ l aliquots respectively and stored at -20°C. The whole cell lysis buffer consisted of: Tris HCL pH 7.4 (50mM),



NaCl (150mM), IGEPAL (1%), sodium orthovanadate (600 $\mu$ M), sodium fluoride (50mM), EDTA (1mM) and SDS (0.1%). 10mls of lysis buffer was prepared and a protease inhibitor tablet (Roche, West Sussex, UK) was added. Prior to protein extraction, 5 $\mu$ l of PMSF and 2 $\mu$ l of DDT was added to 1ml of lysis buffer and the combined solution was then placed on ice until required.

### **3.5.3 Protein extraction**

Hypoxic culture media in the T75 flask was discarded and 5mls of hypoxic PBS was added to each flask. The flasks were individually scraped using a 23cm cell scraper (Nunc, Roskilde, Denmark) and then transferred in to a 15ml sterile tube (Sarstedt, Wexford, Ireland). The above two steps were carried out within the hypoxia chamber to minimise cell exposure to normoxia and potential degradation of HIF-1 $\alpha$  protein. Cells exposed to 0 hrs hypoxia were processed under a Class II laminar flow cabinet (Medical Air Technology, Oldham, UK) using 5mls of non-hypoxic PBS.

Next, the 15ml tubes were removed from the hypoxia chamber and immediately placed on ice. Samples from this point on were always placed on ice until final storage at -80°C. The 15mls tubes were pelleted at 4,000rpm for 5mins at 4°C in a Centra GP8R centrifuge (Thermo IEC, MA, USA). The resulting supernatant was completely discarded and 90 $\mu$ l of lysis buffer containing PMSF and DDT was added to each sample. All samples were kept on ice for 20mins with vortexing every 5 minutes for 10 seconds using a Vortex

Genie-2 (Scientific Industries, NY, USA). Next, samples were transferred in to 1.5ml microfuge tube (TreffLab, Degersheim, Switzerland) and centrifuged at 13,500rpm for 10 minutes at 4°C using a Microlite RF centrifuge (Thermo IEC, MA, USA). The resulting supernatant was collected in 3 x 30µl aliquots and transferred into labeled 0.5ml microtubes (TreffLab, Degersheim, Switzerland) and immediately stored at -80°C.

### **3.5.4 Protein quantification – Bradford assay**

Standard solution of BSA Fraction V (Sigma-Aldrich, Poole, UK), ranging in concentration from 0-32µg/ml was used. Samples were prepared using 500µl of dH<sub>2</sub>O and 1µl of lysis extract. Next, 500µl of Bradford Reagent (Sigma-Aldrich, Poole, UK) was added to each standard and sample. 200µl per well was then transferred to a 96 well plate (Nunc, Roskilde, Denmark) and mean absorbance was calculated at 595nm on a SpectraFlour Plus fluorometer (Tecan, Dorset, UK). An Excel graph (Microsoft) was then plotted to compare µg/ml BSA against the adjusted absorbance of the standards (Figure 3.1). The protein concentration (µg/ml) of each cell lysate sample was interpolated from the standard curve constructed from the BSA solution absorbencies using Excel software.



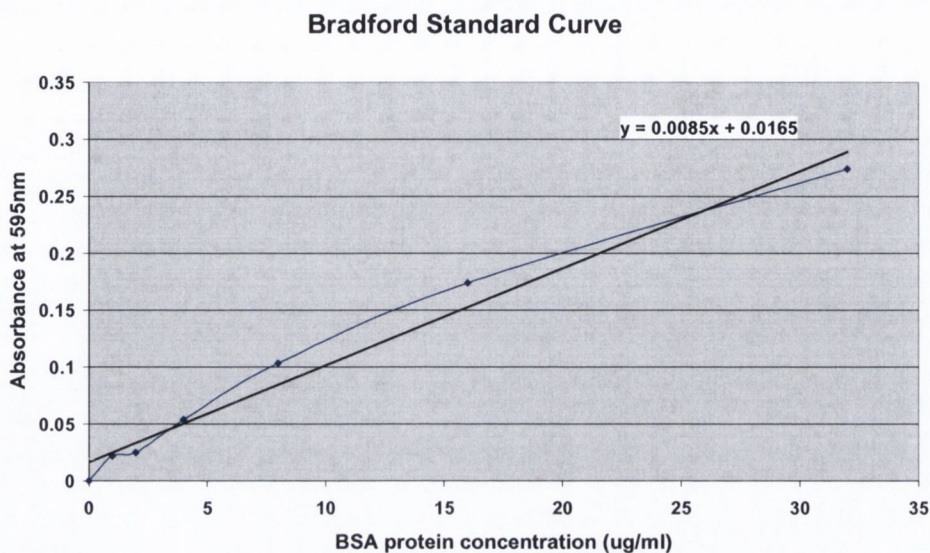


Figure 3.1: Sample Bradford Standard Curve

### 3.5.5 Samples

50 $\mu$ g of protein was prepared into 10 $\mu$ l samples with PBS using the Bradford assay method. 10 $\mu$ l of loading buffer was added to each sample and then boiled for 5 minutes at 97°C. Samples were briefly vortexed and centrifuged for 5 seconds at 13,500rpm.

### 3.5.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a technique used to separate proteins according to their electrophoretic mobility. This method was initially described as the Laemmli Buffering System (Laemmli 1970). Proteins contained in each cell lysate were separated out on a 12% SDS-polyacrylamide gel using the Mini-PROTEAN 3 Electrophoresis System (BioRad, Hertfordshire, UK). The resolving gel and

stacking gel were prepared as outlined in Appendix A - Tables 3 and 4. After mixing, both resolving and stacking gels were degassed by sonication in a water bath for 10 seconds prior to setting the gel. The running buffer consisted of: Tris-HCL (0.025M), glycine (0.192M), and SDS (0.2%). Cell lysate samples (20uL/well) were loaded onto the gel as well as a 10µl protein marker (Precision Plus Protein™ Standards, BioRad, Hertfordshire, UK) in another well. The gel was then run at 200V for 45mins.

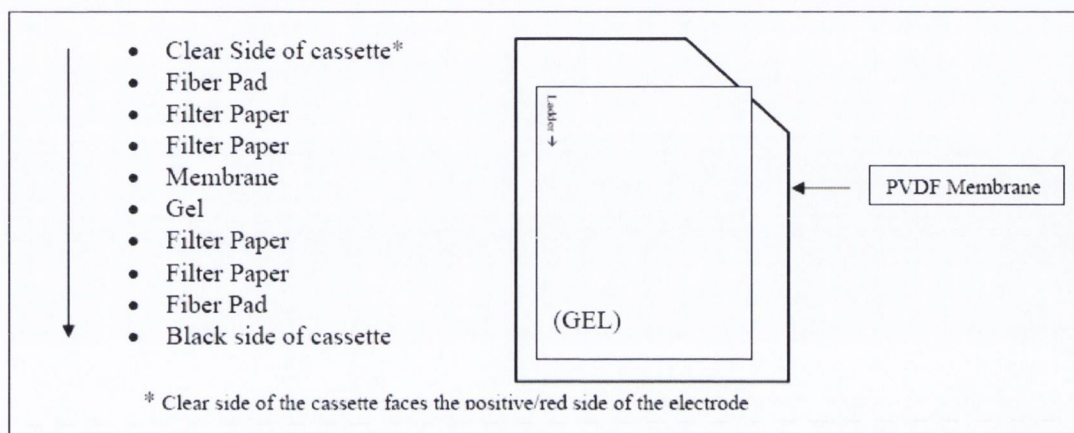
### **3.5.7 Protein transfer**

Following SDS-PAGE the gel was removed from the BioRad rig and the stacking gel was discarded. The gel was equilibrated by soaking in cold (4°C) transfer buffer for 20 minutes. Prior to protein transfer 4 sheet of filter paper and 2 fibre pads were pre-soaked in cold transfer buffer for 20mins. A PVDF membrane (Amersham Biosciences, Buckinghamshire, UK) was briefly activated in 100% methanol for 5-10 seconds and then washed in dH<sub>2</sub>O for 1 minute. The PVDF membrane was then soaked in cold transfer buffer for 10 minutes.

The gel, PVDF membrane, filter paper and fibre pads were arranged in a Mini Trans-Blot Cell (BioRad, Hertfordshire, UK) gel holder cassette (Figure 3.2). The cell was filled with cold transfer buffer and an ice cartridge and the transfer buffer was stirred using a magnetic stirrer bar during the protein transfer. The resolved proteins on the gel were transferred to the PVDF



membrane at 100V for 60mins. After transfer, noted by the complete transfer of the protein ladder from the gel to the PVDF membrane, the membrane was air-dried and stored at 4°C between 2 sheets of filter paper wrapped in cling film.



**Figure 3.2:** Gel and PVDF membrane assembly on Mini Trans-Blot Cell (BioRad, Hertfordshire, UK)

### 3.5.8 Immunodetection

After protein transfer, PVDF membranes (stored at 4°C) were briefly soaked in 100% methanol for 5-10 seconds and washed in dH<sub>2</sub>O for 5 minutes. Membranes were blocked by incubation at room temperature on a shaker for 60 minutes in a blocking solution which consisted of 5% skimmed milk (Marvel, Chivers Ltd., Dublin, Ireland) in PBS containing 0.05% Tween (PBST). Following blocking, membranes were washed in PBST 3 times (5 minutes per wash). Membranes were then transferred into 50ml tubes (Sarstedt, Wexford, Ireland) and incubated overnight at 4°C on a roller with anti-HIF-1 $\alpha$  primary antibody (Cell Signaling Technologies, MA, USA, 1:1000 dilution) in 10mls of

the relevant blocking solution. When blotting for actin, membranes were incubated with anti- $\beta$ -Actin (Clone AC-15, Sigma-Aldrich, Poole, UK, 1:5000 dilution) in 10 mls blocking solution for 60 minutes at room temperature.

Membranes were then washed in PBST 3 times (5 minutes per wash) and incubated as described in the previous paragraph in a 50ml tube for 60 minutes at room temperature in a secondary antibody: Polyclonal Goat Anti-Rabbit IgG HRP-linked Antibody (Cat #7074, Cell Signaling, MA, USA) diluted in 10 mls blocking solution at 1:1000 concentration. Next, membranes were washed again in PBST 3 times (5 minutes per wash) and then incubated with SuperSignal<sup>®</sup> West Pico Chemiluminescent Substrate (Pierce, IL, USA) for 5 minutes according to the manufacturer's instructions. After incubation, the membranes were placed between 2 clear acetate sheets and excess chemiluminescent substrate was removed. The membrane was exposed to x-ray film (AGFA, Middlesex, UK) for the appropriate time (HIF-1 $\alpha$ , 30min;  $\beta$ -actin, 1 second) using an AGFA CP1000 developer (AGFA, Middlesex, UK). After x-ray film development, membranes were air-dried and stored at 4°C between 2 pieces of filter paper wrapped in cling film.

### **3.5.9 Reprobing for $\beta$ -actin**

HIF-1 $\alpha$  blots were always performed prior to  $\beta$ -actin blots. The same PVDF membrane (stored at 4°C) was briefly reactivated in 100 % methanol. Next, the membranes were washed again in PBST 3 times (5mins per wash).



