UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

In vitro analysis of potential anticancer effects associated with watercress

by

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ABSTRACT

FACULTY OF MEDICINE

<u>Doctor of Philosophy</u>

IN VITRO ANALYSIS OF POTENTIAL ANTICANCER EFFECTS ASSOCIATED WITH WATERCRESS

by Breeze Erin Cavell

Epidemiological studies indicate that there is an inverse relationship between consumption of cruciferous vegetables and risk of cancer. As a result there is much interest in understanding the anticancer potential not only of cruciferous vegetables themselves but also of the key phytochemicals contained in them. This project focuses on the potential anticancer properties of watercress, a cruciferous vegetable which is cultivated around the world and eaten raw as a salad vegetable as well as in cooked dishes. Watercress is the most abundant source of gluconasturtiin, a precursor to the phytochemical phenethyl isothiocyanate (PEITC), and is also a rich source of indole-3-carbinol (I3C) and quercetin.

This project addressed the hypothesis that the *in vitro* anticancer activity of watercress can be enhanced by altering the growth environment. To investigate this it was necessary to develop assays that can be used to assess the *in vitro* anticancer activity of watercress-derived compounds, to explore the mechanisms by which PEITC exhibits its anticancer effects, and to determine potential effects of altered growth conditions on *in vitro* anticancer properties.

The activity of watercress-derived phytochemicals was analysed in a series of *in vitro* assays. Based on these results, inhibition of MCF7 cell growth and activation of Nrf2-dependent transcription were selected as potential assays for subsequent analysis of watercress extracts. Mechanistic studies demonstrated that PEITC inhibited the transcriptional activity of hypoxia inducible factor (HIF), a key positive regulator of angiogenesis in malignant cells. Inhibition of HIF function was associated with inhibition of mammalian target of rapamycin complex 1 (mTORC1) activity and decreased $HIF1\alpha$ mRNA translation, and was dependent on the presence of the mTORC1 regulator, tuberous sclerosis complex 2 (TSC2). I also demonstrated that, in addition to effects on $HIF1\alpha$ mRNA translation, PEITC inhibited general protein synthesis and modulated two other key regulators of translation, eukaryotic initiation factor 2 (eIF2) and eukaryotic elongation factor 2 (eEF2). In proof-of-principle experiments, I demonstrated that growth inhibition and Nrf2 activation assays were suitable for the analysis of crude watercress extracts and that a natural variety of "red" watercress displayed approximately 10-fold more potent *in vitro* anticancer activity than standard, commercial "green" watercress. However, in a series of field trials, modulation of time of harvest, water availability or sulfur fertilisation did not alter the *in vitro* effects of watercress extracts in growth inhibition/Nrf2 activation assays.

In conclusion, this study suggests that, rather than altering environmental factors, selective breeding might be a better approach to increase the *in vitro* anticancer activity of watercress. Moreover, my findings have increased our understanding of the mechanisms underlying the anticancer activity of PEITC, uncovering a novel role for PEITC in inhibiting $HIF1\alpha$ and total protein synthesis, and identifying several key regulators of these processes that are modulated by PEITC.

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Wang, X.-H., Cavell, B.E., Syed Alwi, S.S., and Packham, G. (2009). Inhibition of hypoxia inducible factor by phenethyl isothiocyanate. Biochemical Pharmacology *78*, 261-272.

Syed Alwi, S.S., Cavell, B.E., Telang, U., Morris, M.E., Parry, B.M., and Packham, G. (2010). *In vivo* modulation of 4E binding protein 1 (4E-BP1) phosphorylation by watercress: a pilot study. British Journal of Nutrition *104*, 1288-1296.

Cavell, B.E., Syed Alwi, S.S., Donlevy, A., and Packham, G. (2011). Anti-angiogenic effects of dietary isothiocyanates: Mechanisms of action and implications for human health. Biochemical Pharmacology *81*, 327-336.

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Declaration of authorship

I, Breeze Erin Cavell

declare that the thesis entitled

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and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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  Syed Alwi, S.S., Cavell, B.E., Telang, U., Morris, M.E., Parry, B.M., and Packham, G. (2010). *In vivo* modulation of 4E binding protein 1 (4E-BP1) phosphorylation by watercress: a pilot study. British Journal of Nutrition *104*, 1288-1296.

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Abbreviations

4E-BP1	eIF4E binding protein1
5'UTR	5' untranslated region
AMP	5' adenosine monophosphate
Akt	v-Akt murine thymoma viral oncogene
AITC	Allyl isothiocyanate
AMPK	5' adenosine monophosphate-activated kinase
ARE	Antioxidant response element
ATP	5' adenosine triphosphate
BAL	British anti-Lewisite, or 2,3-dimercapto-1-propanol
BITC	Benzyl isothiocyanate
BNIP3	Bcl2/adenovirus E1B 19kDa interacting protein 3
CAIX	Carbonic anhydrase IX
Cdc	Cell division cycle
CDK	Cyclin dependent kinase
CHX	Cycloheximide
CoCl$_2$	Cobalt chloride
CYP	Cytochrome P450 enzymes
DFO	Deferoxamine
DIM	3,3'-diindoylmethane
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DR	Death receptor
EGF	Epidermal growth factor
eIF4E	Eukaryotic translation initiation factor 4E
ER	Oestrogen receptor
ERE	Oestrogen response element
ERK	Extracellular signal-regulated kinase
eEF2	Eukaryotic elongation factor 2
eEF2K	Eukaryotic elongation factor 2 kinase
eIF2	Eukaryotic initiation factor 2
FOX	Forkhead box
GEF	Guanine nucleotide exchange factor
GCN2	General control non-depressible 2
GLS	Glucosinolate

GLUT1	Glucose transporter 1
GPCR	G-protein coupled receptor
GPX	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione-S-transferase
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HAT	Histone acetylase
HER	Human epidermal growth factor receptor
HDAC	Histone deacetylase
HIF	Hypoxia inducible factor
HO1	Heme oxygenase 1
HRE	Hypoxia response element
HRI	Heme-regulated inhibitor
HRP	Horseradish peroxidise
I3C	Indole-3-carbinol
IC_{50}	Half maximal inhibitory concentration
ITC	Isothiocyanate
JNK	Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MRP1	Multidrug resistance protein 1
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(-4-sulfophenyl-2H-tetrazolium inner salt
Mw	Molecular weight
NAC	N-acetylcysteine
NDRG1	N-myc downstream regulated gene 1
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
Nrf2	nuclear factor erythroid 2-related factor 2
NQO1	NAD(P)H:quinone oxidoreductase 1
p53	Protein 53

p70 S6K	70 kDa ribosomal S6 protein kinase
p90RSK	90 kDa ribosomal S6 protein kinase
PAO	Phenylarsine oxide
PBMC	Peripheral blood mononuclear cells
PDK1	Phosphoinositide dependent kinase 1
PEITC	Phenethyl isothiocyanate
PERK	PKR-like endoplasmic reticulum kinase
PH	Pleckstrin homology
PHD	Proyl hydroxylase domain
PI3K	Phosphatidylinositol 3-kinase
PIP$_2$	Phosphatidylinositol (4,5)-bisphosphate
PIP$_3$	Phosphatidylinositol (3,4,5)-trisphosphate
PKR	Protein kinase RNA-dependent
PR	Progesterone receptor
PRAS40	Proline-rich Akt substrate 40 kDa
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
pVHL	von Hippel Landau
Qn	Quercetin
QTL	Quantitative trait locus
Raptor	Regulatory associated protein of mTOR
REDD1	Regulated in development and DNA damage response 1
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S6K	S6 kinase
SAHA	Suberoylanilide hydroxamic acid
SD	Standard deviation
SFN	Sulforaphane
SOD	Superoxide dismutase
tRNA	Transfer RNA
TSA	Trichostatin A
TSC1/2	Tuberous sclerosis complex 1/2
UPR	Unfolded protein response
VEGFA	Vascular endothelial growth factor A
Wx	Watercress

Chapter 1

Introduction

1.1   Overview of introduction

The work described in this thesis was performed as part of an Industrial CASE studentship with Vitacress Salad Leaves Ltd, a major grower of watercress in the UK. Although the potential health benefits of consumption of a diet rich in watercress and other cruciferous vegetables remain unproven, extensive data demonstrate that aqueous extracts of watercress, as well as its key phytochemical constituents, inhibit the growth of human cancer cells *in vitro* and in experimental animals. The primary goal of the project was to investigate molecular mechanisms by which watercress extracts/phytochemicals inhibited cancer cell growth *in vitro*. The secondary goal was to use this knowledge to investigate potential enhancement of the *in vitro* anticancer effects of watercress through manipulation of the crop. The project focused on breast cancer cell lines as a well validated model to investigate effects on human cancer cells.

In this introduction, an overview of the cancer process is provided before the epidemiology and characterisation of human breast cancer is described. Some molecules and pathways that contribute to breast cancer development and progression, and are potential targets for modulation by watercress compounds, including histone deacetylases (HDACs), hypoxia inducible factor (HIF), mammalian target of rapamycin (mTOR) and the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen activated protein kinase (MAPK) pathways, are then described. A background on the cultivation of watercress in the UK, and the potential strategies that could be used to manipulate its potential anticancer properties are also described. Finally, the key phytochemicals of watercress and their known molecular effects, focusing on phenethyl isothiocyanate (PEITC) are detailed.

1.2   Molecular genetics of cancer

Cancer results from genetic and/or epigenetic changes within a cell. Oncogenic mutations occur in two different types of genes, both of which are required for normal cell homeostasis. Gain-of-function mutations in oncogenes act to promote cell division and survival, whereas loss-of-function mutations in tumour suppressor genes inactivate negative regulatory proteins which normally prevent inappropriate cell division and survival. Tumourigenesis is a multistep process whereby normal cells accumulate multiple mutations to acquire a fully malignant phenotype. This is

consistent with the idea that, whilst cancer incidence is high within human populations, the frequency of conversion to a fully malignant phenotype is extremely low for any given cell within an individual. It has been proposed that there are six essential hallmarks that need to be acquired by all cancers, although these may be achieved through different mechanistic strategies; independence from growth signals, insensitivity to growth-inhibitory signals, resistance to apoptosis, limitless replicative potential, angiogenesis, and finally tissue invasion and metastasis (Hanahan and Weinberg, 2000). Several other hallmarks have since emerged, notably the ability to evade the immune system and dysregulate metabolism. In addition, genomic instability and inflammation have been highlighted as enabling characteristics which promote tumour progression (Hanahan and Weinberg, 2011).

1.3   Breast cancer

1.3.1   Epidemiology

In 2008, 48,034 new cases of breast cancer were diagnosed in the UK, with >99% of these being in women (Cancer Research UK, accessed February 2011). Breast cancer is the second most common cause of death from cancer in women following lung cancer. The highest rates of breast cancer occur in the developed world, particularly in Northern and Western Europe, as well as Australia and North America, whereas the lowest rates are found in Asia and parts of Africa.

In the UK the lifetime risk for women of developing breast cancer is 1 in 8. The onset of breast cancer is strongly associated with age and more than 80% of breast cancer cases occur in women over the age of 50, with the highest number of cases diagnosed in the 50-69 age range. Approximately 10-15% of patients with breast cancer develop distant metastasis within 3 years of detection of the primary tumour (Weigelt *et al.*, 2005). Despite a continued increase in the incidence of breast cancer, the number of women dying from breast cancer has steadily declined since 1989. This is likely due to better drug treatments such as tamoxifen, which has been widely used since 1992, and the introduction of the national screening program in the UK in 1988. Current 5-year survival rates for women with breast cancer in the UK are approximately 80% (Cancer Research UK, accessed February 2011).

1.3.2 Familial genetic risk

A predisposition to breast cancer has been identified in people that inherit mutations in the high-risk *BRCA1*, *BRCA2* and *TP53* genes (Lalloo *et al.*, 2006). However, these genes account for only a minority, approximately 5%, of all breast cancers in the population (Key *et al.*, 2001). Most cases of breast cancers are therefore sporadic and more the result of environmental and lifestyle factors.

1.3.3 Subtypes

Breast cancer is a complex and highly heterogeneous disease and as a result there are many different subtypes. The majority of all invasive breast cancers are adenocarcinomas and tend to be either ductal (85%) or lobular (15%) (see Figure 1.1 for structure of the human breast), and both can be further divided according to histological appearance (Table 1.1). In addition to this there are also two types of non-invasive in situ breast cancers, Ductal Carcinoma In Situ (DCIS) and Lobular Carcinoma In Situ (LCIS). LCIS is relatively rare and is believed to be a high risk factor for invasive breast cancer rather than a direct precursor. On the other hand DCIS is the most common type of non-invasive breast cancer and accounts for 20% of all breast cancers detected (Page *et al.*, 1995).

Various markers have also been identified to further characterise breast cancer with an aim to predict prognosis and response to therapy. The most established biomarkers are oestrogen receptor α (ERα) status, progesterone receptor (PR) status and human epidermal growth factor receptor 2 (HER2) expression. Breast cancers that are ERα- and PR-positive are generally associated with a good prognosis and are more likely to respond to endocrine therapy. Alternatively HER2 overexpression is indicative of a poor clinical outcome in patients with axillary-lymph-node metastasis. HER2 expression is particularly important in assessing the potential response to trastuzumab (Herceptin), an antibody directly targeted to HER2 (Weigelt *et al.*, 2005).