Immunodetection for  $\beta$ -actin was then performed as described previously (section 3.5.8). All Western blots for each cell line in this study were performed in triplicate.

### **3.6 Statistical analysis**

All experiments were performed in triplicate. Differences in surviving fraction and relative gene expression were compared using student t-tests or an Analysis of Variance (ANOVA). A p-value of  $<0.05$  was considered statistically significant. Data are presented as Mean  $\pm$  Standard Error of the Mean.





# **CHAPTER 4**

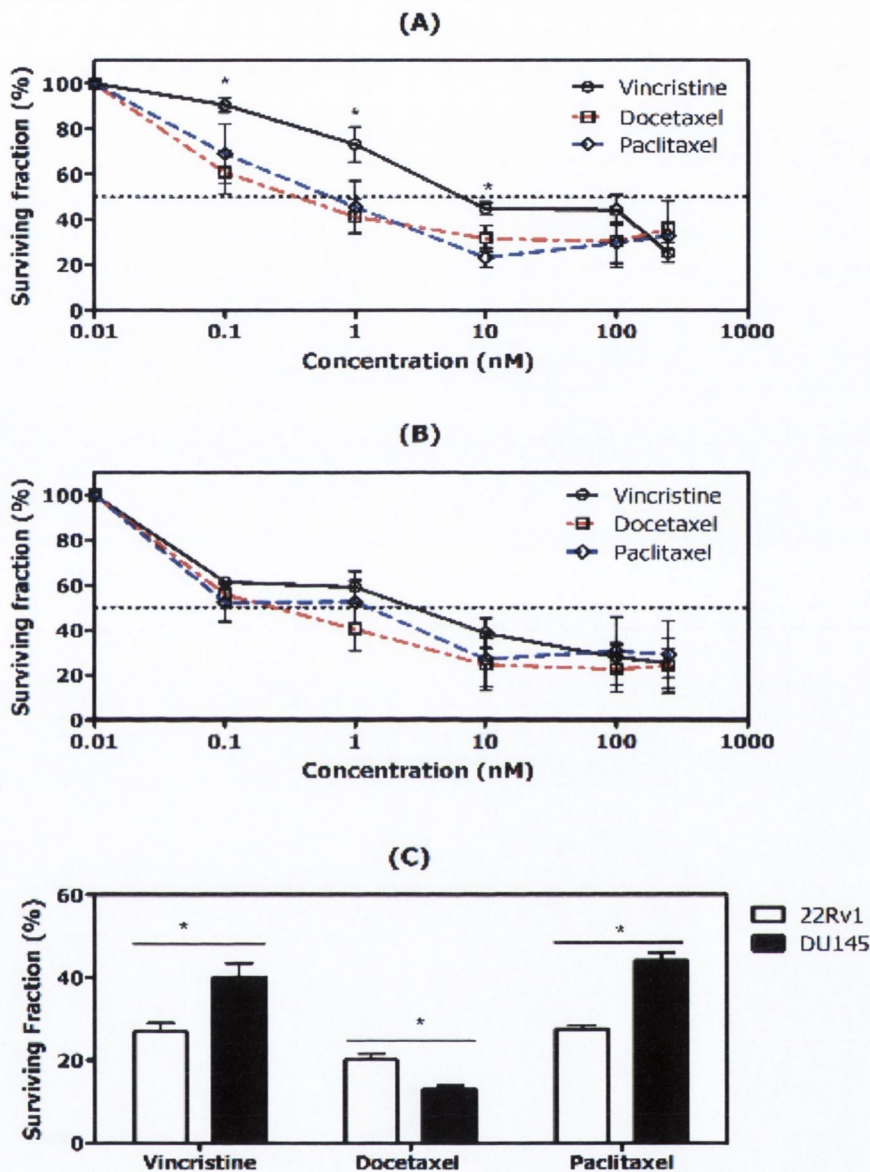
## **Results**

## **4.1 Evaluation of docetaxel as a hypoxic cell sensitizer**

### **4.1.1 Prostate cancer cells are most sensitive to docetaxel**

We initially generated dose response curves of both 22Rv1 and DU145 cells, treated with increasing concentrations of a panel of microtubule-targeting agents or MTAs (vincristine, docetaxel and paclitaxel). Survival was measured using an MTT assay (Figure 4.1 A, B). DU145 cells were as sensitive to all three MTA compounds at all concentrations tested ( $IC_{50} < 1\text{nM}$ ), whereas 22Rv1 cells were significantly more resistant to vincristine at 0.1 ( $p=0.03$ ), 1 ( $p=0.04$ ) and 10 ( $p=0.02$ ) nM concentrations. The cell lines showed similar sensitivity to all three drug tested except for vincristine, which was significantly more cytotoxic to DU145 than 22Rv1 cells at 0.1nM ( $p=0.0008$ ). We next chose a concentration of 1nM for more specific determination of the sensitivity of each cell line to each MTAs using clonogenic assays (Figure 4.1 C). 22Rv1 cells were more resistant to vincristine (1.4-fold;  $p=0.03$ ) and paclitaxel (1.38-fold,  $p=0.01$ ) than to docetaxel. Similarly, DU145 cells were most sensitive to docetaxel (ANOVA,  $p<0.0001$ ). Moreover, DU145 cells were significantly more resistant than 22Rv1 cells to vincristine ( $p=0.028$ ) and paclitaxel ( $p=0.001$ ) but significantly most sensitive to docetaxel ( $p=0.014$ ).





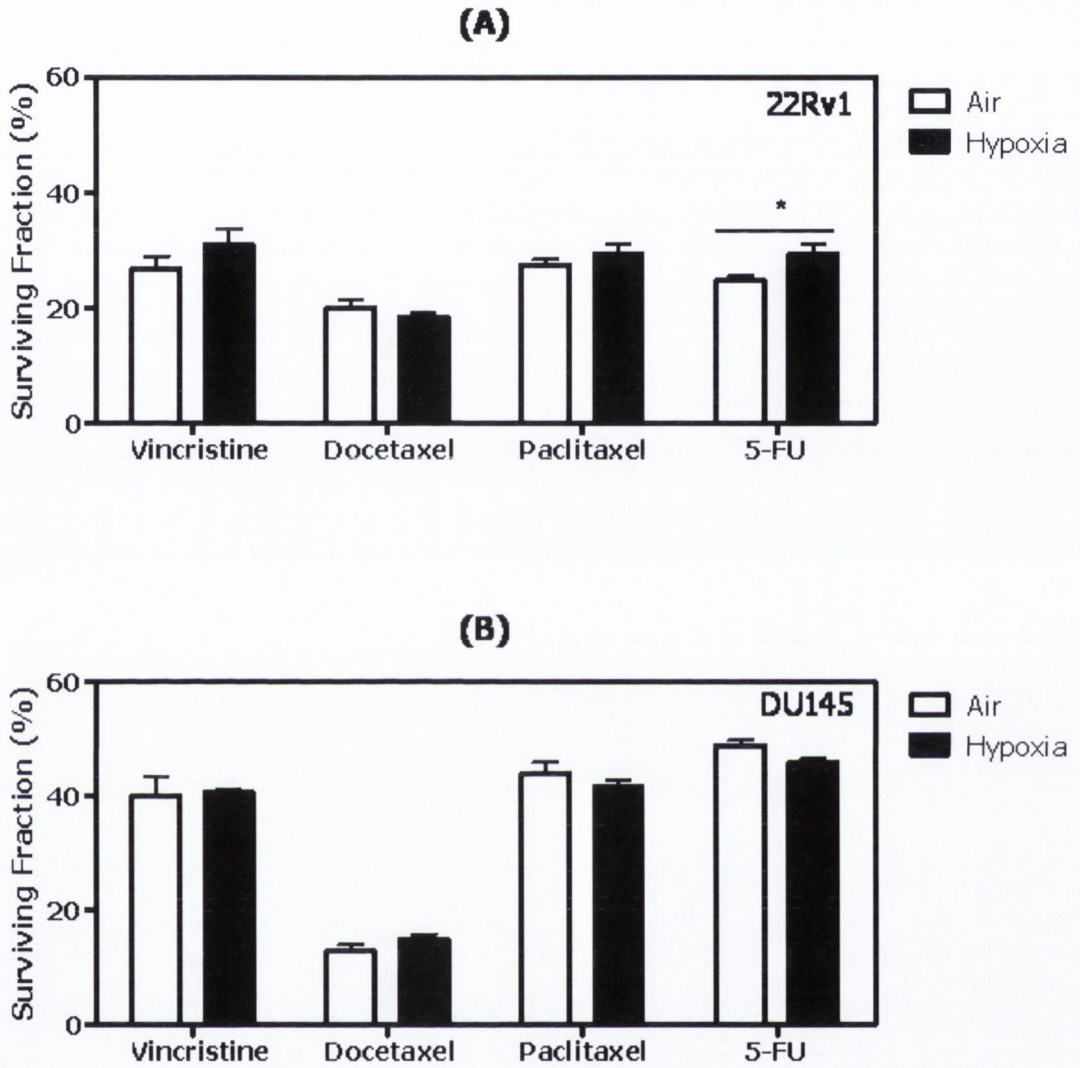
**Figure 4.1: Prostate cancer cells are most sensitive to docetaxel**

Surviving fraction of (A) 22Rv1 and (B) DU145 cells treated with increasing concentrations of vincristine, docetaxel and paclitaxel for 48 hours. (C) Comparison of surviving fractions of 22R1 and DU145 cells treated with 1nM vincristine, docetaxel or paclitaxel for 48 hours. N=3; Mean  $\pm$  SEM; \*,  $p < 0.05$ .

#### **4.1.2 MTAs are not associated with hypoxia-induced chemoresistance**

To determine whether MTAs preferentially maintained their cytotoxicity under hypoxic conditions, both cell lines were treated with each MTA at the same concentration of 1nM for 48 hrs under aerobic or hypoxic conditions and survival was measured using clonogenic assays. The response of cells to treatment with a non-MTA agent, 5-fluorouracil (5-FU, 100nM) was used as a control to determine whether the response was specific to taxanes (Figure 4.2 A, B). Hypoxia-induced chemoresistance was evident in 22Rv1 cells treated with 5-FU only ( $p=0.04$ ). Hypoxic 22Rv1 and DU145 cells remained most sensitive to docetaxel (ANOVA,  $p<0.0001$ ).





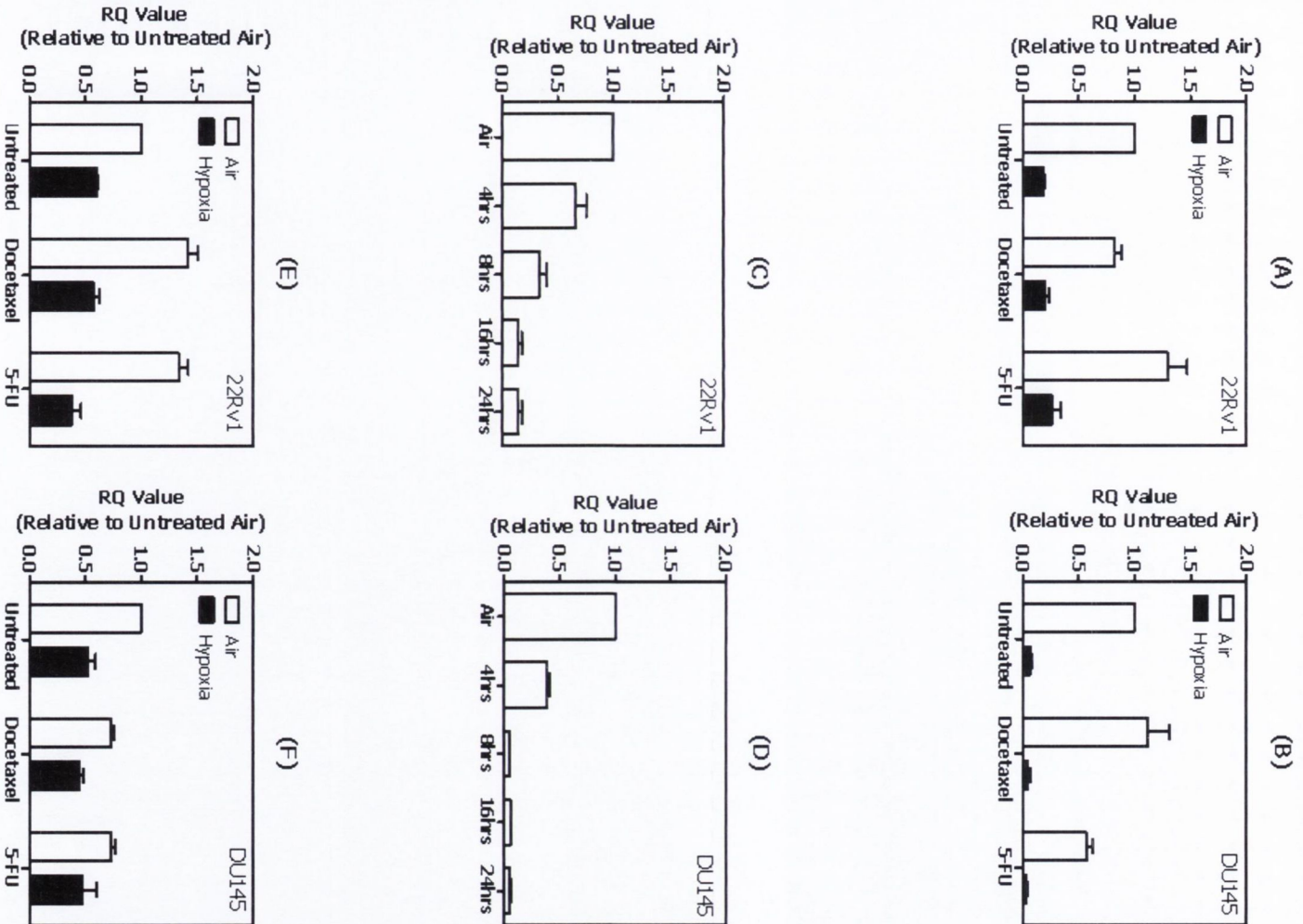
**Figure 4.2: Taxanes maintain their cytotoxic activity in hypoxia**

Surviving fraction of (A) 22Rv1 and (B) DU145 cells treated with 1nM of each vincristine, docetaxel, paclitaxel and 5-FU for 48 hours under aerobic or hypoxic conditions. N=3; Mean  $\pm$  SEM; \*,  $p < 0.05$ .

### **4.1.3 Down-regulation of HIF-1 $\alpha$ gene correlates with hypoxic chemosensitivity**

We next investigated whether loss of hypoxia-induced chemoresistance correlates with HIF-1 $\alpha$  gene expression levels. Basal levels HIF-1 $\alpha$  gene expression levels were 3.5-fold higher in DU145 cells than in 22Rv1 cells. 22Rv1 and DU145 cells were treated with either docetaxel (1nM) or 5-FU (100nM) for 48 hrs under both conditions prior to total mRNA extraction. The response of aerobic untreated cells was used as a control (Figure 4.3 A, B). HIF-1 $\alpha$  gene expression levels were down-regulated in response to 5-FU treatment in aerobic DU145 cells. HIF-1 $\alpha$  gene expression levels were down-regulated 5-fold and 16-fold; respectively in 22Rv1 and DU145 hypoxic untreated samples. Chemotherapeutic treatment did not further modulate HIF-1 $\alpha$  expression in either cell line. Time course experiments confirmed down-regulation of the HIF-1 $\alpha$  gene within 4 hours of hypoxic exposure in both cell lines (Figure 4.3 C, D). At this time point, gene expression levels were not modified in response to drug treatment (Figure 4.3 E, F).



Figure 4.3: Relative quantification of HIF-1 $\alpha$  mRNA levels

**Figure 4.3: Relative quantification of HIF-1 $\alpha$  mRNA levels**

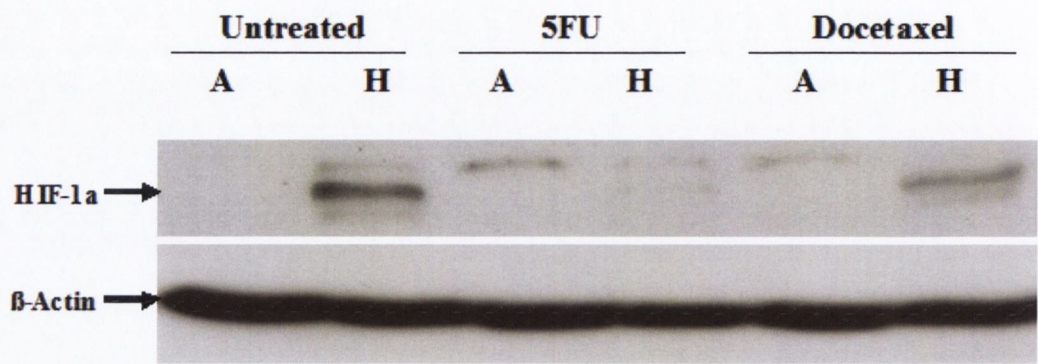
HIF-1 $\alpha$  gene expression levels were initially measured in (A) 22Rv1 and (B) DU145 cells treated with 1nM of either docetaxel or 5-FU for 48 hours under aerobic and hypoxic conditions, relative to untreated aerobic controls. Next, the time course of HIF-1 $\alpha$  gene expression under hypoxic conditions for (C) 22Rv1 and (D) DU145 cells is presented. Finally, HIF-1 $\alpha$  gene expression levels were measured in (E) 22Rv1 and (F) DU145 cells treated with 1nM of either docetaxel or 100 nM of 5-FU for 4 hours under aerobic and hypoxic conditions, relative to untreated aerobic controls. N=3; Mean  $\pm$  SEM; \*,  $p < 0.05$ .



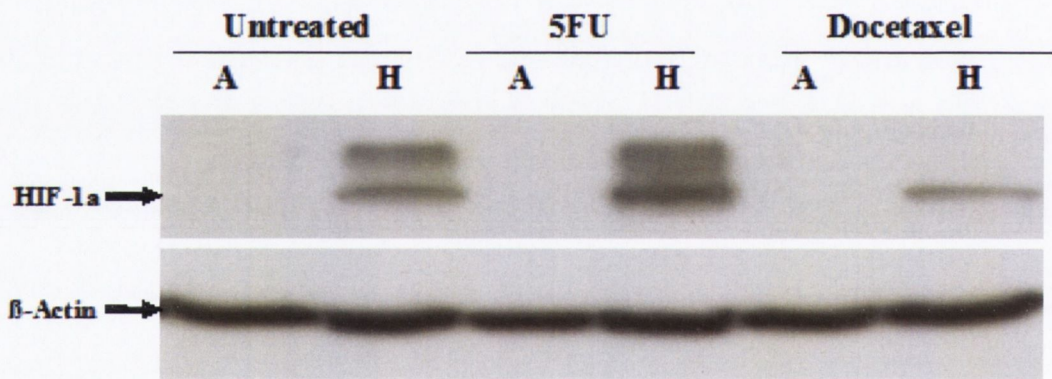
#### **4.1.4 Docetaxel appears to down-regulate HIF-1 $\alpha$ protein**

HIF-1 $\alpha$  protein expression is evident in both 22Rv1 and DU145 cell lines in untreated samples after 6 hours of hypoxic exposure. HIF-1 $\alpha$  protein expression is not evident in any aerobic untreated samples. 22Rv1 and DU145 cells were treated with either docetaxel (1nM) or 5-FU (100nM) for 6 hours under both conditions prior to protein extraction. As expected, HIF-1 $\alpha$  protein expression was not evident in aerobic treated samples of either cell lines. Treatment with docetaxel appeared to down-regulate HIF-1 $\alpha$  protein expression in both cell lines under hypoxic conditions (Figure 4.4), in particular in the DU145 cell line. In addition the non-taxane control 5-FU appeared to down-regulate HIF-1 $\alpha$  protein expression in 22Rv1 cells while up-regulating HIF-1 $\alpha$  protein DU145 cells (Figure 4.4).

A) 22Rv1



B) DU145



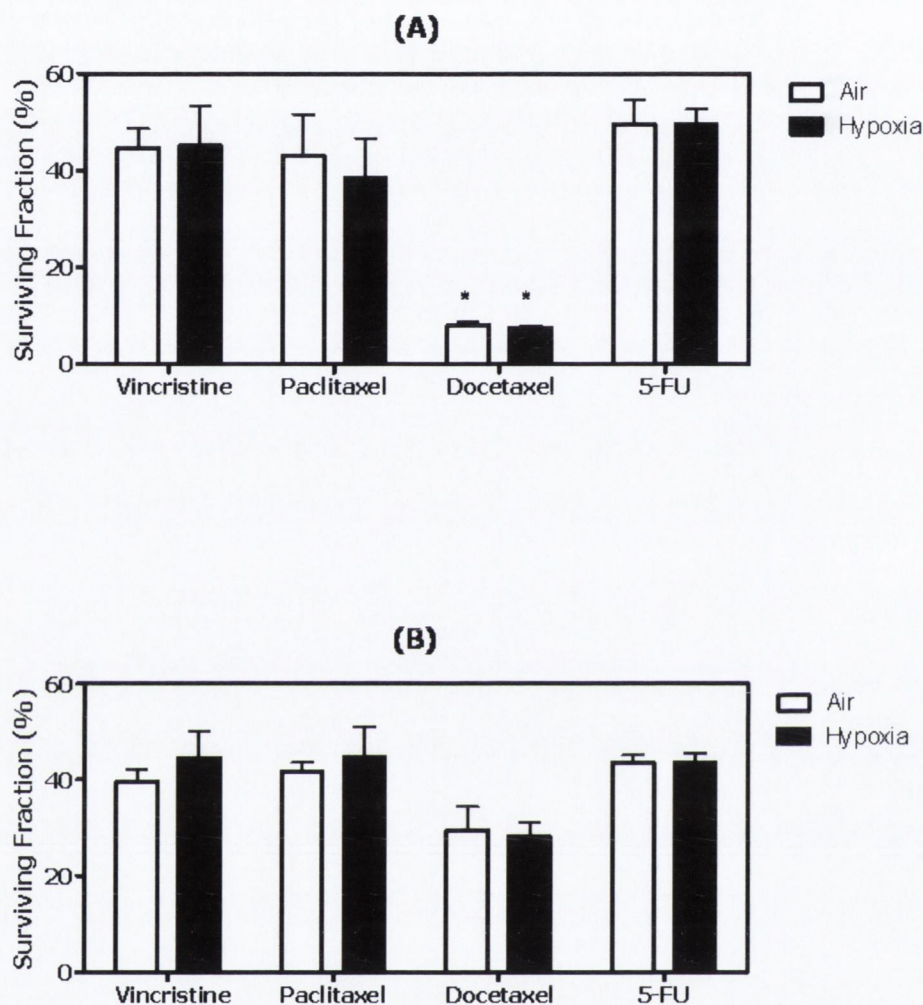
**Figure 4.4: Docetaxel appears to down-regulate HIF-1α protein**

Representative HIF-1 alpha and  $\beta$ -actin immunoblots of (A) 22Rv1 and (B) DU145 cells both treated with 1nM of either docetaxel or 100 nM of 5-FU for 6 hours under aerobic and hypoxic conditions, relative to untreated aerobic and hypoxic controls.