Gene expression arrays have expanded upon characterisation of breast cancer and identified various subtypes of breast cancer based on the presence of several prognostic markers together. ERα negative tumours can be further divided into basal-like group which exhibit high expression of basal epithelial markers such as basal keratins 5/6, HER2 positive group which overexpresses HER2, and
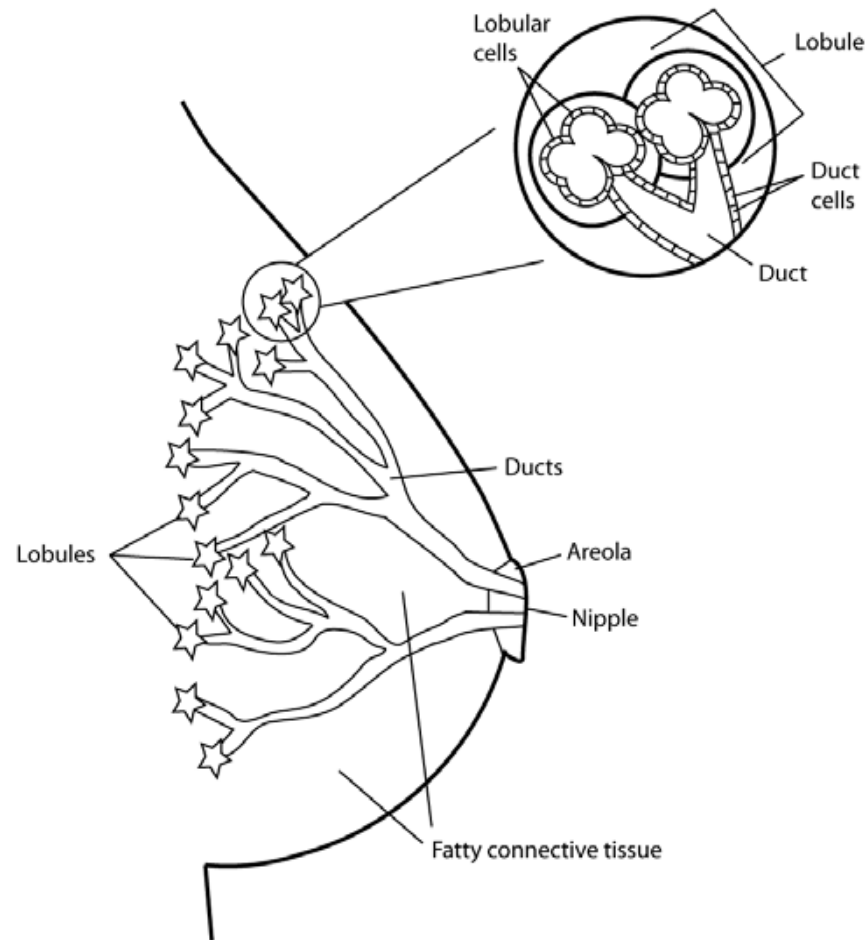
Figure 1.1: Structure of the normal adult human breast.
The ducts connect the milk-producing lobules to the nipple. The lobules and ducts are surrounded by fatty connective tissue and ligaments (Adams, 2008).

Histopathological type of invasive breast cancer	Frequency	10-year survival rate
Invasive ductal carcinoma of no special type	50-80%	35-50%
Invasive lobular carcinoma	5-15%	35-50%
Mixed type, lobular and ductal features	4-5%	35-50%
Tubular/invasive cribiform carcinoma	1-6%	90-100%
Mucinous carcinoma	<5%	80-100%
Medullary carcinoma	1-7%	50-90%
Invasive papillary carcinoma	<1-2%	60%
Invasive micropapillary carcinoma	<3%	Unknown
Metaplastic carcinoma	<5%	Unknown
Adenoid cystic carcinoma	0.1%	Unknown
Invasive aprocrine carcinoma	0.3-4%	Unknown
Neuroendocrine carcinoma	2-5%	Unknown
Secretory carcinoma	0.01-0.15%	Unknown
Lipid-rich carcinoma	<1-6%	Unknown
Acinic-cell carcinoma	7 cases	Unknown
Glycogen-rich, clear-cell carcinoma	1-3%	Unknown
Sebaceous carcinoma	4 cases	Unknown

Table 1.1: Histopathological subtypes of invasive breast cancer, frequency of occurrence and 10-year survival rates.
Information from Weigelt *et al.* (2005).

normal breast like group which resemble the normal breast, expressing genes of adipose-cell and other non-epithelial-cell origin (Perou *et al.*, 2000). Nearly all basal-like breast cancers are triple-negative in that they are negative for both ERα and PR, and do not overexpress HER2. This type of breast cancer has been reported to be particularly aggressive and less responsive to treatment resulting in poor prognosis (Dent *et al.*, 2007). Perou *et al.* (2000) classified all ERα positive tumours in a luminal-type group as they all overexpressed breast luminal cell markers, however, this group has since been divided into luminal A which overexpress HER2 and luminal B which do not (Sorlie *et al.*, 2003).

1.3.4 Oestrogen receptor

Oestrogen receptor α (ERα) positive breast cancer is seen in a third of patients under 50, and this rises to approximately 80% in women over 50 (Clarke *et al.*, 1998 and see Table 1.2). ERs are transcription factors which mediate the response to the hormone oestrogen. In humans there are two ER subtypes, ERα and ERβ, which exhibit different expression patterns in specific tissues. Both receptors share a high level of homology and belong to the nuclear hormone receptor superfamily. Although ERα and ERβ bind to oestrogen with equal affinity, other ligands preferentially bind to one or the other (Enmark and Gustafsson, 1999). In the context of breast cancer ERα is thought to be the most important and it is the status of this subtype that is assessed in clinical practice (Badve and Nakshatri, 2009). However, there is increasing interest in the potential clinical significance of ERβ. Homna *et al.* (2008) found ERβ1-positive breast cancers treated with adjuvant tamoxifen were associated with significantly better survival independent of ERα expression. This is of particular interest when considering triple-negative breast cancer (negative for ERα, PR, and HER2), that might still be positive for ERβ.

ERα+ve/PR+ve	ERα-ve/PR-ve	ERα+ve/PR-ve	ERα-ve/PR+ve
54.8%	22.1%	19.8%	3.2%

Table 1.2: Frequency of progesterone and oestrogen receptor α phenotypes in breast cancer.

Information from Rhodes *et al.* (2000).

Oestrogen is a lipophilic steroid and able to diffuse through the phospholipid plasma membrane to the cytoplasm where it can bind to intracellular ERα or ERβ. The binding of oestrogen to these receptors induces phosphorylation, dissociation from chaperones such as heat shock protein 90 (Hsp90), and a change in protein conformation, ultimately allowing the ERs to dimerise and recruit co-activators to enhance transcription. The ligand bound receptor complex activates transcription either through direct binding to ERE (oestrogen response elements) in the promoter region of ER-dependent genes or through interactions with other transcription factors such as SP1 (Salvatori *et al.*, 2003). It has also been reported that ERs at the cell membrane can mediate non-genomic effects of oestrogen, termed membrane-initiated steroid signalling (MISS), which results in the activation of a variety of cell signalling cascades such as the MAPK pathway (discussed later) (Song *et al.*, 2002) .

In women oestrogen is primarily produced in the ovaries and is responsible for controlling sexual development. One role of oestrogen is to promote the growth of breasts by stimulating cell division. ER positive breast cancers are dependent on oestrogen to grow and as a result this pathway is an important target for therapeutic therapy (Anderson, 2002).

1.3.4.1 Tamoxifen

Tamoxifen is the most widely used treatment of ERα-positive breast cancer, and when given as adjuvant therapy in early stage breast cancer it has been shown to improve survival rates (Clarke *et al.*, 1998). Tamoxifen is a selective oestrogen receptor modulator (SERM) in that it acts as an antagonist in breast tissue, but partial-agonist in the uterus and bone. Through direct binding with ERs in breast tissue tamoxifen induces a conformational change which, in contrast to oestrogen, favours recruitment of co-repressors to the ER, inhibiting transcriptional activity (Shou *et al.*, 2004).

1.3.4.2 Aromatase inhibitors

A second class of drugs, aromatase inhibitors (AIs), prevent the synthesis of oestrogen by inhibiting the enzyme aromatase that catalyses the conversion of androgens to oestrogens. This therapy is only used in postmenopausal women whose main source of oestrogen comes from converted androgens in the periphery tissues, not the ovaries. Examples of AIs include anastrozole, letrozole, and exemestane, and they

tend to be used in preference to tamoxifen in postmenopausal women with ERα positive breast cancer due to better efficacy and less adverse effects (Mokbel, 2002).

1.3.5 Human epidermal growth factor receptor 2

Aberrant expression of epidermal growth factor (EGF) receptors has been linked to many cancers. In particular, human epidermal growth factor receptor 2 (HER2; also known as HER2/neu and ErbB2) gene amplification or protein overexpression is found in 20-30% of human breast cancers and is indicative of poor prognosis (Slamon *et al.*, 1987; Slamon *et al.*, 1989). HER2 along with HER1 (EGFR, ErbB2), HER3 (ErbB3), and HER4 (ErbB4) form the human epidermal growth factor receptor (HER) family of tyrosine kinase receptors. Activation of these receptors by ligands such as EGF causes homo- or heterodimerisation and subsequent phosphorylation. This initiates a signal cascade through activation of both the PI3K/Akt and MAPK pathways (discussed later) that ultimately results in altered gene expression, with biological responses ranging from proliferation to migration (Bazley and Gullick, 2005). No ligand has yet been identified to bind HER2 directly, although the receptor has been shown to act as the preferred heterodimerisation partner to all other ligand-bound members of the HER family (Graus-Porta *et al.*, 1997). It seems HER2 overexpression causes transformation of malignant breast cancer cells by amplifying the signals of other receptors, with Akt phosphorylation being shown in HER1 and HER2 overexpressing cells even in the absence of ligands (Longva *et al.*, 2005).

1.3.5.1 Herceptin

The humanised monoclonal antibody Trastuzumab (more commonly known as Herceptin) is used as a treatment for patients with breast cancer that overexpress HER2. Herceptin is targeted to the extracellular domain of HER2 with treatment being shown to induce regression of HER2 overexpressing breast cancers (Vogel *et al.*, 2002). Although the mechanism is not fully understood it is thought to involve inhibition of Akt phosphorylation, a target of the PI3K pathway activated by HER2 that regulates many processes including cell proliferation, apoptosis, and glycogen metabolism (Longva *et al.*, 2005; Yakes *et al.*, 2002). In particular, Herceptin treated cells have been shown to arrest in the G_1 phase of the cell cycle, believed to be the result of Akt inhibition stabilising the cyclin-dependent kinase 2 (CDK2) inhibitor p27 (Le *et al.*, 2005; Yakes *et al.*, 2002).

1.4   Key molecular pathways in breast cancer

1.4.1 Hypoxia inducible factor pathway

Tumours are only able to grow to approximately 1-2 mm^2 in size before they outgrow the existing vasculature and lack of oxygen and nutrients become a limiting factor. Therefore, in order for solid tumours to progress they must adapt to hypoxic conditions, or increase vascularisation, or both. Hypoxia inducible factors (HIFs) are transcription factors that are critical for this adaptation as they increase expression of genes that enable cells to survive in hypoxic conditions and promote angiogenesis (the growth of new blood vessels), such as glucose transporter 1 (GLUT-1) and vascular endothelial growth factor-A (VEGF-A) (Carmeliet and Jain, 2000).

HIFs are heterodimeric complexes composed of an α-subunit and a β-subunit and are members of the basic helix-loop-helix (bHLH)-PER-ARNT-Sim (PAS) family of transcription factors. Both subunits are constitutively expressed. However, while HIF1β is maintained at relatively constant levels, the HIF1α protein has a very short half life ($T_{1/2} \sim 5$ min) in normoxia, due to oxygen-dependent proteolysis (Figure 1.2). In an oxygen rich environment, proline residues 402 and 577 of HIF1α are hydroxylated by prolyl hydroxylase domain-containing (PHD) proteins. This hydroxylation triggers association with the von Hippel-Lindau tumour suppressor protein (pVHL), a member of the E_3 ubiquitination complex, which subsequently ubiquitinates HIF1α and targets it for degradation by the 26S proteasome. The significance of this tumour suppressor protein and importance of normal HIF regulation is evident in individuals who inherit a defective *VHL* allele. VHL disease is characterised by the presence of hypervascular tumours in multiple organs, although kidney cancer is the primary cause of death (Ohh, 2006). The PHD proteins, which contain iron, require oxygen as a co-factor for activity. In hypoxia when there is a low availability of oxygen the activity of the prolyl hydroxylases is inhibited, HIF1α is no longer targeted for ubiquitination and is stabilised. HIF1α is translocated to the nucleus where it dimerises with HIF1β. The transcriptionally active HIF complex binds to hypoxic response elements (HRE) in the promoter region of hypoxia regulated genes such as those involved in angiogenesis (VEGF-A) and pH regulation (carbonic anhydrase IX; CAIX) (Ke and Costa, 2006; Lee *et al.*, 2004).
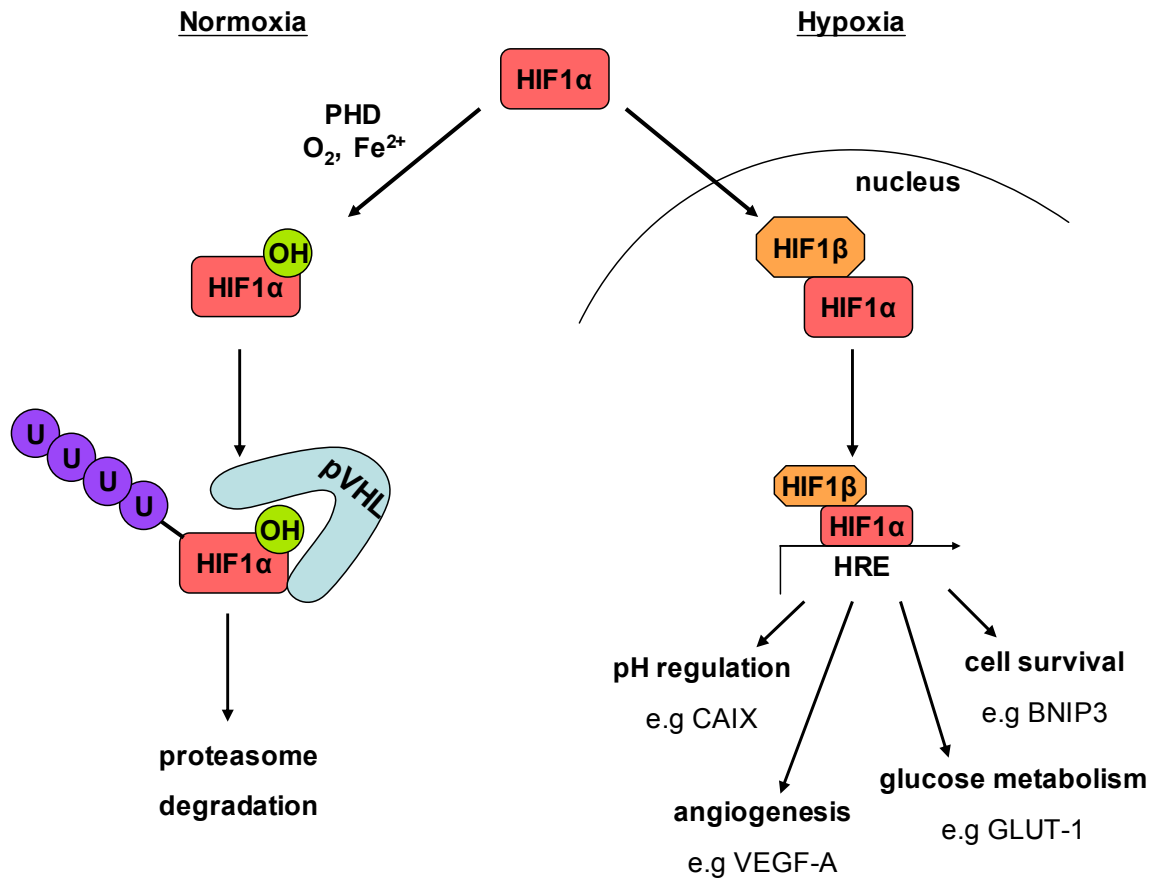
Figure 1.2: Oxygen-dependent regulation of hypoxia inducible factor 1α.
In normoxia where there is an abundance of oxygen (O_2) HIF1α is hydroxylated by
proyl hydroxylases (PHDs). This triggers association with von Hippel-Lindau tumour
suppressor protein (pVHL) which acts to polyubiquitinate HIF1α and subsequently
targets it for degradation by the 26S proteasome. However, in hypoxia PHDs are
unable to hydroxylate HIF1α. Consequently it is no longer targeted for degradation and
is free to translocate to the nucleus. In the nucleus HIF1α forms heterodimers with
HIF1β and the active transcription factor complex can then bind to hypoxia response
elements (HRE) in the promoter region of target genes to initiate transcription. BNIP3
(Bcl2/adenovirus E1B 19kDa interacting protein 3), CAIX (carbonic anhydrase IX),
GLUT-1 (glucose transporter 1), VEGF-A (vascular endothelial growth factor A); U
(ubiquitin).

While only one β-subunit has been identified (HIF1β, also known as aryl hydrocarbon nuclear translocator; ARNT), three α-subunits have been identified; HIF1α, HIF2α (also termed endothelial PAS protein; EPAS1/HIF-like factor; HRF/HIF-related factor; HRF/member of PAS superfamily 2; MOP2) and HIF3α. HIF1α and HIF2α are implicated in the induction of hypoxia responsive genes. HIF1α is ubiquitously expressed whereas HIF2α has been found to be predominantly expressed in the lung, endothelium and carotid body (Ke and Costa, 2006; Wiesener *et al.*, 2003). Little is known about HIF3α but it is known to act as a dominant-negative regulator of HIF1 (Makino *et al.*, 2002).

Due to the importance of hypoxia adaption in the progression of tumour development, HIF1α and HIF2α have been found to be overexpressed in a variety of cancers, including breast cancer (Zhong *et al.*, 1999), and are often associated with resistance to cancer therapies (Teicher, 1994)

1.4.2 Histone deacetylases

DNA is not free within the nucleus but is present in the form of chromatin, where sections of DNA are wrapped around nucleosomes. These nucleosomes are composed of two molecules each of the core histones, H2A, H2B, H3, and H4 that initially form two heterodimers, H2A-H2B and H3-H4, which then combine to produce an octamer barrel structure that the DNA wraps around twice. The fifth class of histone, H1, is the largest with a molecular weight of 23-kDa, and is believed to link the nucleosomes together. In total 200bp of DNA are associated with each nucleosome, with approximately 146bp making up two turns of the DNA around the histone core, with the remaining DNA acting as a linker, connecting the nucleosomes. All the histone proteins exhibit a net positive charge, as approximately 20-30% of their amino acid sequence comprises of the basic amino acids arginine and lysine. This characteristic enables the histones to have a strong charge attraction to the negatively charged phosphate groups in the backbone of DNA. This not only results in the DNA being tightly associated with nucleosomes, but by neutralising the negative charge on DNA the histones also facilitate DNA folding (Geiman and Robertson, 2002; Hames, 2000; Latchman, 2005).

How tightly chromatin is organised correlates with the extent of transcriptional activity in that region of the genome as a result of changes to the accessibility of

transcriptional machinery to the DNA. Therefore tightly packaged chromatin, heterochromatin, is associated with transcriptional repression, and loosely packaged chromatin, euchromatin, is indicative of transcriptional activity. This more relaxed state of chromatin is the result of modifications to either the DNA or histones that decrease the ionic bond between the negatively charged DNA and the positively charged histones (Latchman, 2005).

There are various forms of histone modification that can alter transcriptional activity in different ways and they predominantly occur on the amino tails of the histones that extend out from the nucleosome, typically the amino-terminal tails of the core histones H3 and H4. Methylation, phosphorylation and ubiquitination, are all known modifications that alter transcriptional activity, but perhaps the most characterised form is acetylation (Latchman, 2005). Patterns of acetylation are determined by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs transfer an acetyl group to lysines present on the amino-terminal tails of histones, neutralising the positively charged histones and thus resulting in a decreased affinity of the nucleosomes to the DNA (Figure 1.3). As a result acetylation of histones H3 and H4 correlates with transcriptional activity with acetylation positions including K9, K14, K18, and K27 on histone H3, and K5, K8, K12, and K16 on histone H4 (Salozhin *et al.*, 2005). Conversely HDACs remove the acetyl group, restoring the ionic interaction between the histones and the DNA promoting heterochromatinisation.

Aberrant histone acetylation is found in many cancers and results in irregular gene expression patterns. As a result molecules that are able to inhibit HDACs are providing a promising new way to treat cancer (Ropero and Esteller, 2007).

1.4.3 PI3K/Akt pathway

As already touched upon, the phosphatidylinositol (PI3K)/Akt pathway lies downstream of HER2 signalling. However, PI3K can also be activated by other receptor tyrosine kinases (RTKs) as well as G protein-coupled receptors (GPCR) in response to a broad range of stimuli including growth factors and hormones. PI3K catalyses the addition of a phosphate group to phosphatidylinositol (4,5)-bisphosphate (PIP_2) giving rise to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_3 is required to recruit pleckstrin homology (PH) domain-containing proteins, such as Akt and phosphoinositide
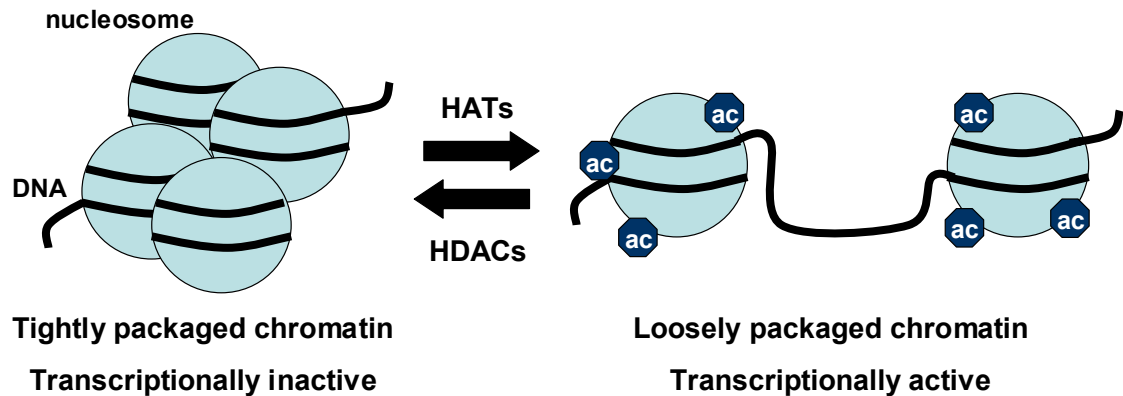
Figure 1.3: Balance between HDACs and HATs in chromatin modelling.
Histone acetyl transferases (HATs) transfer acetyl groups (ac) to lysines on the tails of histones in the nucleosome, decreasing the ionic bond between DNA and the histones. This results in more loosely packaged chromatin that is accessible to transcriptional machinery. Conversely histone deacetylases (HDACs) remove acetyl groups, returning the chromatin to a tightly packaged and transcriptionally inactive state.

dependent kinase 1 (PDK1) to the cell membrane. This acts to colocalise enzymes and their substrates. The tumour suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN) reverses this action by dephosphorylating PIP_3, inhibiting the PI3K/Akt signalling pathway. Aberrant activation of Akt as a result of PI3K or Akt gene amplification, or mutation/loss of PTEN is commonly seen in a variety of cancers (Osaki *et al.*, 2004). Once at the cell membrane Akt (also known as protein kinase B; PKB) is phosphorylated at multiple sites, threonine 308 (Thr308) located in the activation loop by PDK1 (Alessi *et al.*, 1997) and serine 473 (Ser473) present in the hydrophobic motif by mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov *et al.*, 2005). While it seems phosphorylation at both sites is required for full activation of Akt (Alessi *et al.*, 1996), the disruption of mTORC2 stability and subsequent loss of Ser473 phosphorylation of Akt has been seen to only effect a small number of Akt targets, namely the forkhead box (FOX) family of transcription factors (Jacinto *et al.*, 2006).

There are numerous downstream targets of Akt that are involved in the regulation of cell survival and proliferation (Figure 1.4) (Osaki *et al.*, 2004). For example, Akt exerts its anti-apoptotic effects by inhibiting the FOX transcription factors, FOXO1, FOXO3a, and FOXO4, preventing expression of pro-apoptotic proteins such as Fas ligand
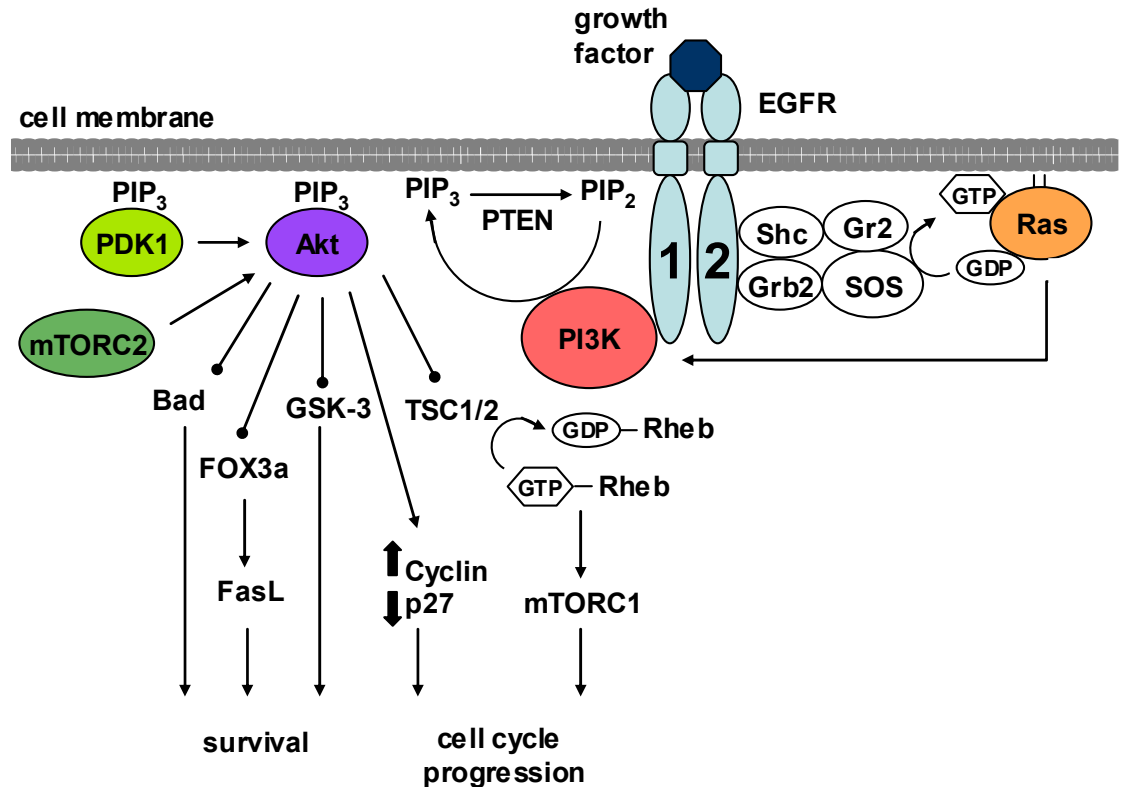
Figure 1.4: Overview of the PI3K/Akt pathway.
PI3K (phosphatidylinositol 3-kinase) can be activated directly by binding to phospho-sites on activated tyrosine kinase receptors or indirectly through adaptor proteins activating small G protein Ras. PI3K converts PIP_2 (phosphatidylinositol (4,5)-bisphosphate) to PIP_3 (phosphatidylinositol (3,4,5)-trisphosphate), which can recruit proteins such as Akt and PDK1 (phosphoinositide dependent kinase 1) to the cell membrane. Akt is activated following phosphorylation by PDK1 and mTORC2 (mammalian target of rapamycin complex 2) and has many downstream effectors involved in the regulation of cell survival and proliferation. PTEN (phosphatase and tensin homolog deleted on chromosome 10) reverses the action of PI3K. EGFR (epidermal growth factor receptor), FOX3a (forkhead box transcription factor 3a), GSK-3 (glycogen synthase kinase 3), Rheb (Ras homolog enriched in brain), TSC1/2 (tuberous sclerosis complex 1/2).