#### **4.1.5 Docetaxel-resistance is not associated with hypoxia-induced chemoresistance**

We next investigated whether transition to docetaxel resistance is also associated with increased chemoresistance under hypoxic conditions. Docetaxel sensitive (PC3) and docetaxel resistant (PC3-D12) cells were treated with MTAs and 5-FU under aerobic and hypoxic conditions for 48 hours and clonogenic survival measured (Figure 4.5). PC3 cells were significantly more sensitive to docetaxel than vincristine, paclitaxel and 5-FU under both aerobic and hypoxic conditions (ANOVA,  $p < 0.04$ ). Hypoxic exposure was not associated with increased docetaxel resistance. PC3-D12 were significantly more resistant to docetaxel than PC3 cells under both aerobic ( $p = 0.01$ ) and hypoxic ( $p = 0.001$ ) conditions, but remained significantly more sensitive to docetaxel than any other drugs tested under aerobic conditions only (ANOVA  $p = 0.04$ ).



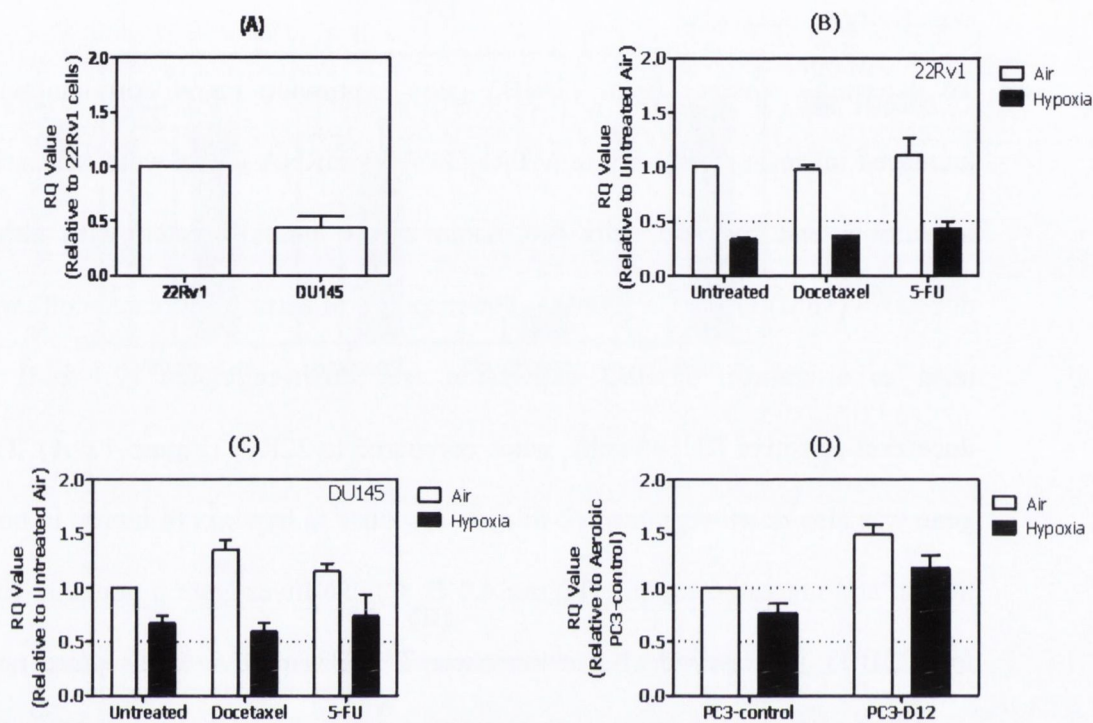
**Figure 4.5: Docetaxel resistance is not associated with hypoxia-induced chemoresistance**

Clonogenic survival of age-matched docetaxel sensitive (PC3) and isogenic docetaxel-resistant (PC3-D12) cells treated with 1nM vincristine, paclitaxel, docetaxel and 100nM 5-FU under aerobic or hypoxic conditions for 48 hours. N=3; Mean  $\pm$  SEM; \*,  $p < 0.05$ .



#### **4.1.6 Down-regulation of the TUBB3 gene correlates with intrinsic sensitivity to docetaxel**

To determine whether basal TUBB3 gene expression could correlate with increased intrinsic sensitivity to MTAs, TUBB3 mRNA levels were measured in aerobic and hypoxic cells (48 hours and 4 hours) treated with either docetaxel (1nM) or 5-FU (100nM). The response of aerobic untreated cells was used as a control. TUBB3 expression was down-regulated (2.1-fold) in docetaxel-sensitive DU145 cells, when compared to 22Rv1 (Figure 4.6 A). The gene was also down-regulated (2-fold) in response to hypoxia (4 hours) in both treated and untreated sample (Figure 4.6 B, C). To investigate a potential role for TUBB3 in acquired chemoresistance, TUBB3 mRNA levels were next measured in docetaxel resistant (PC3-D12) and docetaxel-sensitive (PC3) cells. TUBB3 expression levels were not elevated in docetaxel-resistant cells, when compared to the matched docetaxel sensitive control (Figure 4.6 D).



**Figure 4.6: Relative quantification of TUBB3 mRNA levels**

Basal TUBB3 gene expression levels were (A) initially measured in DU145 cells, relative to that of 22Rv1 cells; next gene expression was determined in (B) 22Rv1 and (C) DU145 cells treated with 1nM of either docetaxel or 5-FU for 4 hours under aerobic and hypoxic conditions, relative to untreated aerobic controls and finally (D) expression as tested in aged-matched PC3 and isogenic docetaxel-resistant PC3-D12 cells. N=3; Mean  $\pm$  SEM; \*,  $p < 0.05$ .



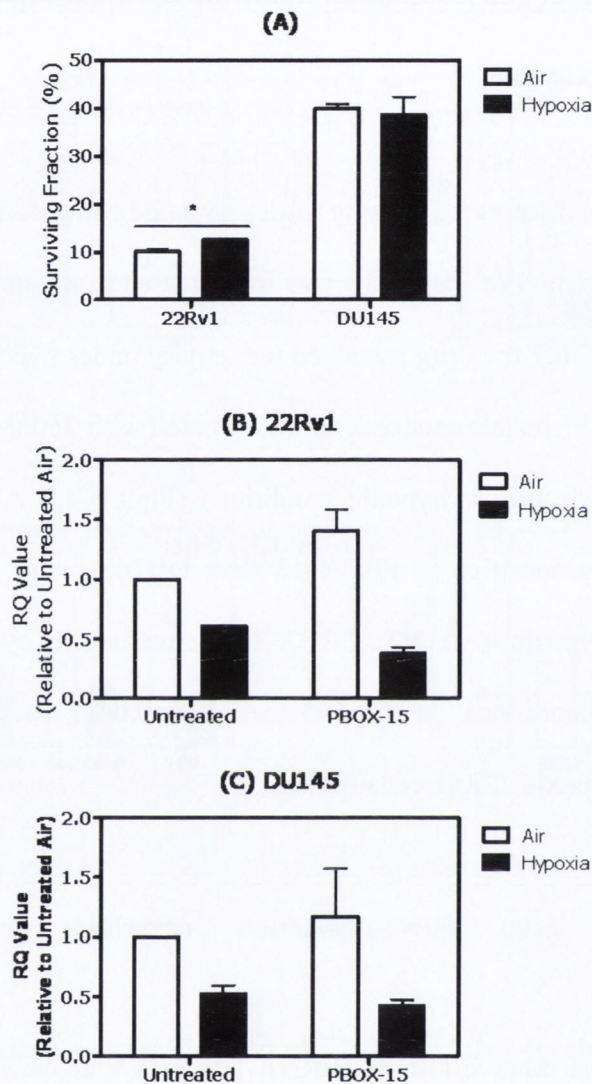
## **4.2     Evaluation of PBOX-15 as an MTA for the management of hypoxic prostate tumours**

### **4.2.1 PBOX-15 maintains its activity under hypoxic conditions**

The cytotoxic potential of PBOX-15 was investigated in prostate cancer cells. We evaluated whether the drug sustained its activity under hypoxic conditions. 22Rv1 and DU145 prostate cancer cells were treated with 250nM PBOX-15 for 48 hours under aerobic or hypoxic conditions (Figure 4.7 A). 22Rv1 were significantly more sensitive to PBOX-15 than DU145 under both condition (Air,  $p=0.001$ ; Hypoxia,  $p=0.002$ ). PBOX-15 maintained its cytotoxic activity under hypoxic conditions in DU145 cells ( $p=0.041$ ), but had reduced cytotoxicity in hypoxic 22Rv1 cells ( $p=0.035$ ).

### **4.2.2    HIF-1 $\alpha$    gene    down-regulation    correlates    with    hypoxic chemosensitivity**

We next measured relative HIF-1 $\alpha$  mRNA levels in untreated and PBOX-15 (250nM) treated aerobic and hypoxic cells to examine whether the difference in hypoxic sensitivity could be correlated with reduced HIF-1 $\alpha$  gene expression. Loss of expression was evident within 4 hours of hypoxic exposure in both untreated and PBOX-15 treated cell lines (Figure 4.7 B, C).



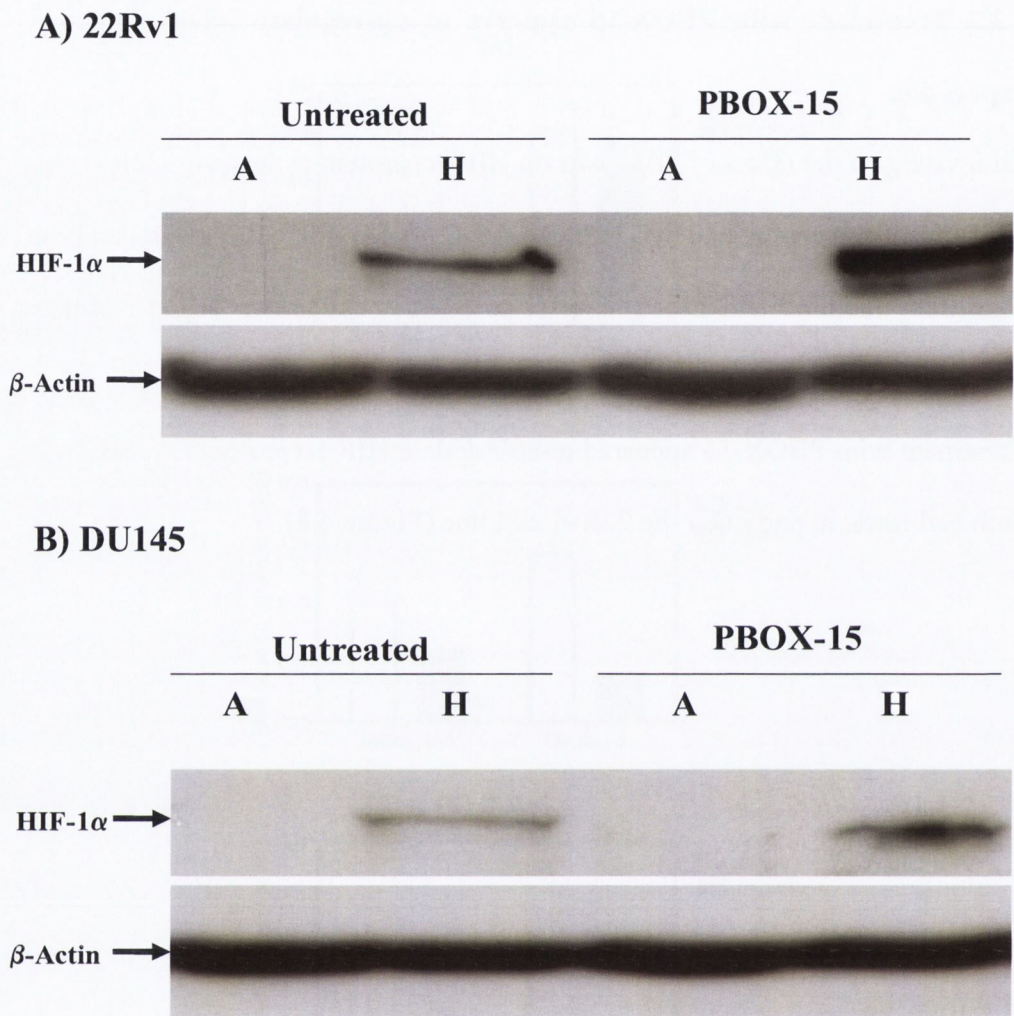
**Figure 4.7: PBOX-15 maintains its activity under hypoxic conditions**

(A) Surviving fraction of 22Rv1 and DU145 prostate cancer cells treated with PBOX-15 (250nM) for 48 hours under aerobic or hypoxic conditions. The relative quantification (RQ Value) of HIF-1 $\alpha$  mRNA levels in untreated and PBOX-15 treated aerobic and hypoxic 22Rv1 cells (B) and DU145 cells (C) is presented. N=3; Mean  $\pm$  SEM; \*,  $p < 0.05$



#### **4.2.3 Treatment with PBOX-15 appears to up-regulate HIF-1 $\alpha$ protein expression**

To investigate the effect of PBOX-15 on HIF-1 $\alpha$  protein expression, 22Rv1 and DU145 cells were treated with PBOX-15 (250nM) for 6 hours under both conditions (aerobic and hypoxic) prior to protein extraction. HIF-1 $\alpha$  protein expression was not evident in aerobic treated samples of either cell lines. Treatment with PBOX-15 appeared to up-regulate HIF-1 $\alpha$  protein expression in both cell lines, in particular the 22Rv1 cell line (Figure 4.8).



**Figure 4.8: PBOX-15 up-regulates HIF-1 $\alpha$  protein expression.**

Representative HIF-1 alpha and  $\beta$ -actin immunoblots of (A) 22Rv1 cells and (B) DU145 cells, treated with 250nM of PBOX-15 for 6 hours under aerobic and hypoxic conditions, relative to untreated aerobic and hypoxic controls.



#### **4.2.4 PBOX-15 radio-sensitises hypoxic prostate cancer cells**

We next investigated whether PBOX-15 could radio-sensitise hypoxic prostate cancer cells. 22Rv1 cells were treated with the drug for 24 hours prior to the delivery of a 2Gy single dose fraction. Determination of the surviving fraction was assessed using clonogenic assays. While hypoxia-induced radio-resistance was evident in untreated cells, with an increase in SF2 from  $53.82 \pm 2.493\%$  in air to  $77.20 \pm 1.096\%$  in hypoxia ( $p=0.002$ ), pre-treatment with PBOX-15 prevented onset of resistance and significantly reduced the hypoxic SF2 to  $38.59 \pm 2.598\%$  ( $p=0.01$ ) (Figure 4.9 A). The combined effect of PBOX-15 treatment and radiation was additive, with no significant difference in the cytotoxic effect of PBOX-15 in unirradiated and irradiated cells (Figure 4.9 B).

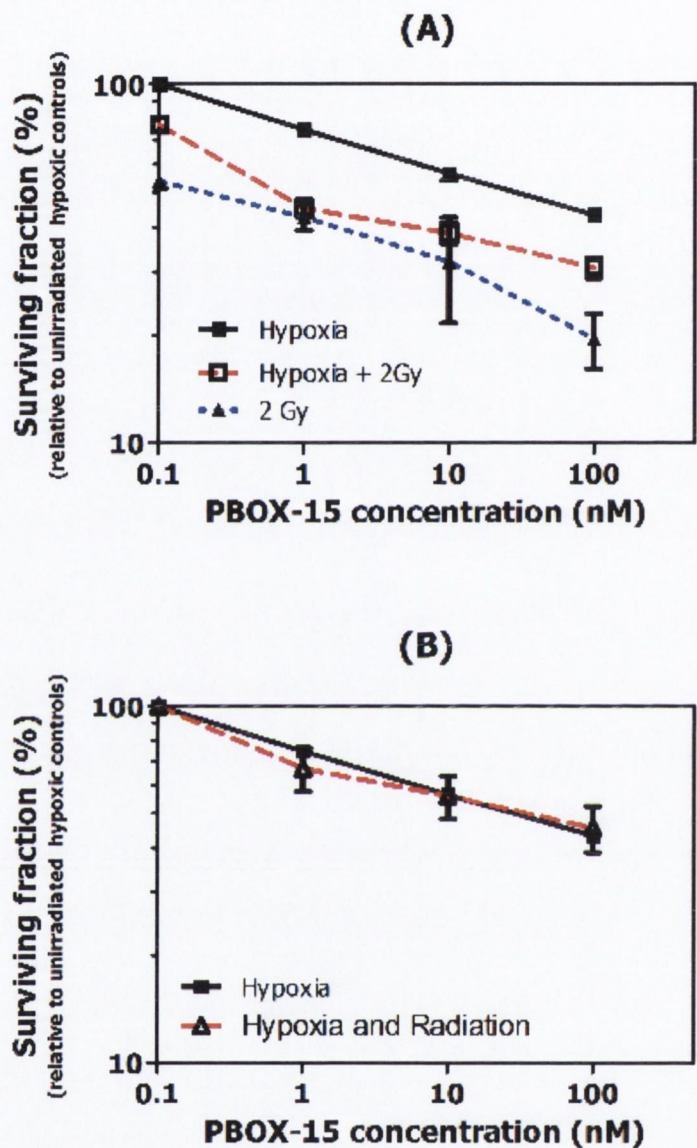


Figure 4.9: PBOX-15 radio-sensitises hypoxic prostate cancer cells.

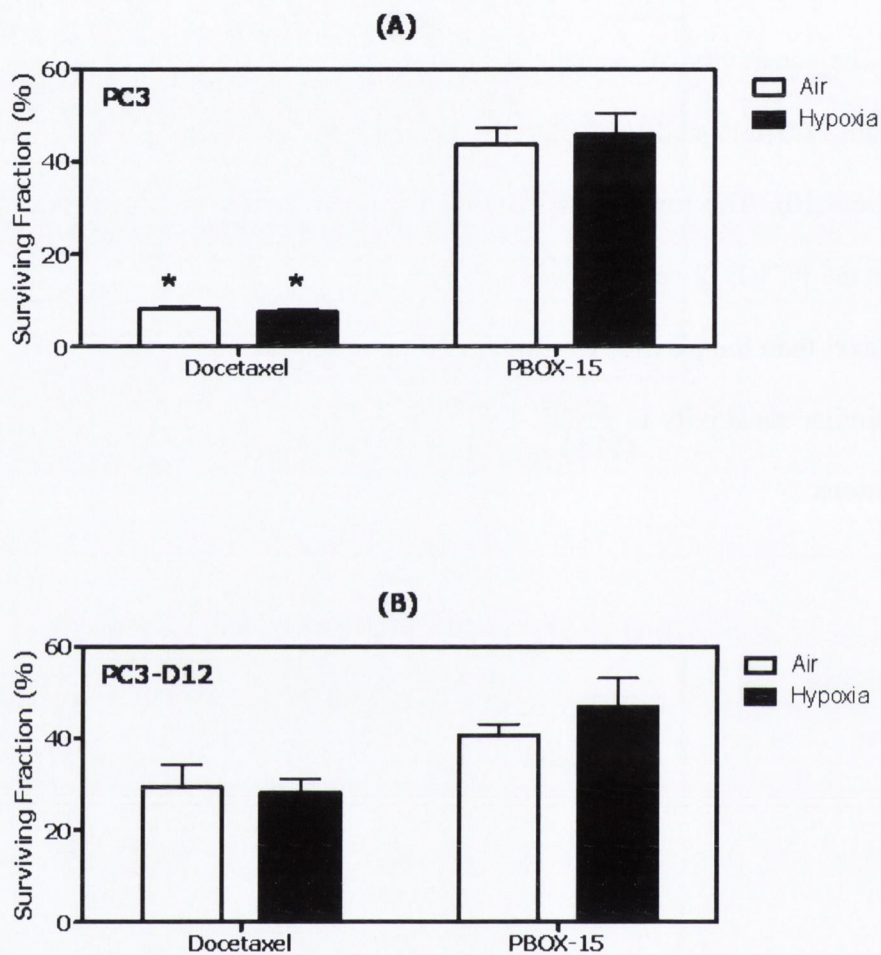
(A) Clonogenic survival of hypoxia 22Rv1 cells treated with PBOX-15, radiation (2Gy) or combination of hypoxia and irradiation.

(B) Clonogenic survival of PBOX-15 treated unirradiated and irradiated (2Gy) hypoxic 22Rv1 cells. N=3; Mean  $\pm$  SEM; \*,  $p < 0.05$ .



#### **4.2.5 PBOX-15 does not overcome docetaxel-resistance**

We finally determined the toxicity of PBOX-15 on a docetaxel resistant cell line. The sensitivity of isogenic docetaxel-sensitive PC3 cells and docetaxel-resistant PC3-D12 cells to PBOX-15 was determined using clonogenic assays (Figure 4.10). The toxicity of PBOX-15 was compared to that of docetaxel. While the PC3-D12 cell line was significantly more resistant to treatment with docetaxel than the parent PC3 line ( $p=0.02$ ), both PC3 and PC3-D12 cell lines had similar sensitivity to PBOX-15 (100nM) under both aerobic and hypoxic conditions.