(FasL) (Brunet *et al.*, 1999) . Whereas, the phosphorylation and inactivation of tuberous sclerosis complex 1/2 (TSC1/2) by Akt enhances protein synthesis via the mammalian target of rapamycin complex 1 (mTORC1) pathway (Cai *et al.*, 2006).

1.4.4 MAPK pathway

There are multiple mitogen activated protein (MAP) kinases which are activated downstream of protein kinase cascades in response to distinct stimuli including growth factors, inflammatory cytokines and environmental stresses. Activation of these pathways mediates diverse cellular responses such as cell proliferation, apoptosis, and the inflammatory response. The signal is transmitted via a series of sequentially activated kinases; MAP kinase kinase kinase (M3K), MAP kinase kinase (MKK) and MAP kinase (MAPK). There are three main MAPK families, the extracellular-signal regulated kinases (ERK1/2, also known as classical MAPK), the C-Jun N-terminal kinase (JNK, also known as stress-activated protein kinase; SAPK), and p38 kinases (Krishna and Narang, 2008).

The ERK1/2 pathway is the most characterised and is preferentially activated by growth factors (Figure 1.5). Signalling is typically initiated by activation of RTKs, resulting in activation of the small G protein Ras. Activated Ras triggers the protein kinase activity of M3K, Raf, which then phosphorylates and activates the MKK, MEK1/2. MEK1/2 then phosphorylate ERK1/2 at two sites, a threonine and a tyrosine residue. Once active, ERK1/2 can translocate to the nucleus and activate transcription factors such as Elk1, resulting in the elevated expression of a variety of proteins associated with proliferation and differentiation. Other downstream targets of ERK1/2 include cytoskeletal and scaffold proteins, and protein kinases such as 90 kDa ribosomal S6 kinases (p90RSK) which further propagate the signal (Yoon and Seger, 2006). Both ERK1/2 and p90RSK can also increase protein synthesis via the phosphorylation of several proteins involved in translational regulation such as negative regulator of mTORC1, TSC2, and eukaryotic elongation factor 2 kinase (eEF2K) (Ma *et al.*, 2005; Wang *et al.*, 2001).

Aberrant activity of the ERK1/2 pathway is observed in multiple cancers, and can be the result of activating mutations in the *ras* or *raf* oncogenes, and overexpression or activating mutations in EGFRs. Consequently many inhibitors of the ERK pathway are currently in clinical trials (Kohno and Pouyssegur, 2006).
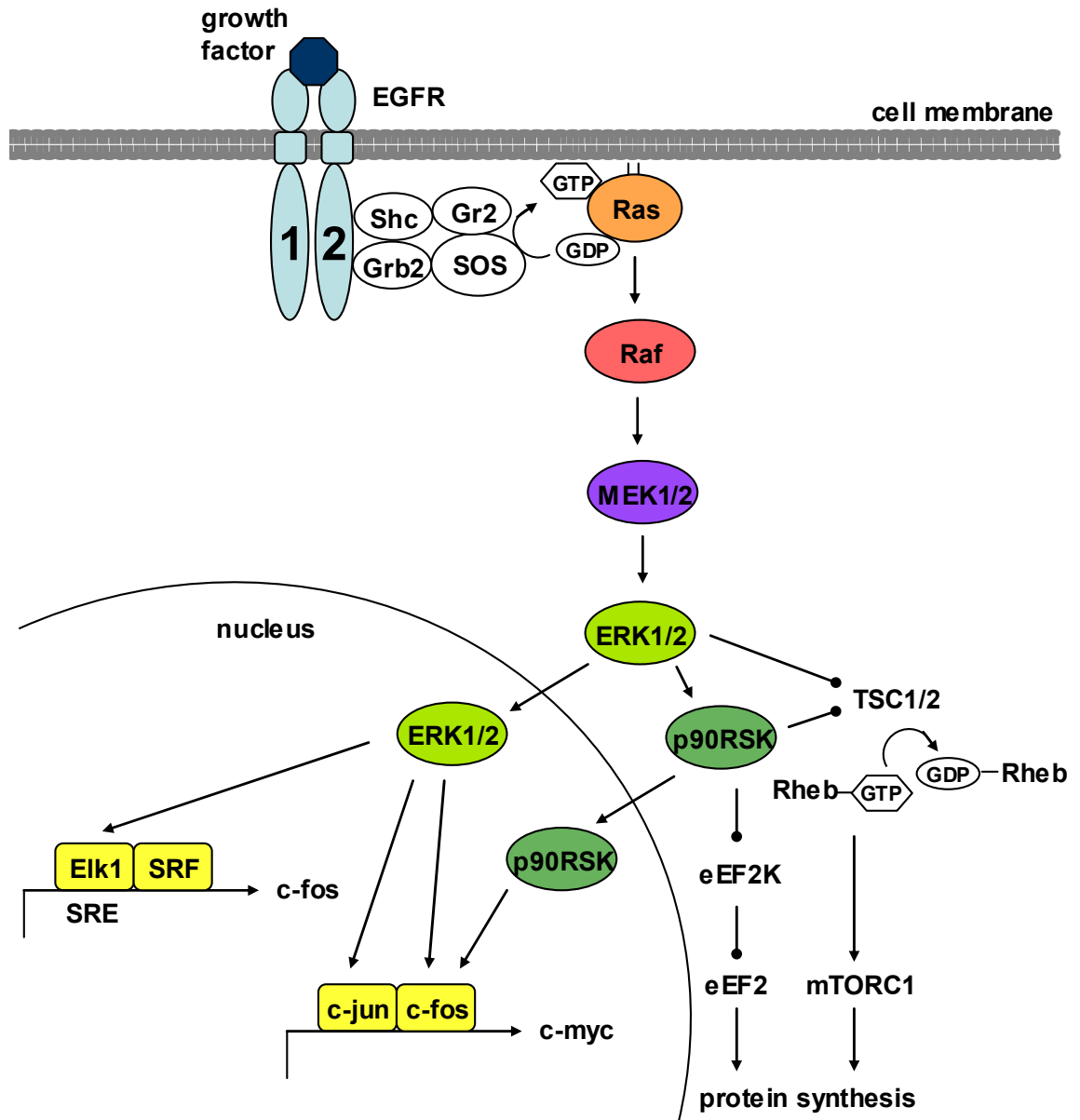
Figure 1.5: Overview of the ERK pathway.
Activation of the small G protein Ras, activates the protein kinase activity of Raf initiating the protein kinase cascade. Raf phosphorylates MEK1/2 (MAP kinase kinase 1/2), which in turn phosphorylates ERK1/2 (extracellular-signal regulated kinases 1/2). ERK1/2 also phosphorylates another protein kinase, p90RSK (90 kDa ribosomal S6 kinase). Both ERK1/2 and p90RSK can translocate to the nucleus and activate several transcription factors, as well as phosphorylating multiple translational regulators to increase protein synthesis. eEF2K (eukaryotic elongation factor 2 kinase), EGFR (epidermal growth factor receptor), mTORC1 (mammalian target of rapamycin complex), SRF (serum response factor), Rheb (Ras homolog enriched in brain), SRE (serum response element), TSC1/2 (tuberous sclerosis complex 1/2).

1.4.5 mTOR

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that forms the catalytic domain of two distinct complexes; mTORC1 and mTORC2. In addition to mTOR, mTORC1 is composed of mLST8 (mammalian lethal with Sec13 protein 8, also known as GβL) and Raptor (regulatory associated protein of mTOR). Two further proteins have also been found to interact with mTORC1, PRAS40 (proline-rich Akt substrate 40 kDa), and Deptor (DEP-domain containing mTOR interacting protein), both of which are believed to have an inhibitory influence on mTORC1 (Haar *et al.*, 2007; Peterson *et al.*, 2009). mTORC2 is less studied than mTORC1 and contains mTOR, mLST8, Rictor (rapamycin-insensitive companion of mTOR), Sin1 (stress-activated protein kinase interacting protein 1) and Proctor. Deptor has also been shown to interact with and inhibit mTORC2 (Peterson *et al.*, 2009). Notably, mTORC1 and mTORC2 can be distinguished by their sensitivity to the immunosuppressant drug, rapamycin. Rapamycin binds to the intracellular protein FKBP12 and together interacts with mTOR only within mTORC1, specifically inhibiting mTORC1 activity (Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004). However, it should be noted that some rapamycin-insensitive functions of mTORC1 have been identified, examples will be given below. As reviewed by Menen and Manning (2008), mTORC1 activity is frequently upregulated in a number of cancers, including breast cancer, and as a result there is much interest in furthering our understanding of this pathway.

1.4.5.1 mTORC1 substrates

The best known substrates of mTORC1 are the eIF4E binding proteins (4E-BP) and 70 kDa ribosomal S6 protein kinases (p70 S6K), both of which are involved in the control of mRNA translation. It seems that these substrates are directed to mTOR by Raptor via Tor signalling motifs (TOS) (Nojima *et al.*, 2003). The 4E-BP family are key negative regulators of the eukaryotic initiation factor 4F (eIF4F) complex required for cap-dependent translation. eIF4F complexes form at the 5' end of mRNA and are composed of three subunits, the scaffold protein eukaryotic initiation factor 4G (eIF4G), the RNA-helicase eukaryotic initiation factor 4A (eIF4A) and the cap-binding protein eukaryotic initiation factor 4E (eIF4E). Specifically, the 4E-BPs, of which 4E-BP1 is the most prominent, tightly regulates the availability of eIF4E by competing with eIF4G for an overlapping binding site on eIF4E in a phosphorylation-dependent manner. Hypophosphorylated 4E-BP1 binds strongly to eIF4E thus preventing the assembly of the eIF4F complex (Haghighat *et al.*, 1995; Lin *et al.*, 1994). mTORC1 phosphorylates

4E-BP1 at multiple sites in a hierarchical manner. Phosphorylation of Thr37 and Thr46 occur first, in response to amino acids, and although these modifications are not sufficient to inhibit the interaction between 4E-BP1 and eIF4E, they act as a priming step for the phosphorylation of Thr70 and then Ser65 when mTORC1 is further stimulated by growth factors (Gingras *et al.*, 2001). However, while phosphorylation of all 4E-BP1 sites is mediated by mTORC1, phosphorylation of Thr37 and Thr46 are insensitive to rapamycin (Wang *et al.*, 2005).

The other main mTORC1 substrate, p70 S6K, is itself a serine/threonine protein kinase, and phosphorylation by mTORC1 increases its activity. Targets of p70 S6K include the S6 ribosomal protein, which forms part of the 40S ribosomal subunit, eukaryotic initiation factor 4B (eIF4B) (Raught *et al.*, 2004) and programmed cell death protein 4 (Pdcd4) (Dorrello *et al.*, 2006), which respectively enhance and impair mRNA helicase eIF4A, and the eukaryotic elongation factor 2 kinase (eEF2K), which inhibits eEF2 (Wang *et al.*, 2001). p70 S6K is known to be an important regulator of cell size (Shima *et al.*, 1998) and considering the known targets of the kinase it seems p70 S6K has a positive influence on ribosome biogenesis and protein synthesis. However, as several p70 S6K targets are also phosphorylated by downstream effectors of the MAPK pathway, its impact on the overall control of protein synthesis is not clear (Shahbazian *et al.*, 2006; Wang *et al.*, 2001).

mTORC1 can also increase the protein synthesis capacity of cells by upregulating specific mRNAs such as those encoding ribosomal proteins. Many of these mRNAs contain a tract of pyrimidines in their 5' untranslated regions (5'UTR) that ordinarily impair translation. The ability of mTORC1 to control translation of 5' tract oligopyrimidine (5'TOP) mRNAs was originally attributed to p70 S6K however, this has since been found not to be the case (Pende *et al.*, 2004). Therefore, despite this process being sensitive to rapamycin, the exact mechanism by which mTORC1 increases translation of these mRNAs remains unknown.