**Figure 4.10: PBOX-15 does not overcome docetaxel resistance**

Surviving fraction of isogenic docetaxel-sensitive PC3 and docetaxel-resistant PC3-D12 cells to docetaxel (1nM) or PBOX-15 (100nM) treatment (48 hours) under aerobic or hypoxic conditions. N=3; Mean  $\pm$  SEM; \*,  $p < 0.05$ .



# **CHAPTER 5**

## **Discussion**

## 5 **Discussion**

From the figures published by the National Cancer Registry of Ireland, the number of cases of prostate cancer in Ireland is expected to reach 6,330 by 2020, which is a 275% increase in incidence between 2000 and 2020 (NCRI 2006). This rise in incidence can attributed to the widespread use of PSA testing in the male population. This trend has led to a stage migration to more localised and curable cancers. However, with this trend comes the over-treatment and over-diagnosis of what many would describe as biologically insignificant disease. The challenge we face is to identify those individuals with more aggressive prostate cancers who will benefit from immediate treatment. By introducing the concept of risk stratification (Thompson 2006) and the use of prostate cancer nomograms (Ross 2001), easier identification of these patients will enable prioritisation of their treatment. One such group of patients are those with hypoxic prostate tumours.

Hypoxia is being increasingly recognised as playing a major role in influencing tumour proliferation and malignant progression. It is associated with poor prognosis mainly due the associated resistance to chemo-radiotherapy regimes. Many of these hypoxia regulated genes are regulated by HIF-1 $\alpha$ , a transcription factor expressed in over 70% of all tumours and their metastases (Zhong, De Marzo et al. 1999). Taxanes have previously been shown to inhibit HIF-1 $\alpha$  accumulation and activity in ovarian cancer cells by disrupting the



microtubular system, thus limiting the hypoxic response (Escuin, Kline et al. 2005). We proposed to identify whether hypoxic prostate tumours may specifically benefit from treatment with microtubule-targeting agents.

### **5.1 Docetaxel sensitivity**

Microtubule-targeting agents (MTAs) are amongst the most commonly employed and most effective chemotherapeutic agents used today in a variety of cancers. Docetaxel is the current standard of care for metastatic hormone refractory prostate cancer. Our first objective was to assess the sensitivity of a primary as well as metastatic prostate cancer line to a variety of MTAs. We confirmed that both cell lines were most sensitive to treatment with docetaxel (Figure 4.1, ANOVA  $p < 0.0001$ ).

Our second objective was to assess whether a selection of MTAs preserve their tumour cell-killing activity under conditions of hypoxia. We concluded that all of the MTAs used (docetaxel, paclitaxel and vincristine) maintained their tumour cell-killing activity in hypoxia in both primary and metastatic prostate cancer cell lines and were not associated with hypoxia induced chemo-resistance (Figure 4.2). We also used a non MTA control 5-FU (Fluorouracil) to determine if the results were specific to MTAs. We found that 5-FU demonstrated reduced cytotoxic activity in hypoxic conditions in the primary prostate cell line 22Rv1 (Figure 4.2,  $p = 0.04$ ). Overall, both 22Rv1 and DU145 prostate cancer cell lines were most sensitive to docetaxel in hypoxic

conditions (Figure 4.2, ANOVA  $p < 0.0001$ ). In particular, the metastatic cell line DU145 was most sensitive to treatment with docetaxel. Previous studies on rat prostate adenocarcinoma cell lines (Dunning R3327-AT1 cell lines) also concluded that the cytotoxicity of docetaxel was not affected by  $O_2$  deficiency despite hypoxia exposure increasing caspase 3-activity and leading to a marked increase in necrosis in the cell line (Thews, Gassner et al. 2007). The preservation of the cytotoxic effect on MTAs in hypoxia may indicate that patients with aggressive hypoxic prostate tumours may benefit from treatment with taxanes such as docetaxel as a neoadjuvant form of treatment.

## **5.2 HIF-1 $\alpha$ gene expression**

To further investigate the potential benefit of taxanes and their effect on hypoxic tumours, we studied the effect on HIF-1 $\alpha$  gene expression after treatment with docetaxel. Down-regulation of HIF-1 $\alpha$  expression, thus limiting the hypoxic response as a result of taxane treatment, may help reduce prostate tumour proliferation and malignant progression. HIF-1 $\alpha$  gene (HIF-1 $\alpha$  mRNA) expression in serum samples from patients with prostate cancer has recently been shown to be up-regulated in the early stages of localised prostate cancer (Pipinikas, Carter et al. 2008). The same study also found that HIF-1 $\alpha$  gene expression levels in patients with metastatic disease to be similar to those with no evidence of disease and considerably lower than levels in localised prostate cancer. The authors concluded that the HIF-1 $\alpha$  gene may be used to distinguish between localised and metastatic disease. In our study, we found the HIF-1 $\alpha$



gene to be expressed in both aerobic cell lines. In fact we found the basal levels of HIF-1 $\alpha$  gene to be 3.5 fold times higher in the metastatic prostate cell line DU145 when compared to primary 22Rv1 cells (Figure 4.3). While previous studies have shown the HIF-1 $\alpha$  gene to be amplified in the PC3 metastatic prostate cell line and not prostate tumour specimens (Saramaki, Savinainen et al. 2001) further studies are necessary to evaluate HIF-1 $\alpha$  gene expression in prostate cancer.

After comparing HIF-1 $\alpha$  gene expression in both cell lines in aerobic conditions we then investigated the effect of hypoxic exposure. Our initial time point of 48 hours of hypoxic exposure showed significant down-regulation of HIF-1 $\alpha$  gene levels in both cell lines, when compared with normoxic controls (Figure 4.3). A subsequent time course experiment over a 24 hour time frame showed the continued degradation of HIF-1 $\alpha$  gene after 4 hours of hypoxic exposure. This rapid degradation may indicate the importance of the HIF-1 $\alpha$  gene in the initial phases of the hypoxic response. HIF-1 $\alpha$  gene expression is necessary in the transcriptional activation of downstream target genes such as vascular endothelial growth factor (VEGF), which plays a major role in the angiogenesis of prostate tumours. Alqawi *et al.* have previously concluded that hypoxia (1% O<sub>2</sub> at 6 and 18 hour time points) had no effect on HIF-1 $\alpha$  mRNA levels in metastatic DU145 prostate cell lines using Northern Blot analysis (Alqawi, Moghaddas et al. 2006). Here, we report that hypoxia (at 0.5% O<sub>2</sub>) decreased HIF-1 $\alpha$  mRNA levels using TaqMan® gene expression assays in

DU145 cells as well as in 22Rv1 cells. This decrease in HIF-1 $\alpha$  mRNA levels after hypoxic exposure using TaqMan® gene expression assays needs to be further investigated.

From our HIF-1 $\alpha$  mRNA time course experiments, we selected a time point of 4 hours of hypoxic exposure to investigate the possible down-regulation of HIF-1 $\alpha$  mRNA in primary and metastatic cell lines as result of treatment with docetaxel and a non-taxane control (5-FU). At this time point, gene expression levels were not modified in response to drug treatment (Figure 4.3). Previous studies investigating the effect of geldanamycin (a benzoquinone ansamycin antibiotic) on HIF-1 $\alpha$  mRNA expression in prostate cancer cells found HIF-1 $\alpha$  mRNA levels to be unchanged after 6 hours of treatment in hypoxic exposure (Alqawi, Moghaddas et al. 2006). Escuin *et al.* also found that MTAs including docetaxel did not effect HIF-1 $\alpha$  mRNA levels in variety of cancer cell lines in either normoxic or hypoxic conditions (Escuin, Kline et al. 2005). We also treated both primary and metastatic cell lines with docetaxel and 5-FU for a longer time interval of 48 hours under both aerobic and hypoxic conditions. While HIF-1 $\alpha$  gene expression levels were down-regulated in response to treatment with 5-FU in aerobic metastatic DU145 cells, MTA treatment did not modulate HIF-1 $\alpha$  gene expression levels in either cell line in hypoxia (Figure 4.3). We can conclude that down-regulation of the HIF-1 $\alpha$  gene in both cell lines correlates with their hypoxic chemosensitivity. Taxanes



such as docetaxel continue to maintain their cytotoxic activity in hypoxia and do not affect HIF-1 $\alpha$  gene expression.

### **5.3 HIF-1 $\alpha$ protein expression**

Previous studies have concluded that MTAs, including docetaxel, inhibit HIF-1 $\alpha$  protein accumulation and activity in ovarian cancer cell lines (Escuin, Kline et al. 2005). We sought to investigate the effect of docetaxel on HIF-1 $\alpha$  protein expression in both primary and metastatic prostate cancer cell lines. Stabilisation of HIF-1 $\alpha$  protein allows activation of over 60 genes associated with tumour progression including VEGF (Semenza 2003). Reduced HIF-1 $\alpha$  protein expression in prostate cancer due to treatment with taxanes would reduce the hypoxic response. This may disrupt the production of mediators of angiogenesis such as VEGF and subsequent tumour survival.

We first confirmed the expression of HIF-1 $\alpha$  protein in hypoxic cell lines only (Figure 4.4). Increased levels of HIF-1 $\alpha$  protein are associated with hypoxic exposure which we had previously seen to reduce HIF-1 $\alpha$  mRNA levels. This indicates that hypoxia does not increase HIF-1 $\alpha$  protein production in prostate cancer cells but stabilises it. HIF-1 $\alpha$  protein is constantly being produced in normoxic conditions but in the presence of oxygen is continuously degraded by the ubiquitin-proteasome system. Hypoxia prevents this ubiquitination and the subsequent proteasomal degradation of HIF-1 $\alpha$ , thus

stabilising it and allowing promotion transcription of its target genes which allow the cell to survive better in hypoxia.

Previous studies have looked at the reduced expression of HIF-1 $\alpha$  protein in cell lines under longer hypoxic incubation times (Mottet, Dumont et al. 2003). Foley *et al.* found HIF-1 $\alpha$  protein expression in 22Rv1 and DU145 cell lines was no longer detectable after 48 hours of hypoxic exposure. They concluded that maximum HIF-1 $\alpha$  protein expression appeared to be between 4 and 8 hours of exposure (Foley, Marignol et al. 2009). We selected a 6 hour time point as the optimal time to investigate if taxane treatment reduced HIF-1 $\alpha$  protein expression in both prostate cell lines. We concluded that treatment with docetaxel appeared to reduce the expression of HIF-1 $\alpha$  protein in metastatic DU145 cells to a greater extent than primary 22Rv1 cells (Figure 4.4). This may be due to the mitotic index (cells in G<sub>2</sub>-M) of hormone refractory prostate cancer cell lines such as DU145 which has been shown to be less than 2% (Escuin, Kline et al. 2005). Docetaxel has indeed been proposed to exert a greater effect in metastases of prostate cancer through inhibition of HIF-1 $\alpha$ , rather than antimitotic effects in primary tumour systems (Escuin, Kline et al. 2005).