1.4.5.2 Upstream control of mTORC1

The PI3K/Akt and MAPK pathways both converge on mTORC1 to increase protein synthesis in response to hormones and growth factors. mTORC1 is also regulated by other pathways that sense whether or not conditions are favorable for cell growth and proliferation, such as nutrient availability, cellular energy levels and oxygen levels, activating or inhibiting mTORC1 accordingly (Figure 1.6). The response of mTORC1 to many of these cellular stresses and growth factors is orchestrated by the tuberous

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sclerosis complex 1/2 (TSC1/2) (Dibble and Manning, 2010). Although it should be noted that mTORC1 can also be regulated independently of TSC1/2, as observed in amino acid mediated activation of mTORC1 (Smith *et al.*, 2005).TSC1/2 is a heterodimeric complex composed of TSC1 (also known as hamartin) and TSC2 which acts as a negative regulator of mTORC1. Genetic mutations in either gene can cause the genetic disorder of the same name, TSC, with patients presenting with multiple benign tumours (hamartomas) in various organs including the brain and kidneys (Kwiatkowski, 2003). TSC2 acts as a guanosine triphosphatase activating protein (GAP), stimulating the intrinsic GTPase activity of Ras homolog enriched in brain (Rheb), and catalysing its conversion from GTP to GDP bound state. It has been shown that Rheb-GTP activates mTORC1, although the precise mechanism behind this is unknown (Inoki *et al.*, 2003a; Tee *et al.*, 2003).

Many of the pathways shown to act via TSC1/2 to control mTORC1 signalling can also influence mTORC1 in a TSC1/2-independent manner by phosphorylating Raptor (Carrière *et al.*, 2008; Gwinn *et al.*, 2008). In addition, Akt can phosphorylate PRAS40, another protein known to associate with the mTORC1 complex (Haar *et al.*, 2007). Interestingly, PRAS40 has also been identified as a substrate for mTORC1 and it seems that phosphorylation by both Akt and mTOR is required for its dissociation from the mTORC1 complex. However, the functional role of this is not fully understood, as while PRAS40 dissociation has been shown to increase 4E-BP1 binding to mTORC1, it does not promote mTORC1 signalling (Rapley *et al.*, 2011).

1.4.5.3 mTORC2

Although mTORC2 activity is enhanced by growth factors little is known about the signalling events involved in mTORC2 regulation (García-martínez and Alessi, 2008). One of the main functions of mTORC2 is to phosphorylate Akt on Ser473, which along with phosphorylation on Thr308 by PDK1 is required for full Akt activation (Sarbassov *et al.*, 2005). However, while this would suggest mTORC2 acts upstream of mTORC1, the disruption of mTORC2 stability and subsequent loss of Ser473 phosphorylation on Akt did not attenuate phosphorylation of TSC2 (Jacinto *et al.*, 2006). mTORC2 also phosphorylates and activates serum- and glucocorticoid-induced protein kinase 1 (SGK1) which is involved in activating various sodium and potassium channels (García-martínez and Alessi, 2008), and protein kinase Cα (PKCα) which has a role in multiple cellular processes including apoptosis, cell cycle control and regulation of cell shape and mobility (Sarbassov *et al.*, 2004).
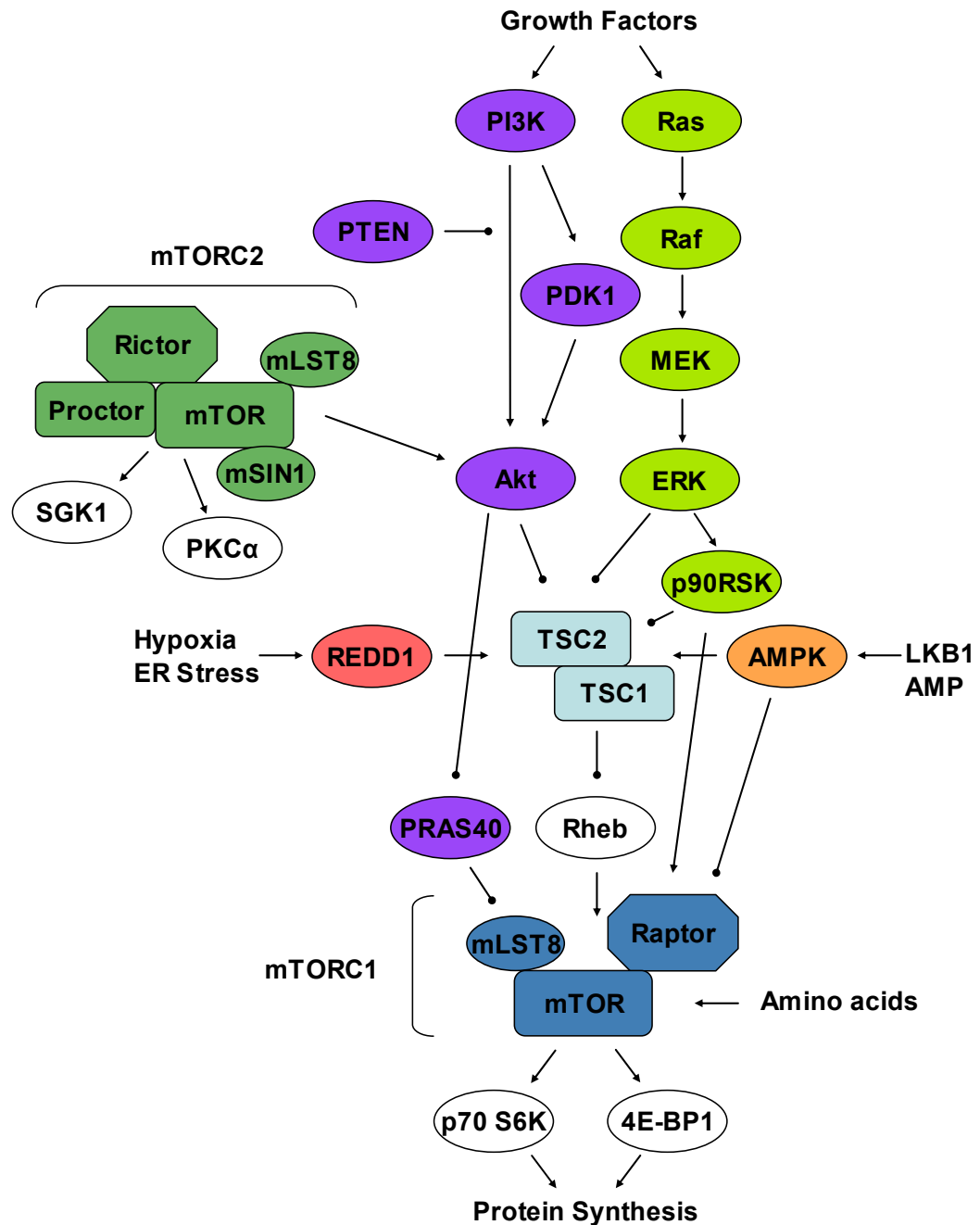
Figure 1.6: Outline of the mTORC1 pathway.
mTORC1 (mammalian target of rapamycin complex 1) is regulated by multiple pathways that sense whether or not conditions are favorable for cell growth and proliferation, such as nutrient availability, cellular energy levels and oxygen levels and the presence of growth factors, activating or inhibiting mTORC1 accordingly. p70 S6K (70 kDa S6 ribosomal protein kinase) p90RSK (90 kDa ribosomal protein kinase), AMPK (5' adenosine monophosphate-activated kinase), 4E-BP1 (eIF4E binding protein 1), ERK (extracellular-signal regulated kinase), PKCα (protein kinase C α), PTEN (phosphatase and tensin homolog deleted on chromosome 10), PI3K (phosphoinositide 3-kinase), PDK1 (phosphoinositide dependent kinase 1), PRAS40 (proline-rich Akt substrate 40 kDa), mLST8 (mammalian lethal with Sec13 protein 8), MEK (mitogen activated protein kinase kinase), Raptor (regulatory associated protein of mTOR), Rheb (Ras homolog enriched in brain), REDD1 (regulated in development and DNA damage response 1), Rictor (rapamycin-insensitive companion of mTOR), SGK1 (Serum- and glucocorticoid-induced protein kinase 1), Sin1 (stress-activated protein kinase interacting protein 1), TSC1/2 (tuberous sclerosis complex 1/2).

1.5   Regulation of mRNA translation

In eukaryotes the majority of mRNAs are translated via cap-dependent translation, which involves three steps; initiation, elongation and termination. This is a tightly controlled process with multiple factors regulating both initiation and elongation. Notably, many of the factors involved in the regulation of mRNA translation have been implicated in downstream oncogenic signalling (Rajasekhar and Holland, 2004).

Initiation begins with the formation of a eukaryotic initiation factor 4F (eIF4F) complex at the 7-methyl guanosine cap at the 5' end of mRNA (Figure 1.7). eIF4F is composed of three subunits, the scaffold protein eIF4G, the cap-binding protein eIF4E, and the RNA-helicase eIF4A which is thought to unwind secondary structure in the 5'UTR. The small 40S ribosomal subunit and associated eIF2-GTP-Met-tRNA$_i$ complex is recruited to the eIF4F complex by ribosome-associated protein eIF3 which interacts with the eIF4G scaffold protein. eIF4G also interacts with the poly(A)-binding protein (PABP) which binds to the poly-A tail present in most eukaryotic mRNAs, resulting in the circularisation of the mRNA molecule (Cormier *et al.*, 2003). A further eukaryotic initiation factor, eIF4B, binds to eIF4A and eIF3, enhancing the helicase activity of eIF4A. eIF4B is a target for p70 S6K phosphorylation which increases its affinity to eIF3 (Raught *et al.*, 2004). Binding of eIF4E to the mRNA 5'-cap is the rate limiting step of cap dependent translation and as a result is tightly regulated. As already mentioned, the availability of eIF4E is controlled by 4E-BPs, which when hypophosphorylated sequester eIF4E away from the eIF4F complex. However, eIF4E can also be regulated by MAPK-signal integrating kinases 1 and 2 (Mnk1/2; also known as MAPK-interacting kinases), which lie downstream of ERK and p38 MAPK, and interact with eIF4G. Phosphorylated eIF4E displays a decreased affinity for the 5'-cap structure, despite the fact signalling pathways that lead to its phosphorylation are associated with increased protein synthesis (Scheper *et al.*, 2002). It is proposed that this facilitated dissociation of eIF4E from 5'-cap of an mRNA molecule already being translated allows eIF4E to bind other mRNAs.

As reviewed in Kimball (1999), after the formation of eIF4F complex at the 5' end of the mRNA molecule and recruitment of the 40S ribosomal subunit, this pre-initiation complex (PIC) scans along the mRNA in the 5' to 3' direction until it locates the AUG start codon. The Met-tRNA$_i$ is then transferred to the 40S ribosomal subunit by hydrolysis of eIF2-GTP to eIF2-GDP facilitated by the GTPase activating protein eIF5.
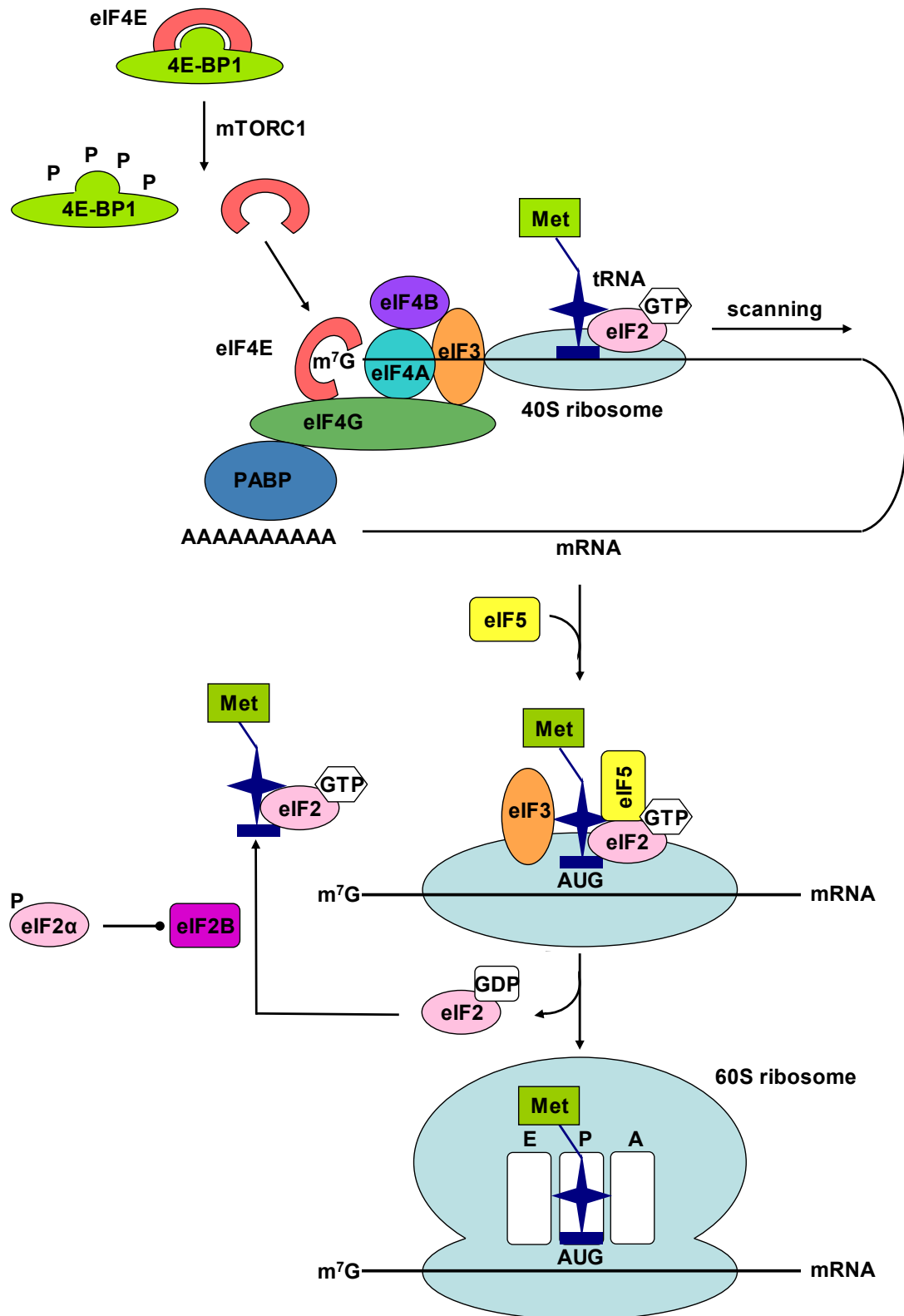
Figure 1.7: Eukaryotic translation: initiation.
Briefly, various eukaryotic initiation factors (eIFs) form a complex at the 5' cap of mRNA and recruit the 40S ribosome. When the start codon is identified Met-tRNA$_i$ is transferred to the 40S ribosomal subunit by hydrolysis of eIF2-GTP to eIF2-GDP facilitated by eIF5. The 60S ribosome then binds and elongation can begin. See text for more details. 4E-BP1, eIF4E binding protein 1; mTORC1, mammalian target of rapamycin 1; PABP, poly(A)-binding protein.

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The 60S ribosomal subunit is then recruited to form the functional 80S ribosome and elongation of the polypeptide chain can begin. The guanine nucleotide exchange factor (GEF) eIF2B is required for the regeneration of eIF2-GDP to its active eIF2-GTP state and continued initiation of translation and protein synthesis. Regulation of this step provides an important mechanism by which the rate of translation can be controlled. eIF2 is comprised of three subunits, α, β and γ, and when Ser51 on the α subunit is phosphorylated it acts as a competitive inhibitor for eIF2B, displaying a higher affinity than unphosphorylated eIF2 (Webb and Proud, 1997).

Elongation requires three eukaryotic elongation factors, eEF1α, eEF2β and eEF2 (Figure 1.8). The aminoacyl-tRNA corresponding to the next codon in the mRNA is recruited by GTP bound eEF1α and transferred to the A (aminoacyl) site of the 80S ribosome by hydrolysis of eEF1α -GTP to eEF1α-GDP. However, before eEF1α can recruit another aminoacyl-tRNA it must first be regenerated to its active eEF1α-GTP state. This is catalysed by the GEF, eEF2β. Once positioned, a peptide bond is formed between the two amino acids, catalysed by the ribosome, and eEF2 is required for the translocation of the peptidyl chain along the ribosome from the A site to the P (peptidyl) site (Proud, 2007). Phosphorylation of eEF2 at Thr56 by eEF2K disrupts eEF2 binding to the ribosome effectively inhibiting its activity and thus translation (Carlberg *et al.*, 1990). eEF2K is a calcium/calmodulin (CAM)-dependent kinase also regulated by multiple phosphorylation sites which have both activatory (e.g. cAMP-dependent protein kinase; PKA) and inhibitory (e.g. p70 S6K) influences (Diggle *et al.*, 2001; Wang *et al.*, 2001).

1.6 Benefits of healthy diet

In 1989, following numerous epidemiological studies that reported diets rich in fruit and vegetables reduced incidence of cancer and heart disease, the National Academy of Sciences' report on diet and health made the recommendation to eat 5 or more servings of a variety of fruit or vegetables every day (National Academy of Sciences, 1989). The 5-a-Day program was subsequently initiated to encourage the consumption of at least five 80 g servings of fruit or vegetables daily, and promote public awareness of the potential health benefits (Liu, 2004). Although it should be noted Boffetta *et al.* (2010) recently reported that while there is an inverse correlation between fruit and vegetable consumption and cancer risk, the effect is not as great as once thought.
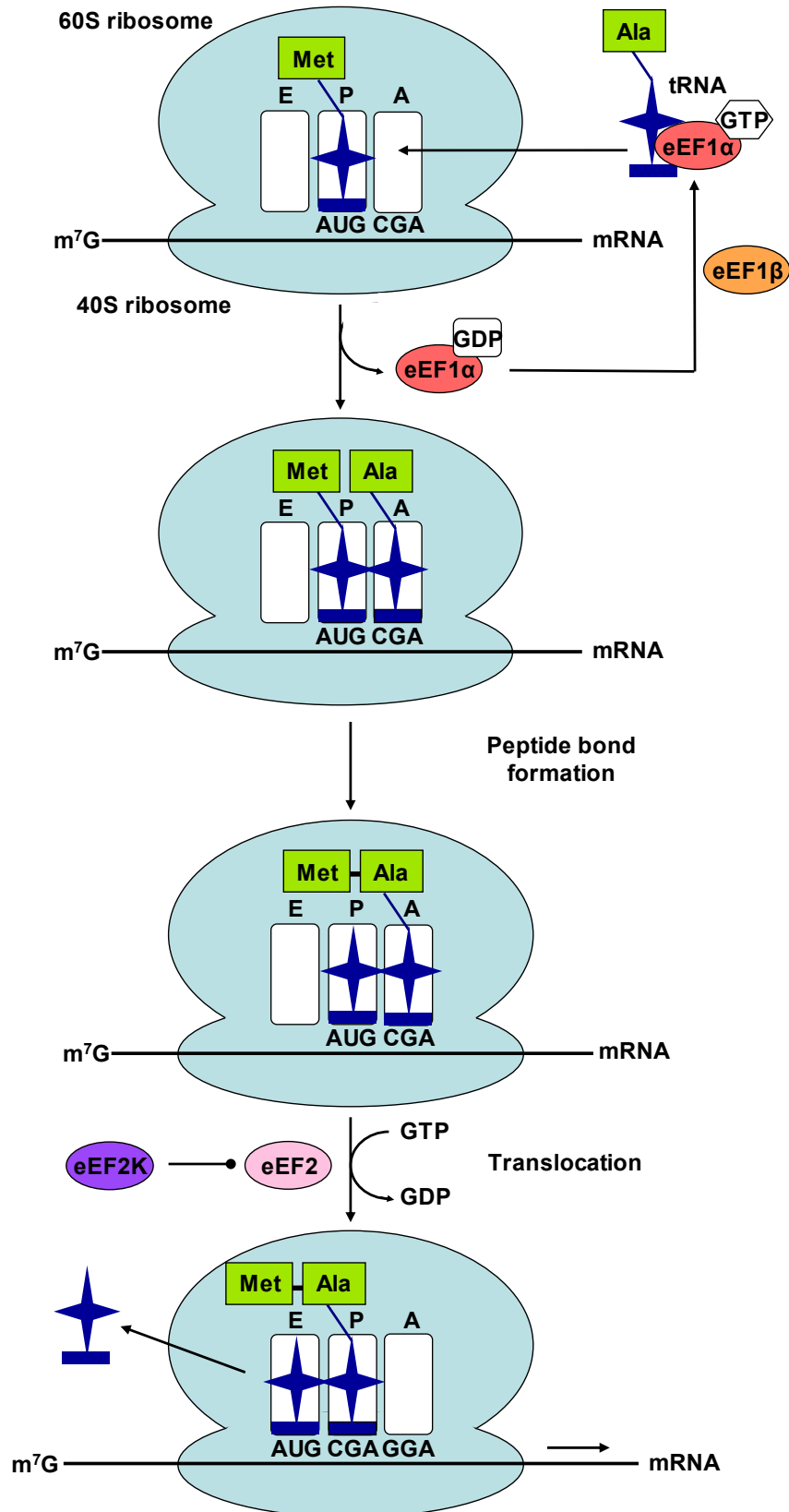
Figure 1.8: Eukaryotic translation: elongation.
Briefly, hydrolysis of eukaryotic elongation factor 1α (eEF1α) transfers the next aminoacyl-tRNA to the aminoacyl (A) site of the ribosome. Following peptide bond formation eEF2 aids in translocation of the tRNA with the peptidyl chain from the A site to the peptidyl (P) site, and the deacylated tRNA moves to the exit (E) site and exits the ribosome. The process can then be repeated. See text for more details.

Specifically, cruciferous vegetable intake has been associated with reduced cancer risk (Verhoeven *et al.*, 1996). In one study, subtype analysis of specific groups of vegetables identified a stronger inverse association with consumption of cruciferous vegetable and reduced risk of colorectal cancer, than overall consumption of fruit and vegetables (Voorrips *et al.*, 2000).

1.7 Watercress

1.7.1 Cultivation

Watercress (*Nasturtium officinale*, formerly *Rorippa nasturtium-aquaticum*) belongs to the Brassicaceae (Cruciferae) family along with Brussels sprouts, broccoli, and cabbage (Figure 1.9). Originating in Europe, watercress has been used since the first century AD for medicinal purposes and consumption as a raw leaf crop. Watercress is now cultivated around the world and, as well as being eaten raw as a salad vegetable, is becoming a popular addition to a variety of cooked dishes (Palaniswamy and McAvoy, 2001).

Watercress is a perennial semi-aquatic crop, thriving in alkaline waters, and can often be found growing wild in and around open-running waterways in chalk or limestone areas. The first recorded commercial cultivation of watercress in Britain dates back to the early 1800s when it was grown along the River Ebbsfleet at Springfield, Kent. In the last 80 years it has been common practice to cultivate watercress in purpose built gravel lined beds that are fed by a constant flow of natural spring water. The temperature of the water is important as it provides a source of heat in the colder months, maintaining growth, and ideally should be 10-11°C. Due to an abundance of uncontaminated alkaline water with a constant temperature at source of 10°C or 11°C, watercress production in Britain tends to be concentrated in Hampshire, Wiltshire and Dorset (Stevens, 1983).

Although watercress can be vegetatively propagated from cuttings this limits genetic variation and means the crop becomes susceptible to diseases, such as fungal crook root disease. As a result watercress is now typically propagated from seed. Commercially, seeds are germinated in polytunnels on a thin layer of soil and at 7-10 days old are transplanted into gravel lined concrete beds. Watercress grows best in spring and autumn, and during this time has a growing period of approximately 4-6

Figure 1.9: Image of green watercress growing in a commercial bed.

weeks from transplanted seedling to mature crop. Watercress flowers under long day conditions, appearing as small white clusters, traditionally meaning that watercress was not sold in the summer months. However, this has now been largely overcome by selecting for late flowering cultivars (Stevens, 1983). In the colder winter months watercress grows much more slowly and in order to maintain annual supply in Britain, the crop is grown abroad in warmer climates and imported back. For example, Vitacress Salad Leaves Ltd. supply the UK with British grown watercress from March through to November, but grows watercress in farms in the USA from November to April to provide watercress over the winter months. In addition, Vitacress Salad Leaves Ltd. grow watercress throughout the year in farms in Portugal.

Up until the Second World War there were two species of watercress being cultivated, the typical green watercress seen cultivated today and brown watercress. Brown watercress, characterised by brown/purple leaves, is a sterile hybrid between *Nasturtium officinale* and a wild watercress, *Nasturtium microphyllum* (formally *Rorippa microphylla*). Although, as brown watercress could only be propagated vegetatively it was susceptible to disease and now green watercress is the only species currently cultivated commercially. Nonetheless, within this species there are multiple cultivars. Most of the commercially available watercress can be characterised by their dark

green circular leaves and are believed to be genetically very similar. However, as every commercial grower carries out their own selections for desirable characteristics each have their own unique cultivar (Stevens, 1983). Interestingly, an American red variety of watercress has recently been commercially cultivated. It is characterised by red/purple leaves and is also sterile so perhaps has similar origins to brown watercress.

1.7.2   Increasing the anticancer activity of watercress

Given the public interest in healthy diet, it might be considered a commercial and health benefit to generate crops with enhanced anticancer activity. Indeed, previous studies have manipulated both genetics and environmental factors to increase glucosinolate concentration in a variety of cruciferous vegetables.

1.7.2.1 Genetics

Conventional breeding involves cross-pollination of two related plants to produce a new variety with favourable genetic traits from both and an absence of any negative traits. This is a laborious process. As per the nature of sexual recombination, progeny inherit a random mix of genes, and hence further selection and cross breeding over several generations is required to identify a variety with all the desired traits. On the other hand, genetic engineering of crops allows for the insertion of a single gene or genes associated with a specific trait into the genome of another plant. This produces a crop with the desired trait without altering the parent genotype. However, there is still public concern on the long term effects of consuming genetically modified (GM) crops and as a result they are often avoided by consumers (Harlander, 2002).

Using plant genomics to identify genetic markers linked to desired traits allows for improved selection of progeny generated from conventional breeding programs, speeding up the process. This marker-assisted breeding was utilised in the generation of an isothiocyanate-enriched variety of broccoli (Mithen *et al.*, 2003). The introgression of three genomic segments from the *Brassica villosa*, a wild relative of broccoli, into the genetic background of commercial broccoli (*B. oleracea* var. *italica*) was shown to increase glucosinolate levels. These three segments were identified as being responsible for the increased glucosinolate content of wild broccoli and while each contains a quantitative trait locus (QTL), the exact function of the gene or genes

remain unknown. Compared to commercial broccoli, the broccoli hybrid was 80-fold more potent at inducing quinone reductase activity. This effect was attributed not only to an increase in 3-methylsulphinylpropyl and 4-methylsulphinylbutyl glucosinolates, but also a greater conversion of the glucosinolate precursors to their respective isothiocyanates, iberin and sulforaphane.