#### **5.4 Docetaxel resistance**

Taxane resistance in hormone refractory prostate cancer is increasingly being observed. Median duration of response to treatment with docetaxel is



limited to between 6 to 9 months (Petrylak, Tangen et al. 2004; Tannock, de Wit et al. 2004). Although various authors have described the mechanisms of taxane resistance pathways, there is little literature regarding clinical studies of taxane resistance in prostate cancer (Mathew and Dipaola 2007). We sought to investigate whether the transition to docetaxel resistance was associated with increased chemo-resistance in hypoxia. We used a docetaxel resistant metastatic cell line (PC3-12) and a docetaxel sensitive age matched control (PC3). We confirmed the PC3-D12 cell line to be significantly more resistant to treatment with docetaxel than the control PC3 line (Figure 4.5) under both aerobic ( $p=0.01$ ) and hypoxic ( $p=0.001$ ) conditions. This resistance was specific to docetaxel as the PC3-D12 line was shown to sensitive to the other taxane paclitaxel. Hypoxia was not associated with increased docetaxel resistance, with the PC3-D12 cell line maintaining its docetaxel resistance in hypoxia. Age matched control PC3 cells were significantly more sensitive to docetaxel than other MTAs (vincristine and paclitaxel) as well as the non-taxane control 5-FU in both aerobic and hypoxic conditions. Novel use of docetaxel sensitizers may be of benefit in enhancing the efficacy of docetaxel, particularly in taxane resistant cell lines. Recently, the use of garlic-derived S-allylmercaptocysteine, in combination with docetaxel, was shown to be 53% more potent than docetaxel alone in metastatic PC3 and DU145 cells as well as primary 22Rv1 cells (Howard, Lee et al. 2008).

### **5.5 TUBB3 expression and its relevance in treatment resistance**

For further progress to be made in the treatment of taxane refractory disease, a greater understanding of the mechanisms of taxane resistance is required. Analogues of taxanes, adapted to overcome the various resistance mechanisms, may be of future benefit. One such mechanism of interest is the role of TUBB3.

Over expression of the class III beta-tubulin (TUBB3) gene has been found in breast (Paradiso, Mangia et al. 2005), lung (Seve, Isaac et al. 2005) and pancreatic cancer (Lee, Cao et al. 2007). Down-regulation of TUBB3 expression in lung cancer cells has been found to increase their sensitivity to treatment with paclitaxel. More recently, Raspaglio *et al.* proposed that hypoxia was a possible inducer of TUBB3 expression in ovarian and cervical cancer cell lines with hypoxia exposure raising expression of the TUBB3 gene more than fivefold after 72 hours of exposure (Raspaglio, Filippetti et al. 2008). We firstly studied basal TUBB3 expression in both primary 22Rv1 cells and metastatic DU145 cells. TUBB3 mRNA levels were measured in aerobic and hypoxic (4 hours exposure) cells treated with either docetaxel or 5-FU. We found that TUBB3 expression was 2 fold lower in the aerobic untreated DU145 cell line when compared to the aerobic primary untreated 22Rv1 cells (Figure 4.6). This reduced expression in DU145 cells may contribute to our observation of a greater sensitivity to treatment with docetaxel in metastatic DU145 cells in our clonogenic survival assays. We subsequently measured TUBB3 mRNA levels



in cells exposed to 4 hours of hypoxia and found that hypoxic exposure down-regulated the gene in both untreated and treated samples and to a greater extent (2 fold) in the primary 22Rv1 cell line (Figure 4.6). This down-regulation of TUBB3 gene expression in primary prostate cell lines correlates with the continued sensitivity of hypoxic prostate cells to docetaxel. These findings may indicate a possible neoadjuvant role for docetaxel in patients with aggressive hypoxic primary prostate tumours. Down-regulation of TUBB3 in hypoxic tumours may provide a greater sensitivity to docetaxel treatment as an initial form of treatment as opposed to the current use of docetaxel for those with hormone refractory metastatic disease.

We also investigated if TUBB3 over expression played a role in the acquired docetaxel resistance in the PC3-D12 cell line. We found TUBB3 mRNA levels not to be elevated in the docetaxel-resistant cells when compared to the docetaxel-sensitive cells (Figure 4.6). Taxane resistance in prostate can be due to a wide variety of mechanisms. In particular there are two mechanisms that may be relevant. The first mechanism is that of the MDR-1 gene which is an integral membrane efflux protein, which actively translocates substrates from the inside of cells to their outside. Over expression of the MDR-1 gene would enable the cell to efflux taxanes out of the cell thus hampering their retention (Horwitz, Lothstein et al. 1986). Another mechanism that may influence docetaxel resistance in the PC3-D12 cell line is presence of a point mutation in tubulin at the taxane binding site causing taxane resistance. This has been

investigated in lung cancer patients where a strong correlation was observed between tubulin point mutations and taxane resistance (Monzo, Rosell et al. 1999). Research into the mechanisms of taxane resistance is important (Galletti, Magnani et al. 2007) and the creation of novel taxane analogues which overcome these mechanisms may be of benefit in treatment of patients with taxane refractory prostate cancer.

Recently it has been found that increase in TUBB3 upon hypoxia was abolished in taxane-resistant cells through methylation of the 3' enhancer (Lee, Cao et al. 2007). If this is true for different cancer types, TUBB3 methylation may represent a potential pre-treatment molecular marker for preferential response to MTAs in patients presenting with hypoxic prostate tumours. We are currently evaluating the TUBB3 methylation status of different prostate cancer cell lines.

## **5.6 PBOX-15**

Novel MTAs with a different mechanism of interaction with tubulin may be of benefit in overcoming taxane resistance. In addition to docetaxel, paclitaxel and vincristine, we proposed to investigate a novel MTA, PBOX-15, which is a member of the pyrrolo-1,5-benzoxazepine family of compounds, in aerobic and hypoxic prostate cancer cell lines. These compounds were shown previously to potently induce apoptosis in a wide variety of human chemotherapy resistant cancer cells including those derived from



haematological malignancies (Bane, Bannon et al. 2009), breast carcinomas (Greene, Fleeton et al. 2005), and chronic myeloid leukemia cells (Mc Gee, Campiani et al. 2002). One compound from this series, known as PBOX-15, has shown novel microtubule-targeting effects by depolymerizing tubulin in vitro in the MCF-7 breast cancer cell line (Mulligan, Greene et al. 2006). We sought to investigate if PBOX-15 would be suitable as a novel chemotherapeutic agent in prostate cancer and in particular hypoxic prostate cancer cell lines.

#### **5.6.1 Sensitivity of prostate cancer cells to PBOX-15**

Our initial objective with PBOX-15 was to compare its effect on prostate cancer cell lines to that of docetaxel in both aerobic and hypoxic conditions. As hypoxia is known to be associated with chemo-resistance, we investigated the effect of PBOX-15 under both aerobic and hypoxic conditions on the primary 22Rv1 and metastatic DU145 cell lines. We firstly found that 22Rv1 cells were significantly more sensitive to PBOX-15 than DU145 cells (Figure 4.7) under both aerobic ( $p=0.001$ ) and hypoxic ( $p=0.002$ ) conditions. However, when both cell lines were treated with docetaxel, DU145 were found in that case to be more sensitive to treatment with docetaxel than 22Rv1 cells. However, although PBOX-15 maintained its cytotoxic activity in hypoxia in both prostate cell lines, it displayed reduced cytotoxic properties in hypoxic 22Rv1 (Figure 4.7,  $p=0.035$ ) cells when compared to DU145.

We then sought to investigate the effect of PBOX-15 on both HIF-1 $\alpha$  gene and protein expression. As we had demonstrated with our time course experiments, HIF-1 $\alpha$  gene expression was down-regulated in hypoxia within 4 hours. PBOX-15 treatment for 4 hours in hypoxic exposure had no effect on the down-regulation of the HIF-1 $\alpha$  gene in either 22Rv1 or DU145 cell lines (Figure 4.7). This was similar to the results seen with docetaxel treatment. The next step was to assess the effect on HIF-1 $\alpha$  protein expression. Treatment with PBOX-15 appeared to up-regulate HIF-1 $\alpha$  protein expression after 6 hours of hypoxia (Figure 4.8). This was observed in both cell lines and differed from the docetaxel data, which appeared to show down-regulation of HIF-1 $\alpha$  protein expression. In addition, there was a more pronounced increase in HIF-1 $\alpha$  protein expression following treatment with PBOX-15 in 22Rv1 cells compared to DU145 cells (Figure 4.8). This up-regulation of HIF-1 $\alpha$  protein may correlate with the reduced cytotoxic activity of PBOX-15 in hypoxic primary 22RV1 cells. While PBOX-15 may not have overcome hypoxia, it may still overall have a role in eradicating prostate cancer cells.

Our final objective in investigating the sensitivity of prostate cancer cells to PBOX-15 was to determine if use of a novel MTA would overcome docetaxel resistance in both aerobic and hypoxic environments. The PC3-D12 cell line showed similar sensitivity to PBOX-15 to that of the docetaxel sensitive age matched control PC3 cell line (Figure 4.10). Further evaluation on the mechanism of reduced activity of PBOX-15 in hypoxia is warranted.



Despite PBOX-15 not overcoming hypoxia, it may still be of use as a novel MTA in the control of prostate tumour growth and progression.

### **5.6.2 Radio-sensitising properties of PBOX-15**

Radiotherapy resistance due to hypoxia exposure is well established (Semenza 2004). Hypoxia has been found to increase radio-resistance by up to 3 fold, due to a decrease in lethal DNA double strand breaks (Bristow 2005). Recent studies have implicated HIF-1 $\alpha$  in radiation resistance with overexpression of HIF-1 $\alpha$  in oropharyngeal cancer associated with an increased risk of failure to achieve complete remission after radiation therapy (Aebersold, Burri et al. 2001). It has been proposed that the addition of chemotherapeutic agents may improve radio-sensitisation. Studies have sought to establish the radio-sensitising properties of the taxane paclitaxel in head and neck cancers with some encouraging results (Herscher and Cook 1999). Other studies have recently investigated novel radio-sensitising agents in prostate cancer. Recently it was found that administration of nutlin-3 (an inhibitor of p53-dependent DNA damage signaling) radio-sensitised hypoxic prostate cancer cells (Supiot, Hill et al. 2008). We sought to investigate if treatment with the novel MTA PBOX-15 could radio-sensitise hypoxic prostate cancer cells. Primary 22Rv1 cells were treated for 24 hours with PBOX-15 in hypoxia and then a 2Gy single dose fraction was administered. We found that while hypoxia induced radio-resistance was evident in the control untreated cells, pre-treatment with PBOX-15 prevented the onset of radio-resistance (Figure 4.9,  $p=0.01$ ). The use of

PBOX-15 as a radio-sensitiser could have benefits in patients identified as having aggressive hypoxic tumours as an initial treatment. Further investigation into the effect of MTAs such as PBOX in combination with radiation on HIF-1 $\alpha$  expression may reveal if combined therapy may reduce the molecular hypoxic response.

## **5.7 Future advances in the treatment of prostate cancer**

### **5.7.1 Neo-adjuvant chemotherapy**

Recent clinical trials have focused on the role of neoadjuvant treatment in patients with high-risk localised prostate cancer (Nakabayashi and Oh 2004). Initial studies have found the use of neoadjuvant docetaxel therapy is safe and feasible with tumor shrinkage, serological response as well as pathological downstaging seen (Mazhar and Waxman 2008). Another study involved combination therapy in locally advanced disease in which docetaxel was given with gefitinib (a selective inhibitor of epidermal growth factor receptor's tyrosine kinase domain). This showed a clinical partial response in the majority of patients, with some patients demonstrating radiologic improvement on MRI (Vuky, Porter et al. 2009). Several other randomized trials are still ongoing with the results keenly awaited.

### **5.7.2 HIF-1 $\alpha$ as a prognostic marker**

Identification of patients with high risk prostate cancer has been enhanced with the use of nomograms and risk stratification approaches.



Hypoxic prostate tumours are associated with aggressive disease and identification of these tumours and their future incorporation into nomograms may further benefit in identifying those most in need of treatment. HIF-1 $\alpha$  may be of use in this regard as a prognostic marker. Previously published work by Vergis *et al.* looked at almost 300 diagnostic biopsies from patients with prostate cancer. They found that increased staining for HIF-1 $\alpha$  expression was associated with a shorter time to biochemical (PSA) failure (Vergis, Corbishley *et al.* 2008). Foley *et al.* found immunostaining intensity for HIF-1 $\alpha$  was significantly enhanced in 75% of prostate cancer specimens when compared to matched benign specimens ( $p < 0.0001$ ) (Foley, Marignol *et al.* 2009). These results further strengthen the argument for incorporating the HIF-1 $\alpha$  expression status into a future type of nomogram.

### **5.7.3 HIF-1 $\alpha$ targeted therapies**

HIF-1 $\alpha$  may not just be of benefit as a prognostic marker. There is an increasing body of evidence to suggest that targeting and inhibition of HIF-1 may be a relevant in anticancer therapeutics. The mechanism of action of a number of drugs in trials at present is the inhibition of angiogenesis in tumours. There is concern that inhibition of tumour angiogenesis may be preferential to cancer cells that survive in hypoxia and are associated with treatment resistance (Semenza 2003). Therefore, inhibition of HIF-1 $\alpha$  instead of direct inhibition of growth factors such as VEGF may represent a more potent form of anticancer therapy. Screens for small molecule inhibitors of HIF-1 $\alpha$  are underway and so

far studies have shown that inhibition of HIF-1 $\alpha$  is feasible. The small molecule YC-1 (3-(5'-hydroxy-methyl-2'-furyl)-1-benzylindazole) was shown to reduce HIF-1 $\alpha$  levels and xenograft growth (Yeo, Chun et al. 2003). YC-1 is known to stimulate soluble guanylate-cyclase activity, but this effect is not required for inhibition of HIF-1 $\alpha$  levels. The mechanism by which YC-1 reduces HIF-1 $\alpha$  levels has not been established. Other studies have investigated geldanamycin, a benzoquinone ansamycin antibiotic, which binds to heat shock protein 90 (HSP90) and interferes with its function (Mabjeesh, Post et al. 2002; Zagzag, Nomura et al. 2003). HIF-1 $\alpha$  has previously been shown to interact with HSP90 (Isaacs, Jung et al. 2002). By interfering with HSP90, geldanamycin induced HIF-1 $\alpha$  degradation in a VHL-independent manner, indicating that HSP90 is required for HIF-1 $\alpha$  stability. In these studies HIF-1 $\alpha$  has not been specifically targeted and further study into finding a direct acting HIF-1 $\alpha$  inhibitor may be of major therapeutic benefit.

## **5.8 Conclusion**

In our hypothesis, we sought to evaluate the effect of MTAs including docetaxel on hypoxic prostate tumours and HIF-1 $\alpha$  gene and protein expression in particular. We concluded that administration of docetaxel may down-regulate HIF-1 $\alpha$  protein expression and the subsequent hypoxic response. With the increasing incidence of prostate cancer in the future, identification of those individuals with disease more likely to progress will be a priority. The possible use of HIF-1 $\alpha$  as a prognostic marker may help in the identification of these



subsets of patients. Neoadjuvant treatment with docetaxel in localised tumours may help reduce the initial hypoxic response and future risk of biochemical failure. With regard to more advanced taxane refractory disease, further research into the mechanisms of taxane resistance may aid in the development of taxane analogues which are more effective in the treatment of the disease. Microtubule targeting agents have long been regarded as one of the most effective anti-cancer agents. Enhancing their efficacy rather than replacing them with a novel agent may be of benefit in treatment of those with prostate cancer.





# **CHAPTER 6**

## **Appendices**

6.1 Appendix A – Solutions

Table 1. Whole Cell Lysis Buffer for Protein Extraction for Western Blotting

| Ingredient                                  | Final concentration<br>in lysis buffer | Amount   |
|---------------------------------------------|----------------------------------------|----------|
| 500mM TRIS, pH 7.5                          | 50mM                                   | 1 mL     |
| 1.5M NaCl                                   | 150mM                                  | 1 mL     |
| 100% IGEPAL                                 | 1%                                     | 100 uL   |
| 10% SDS                                     | 0.1%                                   | 100 uL   |
| 0.5M EDTA                                   | 1mM                                    | 20 uL    |
| 0.06M Sodium Orthovanadate                  | 600µM                                  | 100 uL   |
| 500mM Sodium Fluoride                       | 50 mM                                  | 1 mL     |
| Complete Protease Inhibitor Tablet          |                                        | 1 tablet |
| 0.2M Phenyl Methyl Sulfonyl Fluoride (PMSF) | 1mM                                    | 5 uL     |
| Dithiothreitol (DTT)                        | 1mM                                    | 2 uL     |



Table 2. Solutions used in Western Blotting Protocols

| Solution                     | Ingredients                                                                                                 |
|------------------------------|-------------------------------------------------------------------------------------------------------------|
| 0.5M Tris HCl pH 6.8         | Tris 18g<br>dH2O 300mls<br>(Adjust pH to 6.8 with HCl)                                                      |
| 1.5M Tris-HCl pH8.8          | Tris 54.46g<br>dH2O 300mls<br>(Adjust pH to 8.8 with HCl)                                                   |
| 10% SDS                      | SDS 20g<br>dH2O 200mls                                                                                      |
| APS                          | Ammonium Persulfate 0.1g<br>dH2O 1ml<br>(Make up fresh)                                                     |
| Running Buffer (10X)         | Tris 30.3g<br>Glycine 144.0g<br>SDS 10g<br>dH2O make up to 1 litre<br>(Dilute 1:10 with dH2O prior to use)  |
| Transfer Buffer (1X)         | Tris 3.03g<br>Glycine 14.4g<br>Methanol 200mls<br>dH2O make up to 1 litre<br>(Store transfer buffer at 4°C) |
| 0.05% Tween PBS              | dH2O 1L<br>PBS tablets 2<br>Tween 500µl                                                                     |
| 5% Marvel in 0.05% Tween PBS | Marvel 5g<br>0.05% Tween PBS 100ml                                                                          |
| 5% BSA in 0.05% Tween PBS    | BSA 0.5g<br>0.05% Tween PBS 10ml                                                                            |

Table 3. 12% Resolving Gel for SDS-PAGE

| Solution                | Amount for 2 Gels |
|-------------------------|-------------------|
| dH <sub>2</sub> O       | 3.4 mL            |
| 1.5M Tris Buffer pH 8.8 | 2.5 mL            |
| 10% SDS                 | 100 uL            |
| 30% Acrylamide          | 4 mL              |
| APS*                    | 50 uL             |
| TEMED*                  | 10 uL             |

\*Add APS and TEMED last

Table 4. 6% Resolving Gel for SDS-PAGE

| Solution                | Amount for 2 Gels |
|-------------------------|-------------------|
| dH <sub>2</sub> O       | 3.05 mL           |
| 0.5M Tris Buffer pH 6.8 | 1.25 mL           |
| 10% SDS                 | 50 uL             |
| 30% Acrylamide          | 667 uL            |
| APS*                    | 25 uL             |
| TEMED*                  | 5 uL              |

\*Add APS and TEMED last



## 6.2 Appendix B - Mycoplasma test

All cells lines were tested for mycoplasma infections every 2 months. The cell lines used in this study consistently tested negative for mycoplasma contamination. Cell lines were grown in their own specific antibiotic-free media in T25 culture flasks for 2 passages prior to testing using a mycoplasma PCR ELISA kit (Roche, West Sussex, UK). 1ml of cell culture supernatant was removed and centrifuged at 200 x g for 10 minutes at room temperature. The supernatant was removed and centrifuged at 13,000 x g for 10 minutes at 4°C to sediment mycoplasma. The supernatant was then discarded and any pellet formed was re-suspended in 10µL sterile water (autoclaved deionised, ultraviolet irradiated) and 10µL lysis solution (ELISA kit). 2 positive and negative controls were included in testing. Positive controls consisted of 10µL of positive control DNA and 10µL lysis buffer. Negative controls were made up of 10µL sterile water and 10µL of lysis solution. Both samples and controls were then incubated at 37°C for 1 hour. Then 30µL of neutralization solution was added to each sample.

Next PCR reactions were performed using the cell extracts and controls. 10µL was added to each 25µL PCR mix and 15µL sterile water. Amplification was carried out on PTC - 200 thermocycler as follows:

1. 95°C for 5 minutes
2. 39 cycles:
  - a. 94°C for 30 seconds

- b. 62°C for 30 seconds
  - c. 72°C for 1 minute
3. 72°C for 10 minutes

Positive control PCR products were electrophoresed on a 1% agarose gel to ensure that the 520bp product had been successfully amplified before proceeding to the ELISA step. 40µL denaturation reagent was then added to 10µL PCR product and incubated for 10 minutes at room temperature. 450µL hybridization reagent was added to each sample and mixed thoroughly. 200µL of each sample was transferred into a 96 well plate (Sarstedt, Wexford, Ireland) which was sealed with adhesive foil and aluminum foil. The samples were shaken at 150rpm at 37°C for 3hrs in a Stuart Scientific S150 incubator (Premier Scientific, Belfast, UK). Next wells are washed 3 times with 250µL washing buffer. 200µL of room temperature anti-DIG-peroxidase was added to samples and plate was further shaken for 30 minutes at 150rpm at room temperature. Wells are then washed 5 times with 250µL with washing buffer. Next, 100µL TMB (3,3',5'5-tetramethylbenzidine) is added to each well and again the sample are shaken at 150rpm at room temperature for 20 minutes. 100µL of stop solution is then added.

Absorbance was measured with a SpectraFlour Plus fluorometer (Tecan, Dorset, UK) at an absorbance of 450nm with a reference absorbance at 690nm.



A450-A690 was greater than 1.2 for positive controls and less than 0.25 for negative controls. For controls that fell outside this range the test was repeated.

Samples were considered mycoplasma negative based on the following formula:

$(A450-A690) - (\text{mean } A450-A690 \text{ of negative controls})$





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