1.7.2.2 Environment

Previous groups have reported that glucosinolate concentration in a variety of cruciferous vegetables was effected by temperature, photoperiod, light quality, water availability, and sulfur and nitrogen fertilisation (Bouchereau *et al.*, 1996; Engelen-Eigles *et al.*, 2006; Rosen *et al.*, 2005).

In studies looking at watercress, crops grown under long days (16 h), low night temperatures (10°C or 15°C), and supplemented with red light (light quality) were found to have higher gluconasturtiin concentrations, the glucosinolate precursor of PEITC, compared to those grown under short days (8 h), higher night temperatures (20°C or 25°C), and far-red light supplementation (Engelen-Eigles *et al.*, 2006). Increasing either sulfur or nitrogen fertilisation in watercress was also found to enhance glucosinolate concentration. However, while sulfur fertilisation increased glucosinolate levels in a linear manner, there was an optimal concentration of nitrogen with the highest nitrogen concentration causing a drop in glucosinolate content (Kopsell *et al.*, 2007).

1.7.3   Key active components in watercress

1.7.3.1 Glucosinolates

Cruciferous vegetables are characteristically rich in glucosinolates, nitrogen and sulfur containing glycosides, which make up a class of relatively inert phytochemicals. However, upon tissue damage, such as bruising, cutting or mastication, glucosinolates are hydrolysed by the plant β-thioglucosidase enzyme, myrosinase, to generate various bioactive compounds. The disruption to the tissue acts to break the physical barrier between the glucosinolates and myrosinase, with the latter being sequestered within aqueous vacuoles (Fahey *et al.*, 2001; Holst and Williamson, 2004). Microflora present in the gut also have myrosinase activity although it is relatively limited. Thus,

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individuals consuming watercress that has been cooked to completely denature all the plant myrosinase have considerably less glucosinolate metabolites in their urine (Getahun and Chung, 1999).

1.7.3.1.1 Glucosinolate metabolism

Structurally, glucosinolates are (cis)-N-hydroximinosulphate esters with a sulfur-linked β-[D]-glucopyranose group and a highly variable side chain (R) that may include aliphatic, aromatic or heterocyclic groups. Myrosinase cleaves off the β-glucosyl moiety by hydrolysing the thioglycosidic bond to generate glucose, sulfate and the unstable aglycone, thiohydroxamate-O-sulfonate (Fahey *et al.*, 2001). Depending on the R side chain, reaction conditions and the presence of specifier proteins, thiohydroxamate-O-sulfonate rapidly converts to a series of hydrolysis products, principally isothiocyanates, nitriles, or thiocyanates (Figure 1.10). The generation of isothiocyanates from glucosinolates is believed to be adapted as a defence mechanism, making the plant bitter and unpalatable to predators (Rask *et al.*, 2000).

1.7.3.1.2 Isothiocyanates

Watercress contains several glucosinolates which can be subsequently converted to their constituent isothiocyanate (Table 1.3). The most abundant isothiocyanate derived from watercress is phenethyl isothiocyanate (PEITC), with watercress being the richest dietary source of this compound.
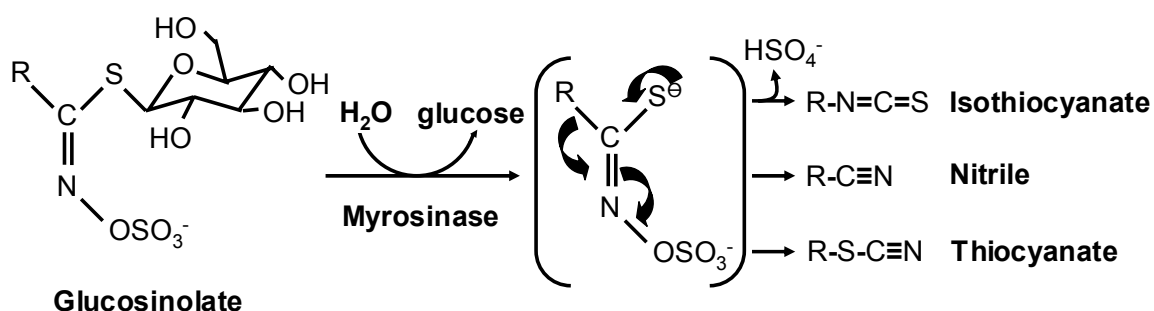
Figure 1.10: General schematic of the hydrolysis of a glucosinolate to an isothiocyanate, a nitrile or a thiocyanate by myrosinase.

The exact product of hydrolysis depends on the R side chain of the glucosinolate. Adapted from Fahey *et al.* (2001).

Glucosinolate		Isothiocyanate		
Chemical Name	**Common Name**	**Chemical Name**	**μmol/g fresh weight**	**μmol/g dry weight**
2-Phenylethyl-GLS	Gluconasturtiin	2-Phenylethyl-ITC	1.53[1]	17.98[1], 23.7[2]
6-Methylsulfinylhexyl-GLS	Glucohesperin	6-Methylsulfinylhexyl-ITC		0.2[2]
7-Methylsulfinylheptyl-GLS	Glucoibarin	7-Methylsulfinylheptyl-ITC	0.1[1]	1.07[1], 3.9[2]
8-Methylsulfinyloctyl-GLS	Glucohirsutin	8-Methylsulfinyloctyl-ITC	0.06[1]	0.68[1], 2.1[2]
7-Methylthioheptyl-GLS	-	7-Methylthioheptyl-ITC		1.8[2]
8-Methylthiooctyl-GLS	-	8-Methylthiooctyl-ITC		0.8[2]
4-Methoxy-3-indolylmethyl-GLS	4-Methoxyglucobrassin	4-Methoxy-3-indolylmethyl-ITC	0.065[1]	0.791[1] 0.7[2]
3-Indolylmethyl-GLS	Glucobrassin	Indole-3-carbinol	0.04[1]	0.43[1]

Table 1.3: The isothiocyanate and glucosinolate content of watercress.

Information taken from [1]Gill *et al.* (2007) and [2]Rose *et al.* (2000)

Isothiocyanates are generally very electrophilic and as a result rapidly conjugate with thiols. This can occur either via enzymatic or non-enzymatic processes, and subsequently the issue of enzyme availability is not rate-limiting for the formation of thiol conjugates. As the most abundant thiol in cells is glutathione (GSH), most isothiocyanates after diffusion into cells form S-(N-phenylethylthiocarbamoyl) glutathione. These dithiocarbamate conjugates can then be exported out of the cell by various transporters such as multidrug resistance protein 1 (MRP1) and enter the mercapturic acid pathway. Here they are metabolised to cysteine conjugates and finally acetylated to mercapturic acids, which are isothiocyanate-*N*-acetylcysteine conjugates (ITC-NAC) in the kidney and excreted in the urine (see Figure 1.11 for metabolism pathway). ITC-NACs are the main ITC metabolite excreted by humans in the urine, and have been used to study the bioavailability of ITCs (Callaway *et al.*, 2004). Chung *et al.* (1992) determined that after consumption of 30 g watercress, containing 21.6 mg gluconasturtiin, 30-67% of the gluconasturtiin was recovered as the mercapturic acid metabolite NAC-PEITC in the urine. Assuming that there was quantitative hydrolysis to PEITC, 21.6 mg gluconasturtiin would yield 7.6 mg PEITC. Furthermore, the excretion of NAC-PEITC in the urine peaked between 2-4 h and was completely absent by 24 h, suggesting PEITC is rapidly metabolised.
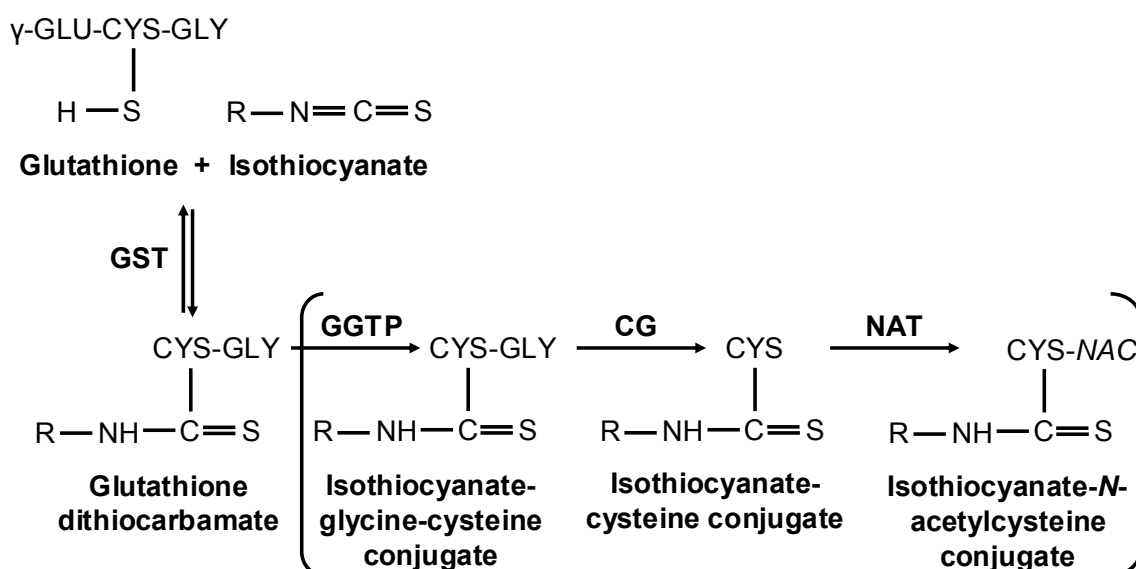
Figure 1.11: The metabolism of isothiocyanates.

After conjugation of glutathione and isothiocyanate to form glutathione dithiocarbamate, glutathione dithiocarbamate enters the mercapturic acid pathway ([ ]) to generate an isothiocyanate-N-acetylcysteine (GST, glutathione-S-transferase; GGTP, γ-glutamyltransferase; CG, cysteinylglycinase; NAT, N-acetyltransferases). Adapted from Holst and Williamson (2004).

However, following export of GSH-ITC conjugates out of the cell, extracellular hydrolysis can also occur, releasing free ITCs which are able to re-enter the cell and again react with GSH. This results in cellular depletion of GSH and rapid accumulation of intracellular ITCs, 100-200-fold over extracellular concentrations (Zhang, 2001). Once GSH levels are depleted, ITCs can conjugate to other protein cysteinyl thiols and this is thought to be important in ITC-mediated apoptosis and cell cycle arrest (Figure 1.12). In human non-small lung cancer A549 cells treated with PEITC, intracellular levels of PEITC–GSH conjugates reached a maximum at 30 min post treatment and dropped rapidly thereafter. By contrast, PEITC–protein content gradually increased, accounting for 87% of total cellular content by 4 h. Interestingly, SFN–protein conjugates accounted for only 12% of total SFN uptake at the same time point. PEITC appears to bind to proteins more readily than SFN, perhaps accounting for its more potent apoptosis promoting activity (Mi *et al.*, 2007). However, the critical targets involved in growth inhibition by ITCs are not known and only a few proteins have been shown to be direct targets for ITCs, including negative regulator of Nrf2, Keap1, cytoskeletal protein α-tubulin and protein kinase MEKK1 (Brown and Hampton, 2011).

Furthermore, as GSH is a major antioxidant its depletion by ITCs reduces the redox buffering capacity of the cell and causes an increase in intracellular reactive oxygen species (ROS). Generation of ROS is thought to contribute to ITC-mediated apoptosis, as the addition of hydrogen peroxide-scavenging enzyme catalase attenuated PEITC-induced cell death (Trachootham *et al.*, 2006).

Interestingly, the indole glucosinolates such as glucobrassin, which is present in watercress, generate very unstable isothiocyanates that readily undergo hydrolysis leaving the subsequent alcohol indole-3-carbinol (I3C), in the case of glucobrassin, and a thiocyanate ion. Furthermore, I3C can further condense, and in an acidic pH such as that provided by the gut, form various condensation products including 3,3'-diindoylmethane (DIM) that can then form dimers, trimers and tetramers (Grose and Bjeldanes, 1992).

1.7.4 Carotenoids

Carotenoids are another class of photochemical that can be further split into two groups of compounds, the carotenes such as β-carotene and α-carotene that are unoxygenated, and the xanthophylls like lutein and zeaxanthin that contain oxygen. Watercress contains relatively high concentrations of β-carotene, a precursor to
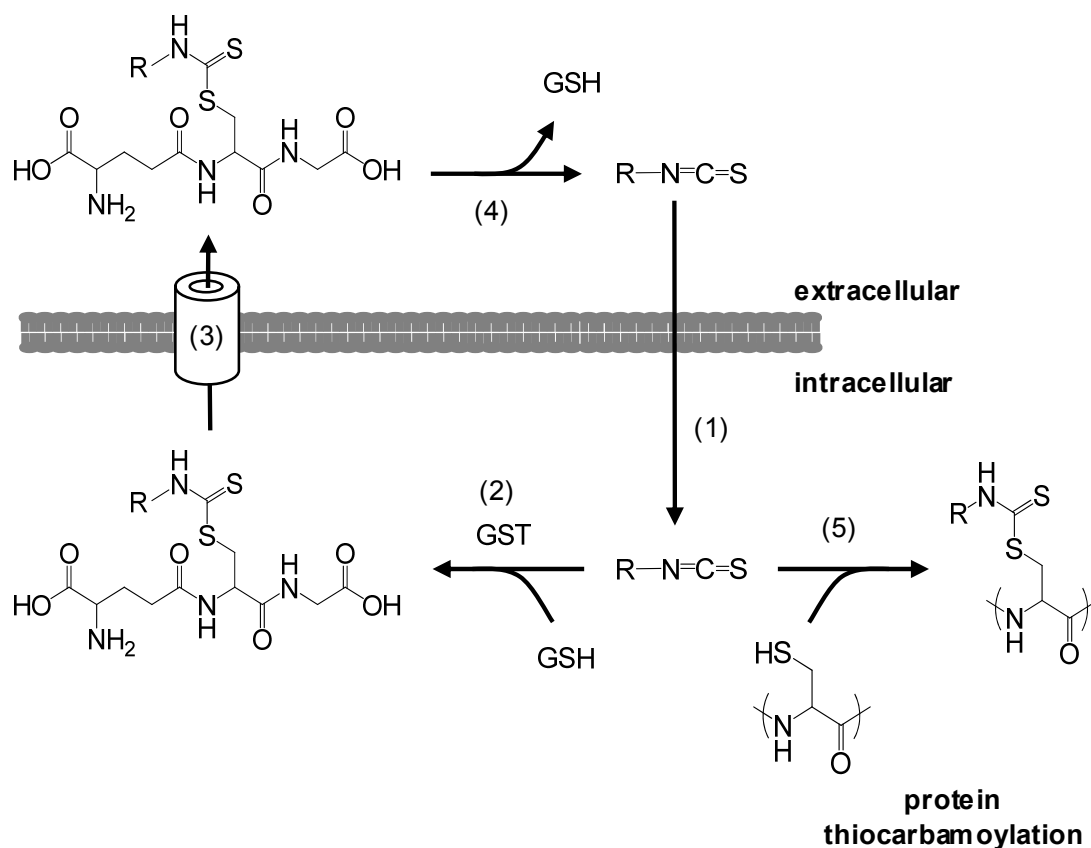
Figure 1.12: Intracellular accumulation of isothiocyanates.

Following their initial diffusion into cells (1), isothiocyanates (ITCs) are rapidly conjugated to glutathione (GSH) via the action of glutathione-S-transferases (GST) (2). The GSH conjugates are exported from the cells via efflux pumps (3). Extracellular hydrolysis of the GSH conjugate (4) gives rise to the free ITC which is able to re-enter the cell. The net effect of this cycle is the rapid (1–3 h) and profound (100- to 200-fold) accumulation of ITC and the concomitant depletion of GSH. Once GSH levels are reduced, ITCs are able to conjugate to intracellular protein cysteinyl thiols. From Cavell *et al.* (2011).

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vitamin A, and lutein. Despite there being variation between sources on the exact content of both β-carotene and lutein in watercress the amount of lutein is generally twice that of β-carotene (Table 1.4). Following the consumption of 85g of raw watercress daily for a period of 8 weeks, plasma concentration of β-carotene increased by 33% and that of lutein increased by 100% (Gill *et al.*, 2007).

1.7.5 Phenolics

Flavonoids are antioxidant polyphenolics consisting of five subclasses of which watercress contains compounds present in two, the flavones and the flavonols (Table 1.5). In plants these compounds are typically glycosylated, the majority thought to be converted to the more active aglycone by cleavage of the glycoside in the gastrointestinal tract. Of particular interest is quercetin which is present in considerably higher amounts than any of the other flavonoids. Quercetin is present in watercress in the form of various glycosides such as rutin, consisting of quercetin and the disaccharide rutinose (Rice-Evans *et al.*, 1996). Watercress also contains a relatively large concentration of the phenolics, hydroxycinamic acid derivatives (Gill *et al.*, 2007).

1.8   Anticancer effects of PEITC

The breakdown products of glucosinolates were initially recognised as potential agents in preventing cancer by their ability to alter the activity of phase I and phase II drug metabolising enzymes. More recent studies point to there being a direct anticancer role for ITCs, by inducing apoptosis and inhibiting cell cycle progression, angiogenesis and metastasis. However, despite this, the upstream triggering events remain largely unknown.

The anticancer activity of ITCs has been the topic of multiple reviews (Cavell *et al.*, 2011; Cheung and Kong, 2010; Zhang *et al.*, 2006). As a result, this introduction will focus on the effects of ITCs on Nrf2 activity and angiogenesis as this relates specifically to the work within this study. Nonetheless, a brief overview of the potential mechanisms contributing to the anticancer effects of PEITC will be given in order to provide an insight into the scope of downstream effectors.

Carotenoid	µg/100g fresh weight
β-carotene	4777[1], 2520[2]
Lutein	10713[1], 5767[2] (lutein and zeaxanthin)

Table 1.4: The carotenoid content of watercress.

Information taken from [1]Hart and Scott (1995) and [2]Food Standards Agency (2002).

Flavonoids	µg/100g fresh weight
Flavones	
Apigenin	10
Luteolin	20
Flavonols	
Kaempferol	1400
Myricetin	200
Quercetin	7440

Table 1.5: The flavonoid content of watercress.

Information from the USDA Database for the Flavonoid Content of Selected Foods, Release 2 (2006).

1.8.1 Chemopreventive effects

Phase I enzymes include cytochrome P-450 enzymes (CYP), reductases, and hydrolases, the former being the most influential, which are involved in the biotransformation and subsequent activation of many chemical carcinogens. On the other hand phase II enzymes like glutathione-S-transferase (GST), N-acetyltransferase (NAT) and NAD(P)H:quinone oxidoreductase 1 (NQO1) are detoxification enzymes that convert carcinogens to polar conjugates so that they can be easily excreted in the urine or bile and therefore removed from the body (Conaway *et al.*, 2002).

Inhibition of carcinogen-induced tumourigenesis in rodents by PEITC has been demonstrated in a variety of tissues and is associated with a reduction of DNA adducts and carcinogen metabolism, as well as increase in carcinogen excretion (Huang *et al.*, 1993; Morse *et al.*, 1989; Stoner *et al.*, 1991). For example, PEITC has been shown to inhibit lung tumourigenesis induced by key tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in rats. F344 rats were fed approximately 49 µM/d PEITC for 1 week prior and for the following 111 weeks during which NNK was also given. An increase in NNK metabolites, NNAL and NNAL-Gluc, in the urine of rats treated with PEITC and NNK compared to just NNK, suggests that PEITC not only enhances detoxification but also interferes with the metabolic activation of NNK that otherwise would give rise to more harmful intermediates (Hecht *et al.*, 1996). As a result these chemopreventive effects of PEITC are believed to be due to modulation of the activity of phase I and II drug metabolising enzymes.

Indeed, PEITC has been shown to induce activity of several phase II drug metabolising enzymes including GST and NQO1 *in vitro* and *in vivo* (Dingley *et al.*, 2003; Rose *et al.*, 2000). GST catalyses the conjugation of GSH to electrophilic moieties on a variety of substrates (Armstrong, 1991), while NQO1 catalyses two electron reduction of quinones, effectively preventing one electron reduction that would give rise to harmful radical species (Li *et al.*, 1995). Observations that PEITC caused an increase in the mRNA levels of GST, NQO1 and antioxidant enzyme heme oxygenase 1 (HO1) indicates that PEITC acts at the level of transcription (Ernst *et al.*, 2011; Keum *et al.*, 2003; Xu *et al.*, 2006b).

Phase II metabolising and antioxidant enzymes are transcriptionally upregulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) binding to antioxidant response elements (ARE) present in their promoter regions. Nrf2 is

negatively regulated by Kelch-like ECH-associated protein 1 (Keap1), which binds to Nrf2 and prevents it from translocating to the nucleus as well as targeting Nrf2 for degradation via the ubiquitin-proteasome pathway (Itoh *et al.*, 2003). Keap-1 dissociates from Nrf2 in response to ROS and electrophiles, allowing Nrf2 to translocate to the nucleus where along with small Maf proteins it can bind to AREs and enhance transcription of responsive genes (Figure 1.13). In mice genetically ablated for Nrf2, protection against benzo(a)pyrene-induced gastric tumour formation by sulforaphane (SFN), an isothiocyanate found in broccoli, was lost (Fahey *et al.*, 2002). Furthermore, induction of NQO1 gene expression by SFN was abolished in Nrf2 null MEFs (Nioi *et al.*, 2003). These studies demonstrate the importance of Nrf2 in ITC-mediated induction of phase II drug metabolising enzymes and protection from carcinogen-induced tumourigenesis.

ITCs have been shown to induce transcription of phase II drug metabolising enzyme by binding directly to several key cysteine residues in Keap1 (Ahn *et al.*, 2010; Dinkova-Kostova *et al.*, 2002). Conjugation of Keap1 with ITCs was initially thought to trigger the release of Nrf2, however it is now believed that ITCs inhibit Keap-1 dependent ubiquitination and proteasome mediated degradation of Nrf2 (Zhang and Hannink, 2003). As ROS can also react with Keap1 to activate Nrf2, PEITC may also be able to activate Nrf2 indirectly. However, while Ernst *et al.* (2011) found induction of ARE-driven expression by hydrogen peroxide to be attenuated by the antioxidant catalase, induction by allyl isothiocyanate (AITC) was not. This suggests that thiocarbamoylation rather than ROS generation is responsible for ITC-induced Nrf2 dependent transcription. Although, activation of the MAPK pathway by ITCs may also contribute to the upregulation of phase II drug metabolising enzymes, via downstream phosphorylation of Nrf2 (Keum *et al.*, 2003; Xu *et al.*, 2006b). Indeed, loss of PEITC-induced ERK phosphorylation by an inhibitor of the MAPK pathway was found to attenuate the rise in mRNA levels of drug metabolising enzyme HQO1 (Ernst *et al.*, 2011).

1.8.2 Anticancer effects

The potential mechanisms contributing to the anticancer effects of PEITC are outlined in Table 1.6. While the underlying mechanisms are unknown, inhibition of cancer cell proliferation has been shown to be due to the ability of isothiocyanates to induce apoptosis and/or cause cell cycle arrest, and an overview of the signalling pathways implicated is provided in Figure 1.14.
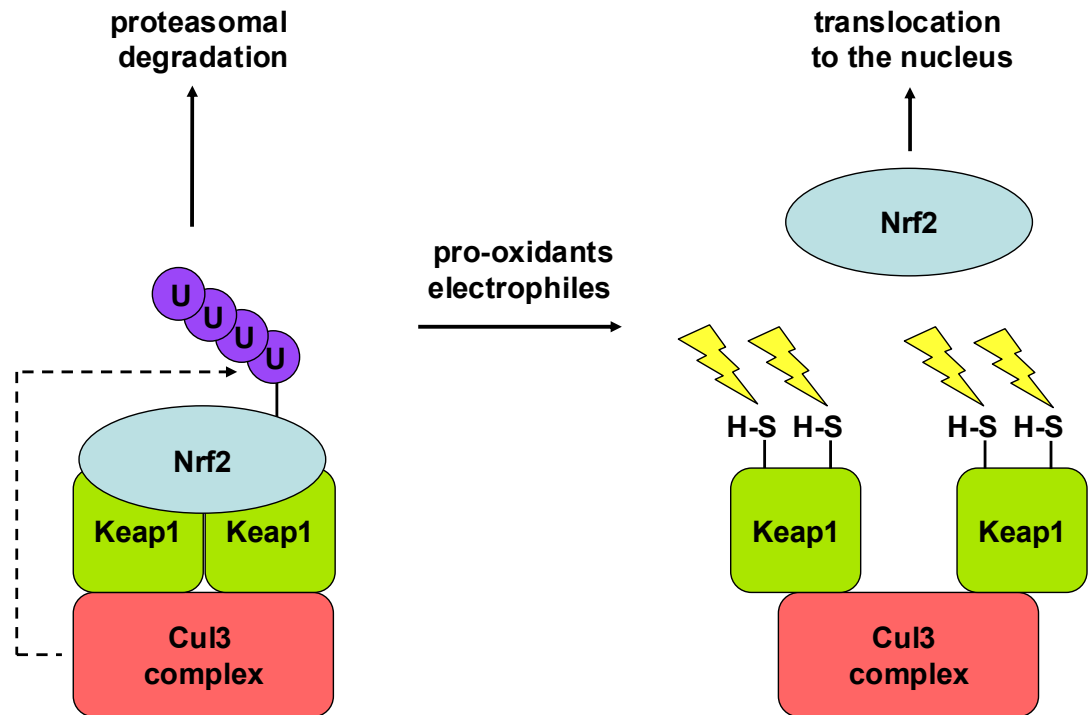
Figure 1.13: Activation of Nrf2.
Keap1 (Kelch-like ECH-associated protein 1) binds to Nrf2 (nuclear factor erythroid 2-related factor 2), targeting it for ubiquitination (U) by Cul3 (Cullin 3)-based E_3 ligase. Electrophiles, such as isothiocyanates, and pro-oxidants, like ROS, attack (✎) the thiol group (S-H) of several key cysteine residues on Keap-1, allowing accumulation of Nrf2.

Biological response	Effect	Concentration[1]
Cell cycle arrest	Inhibition of cell cycle progression has been observed in a wide variety of cell lines derived from both solid and haematological malignancies. Arrest in S or G_2/M phases of the cell cycle has been described.	5 µM (Xiao *et al.*, 2004) 10 µM (Mi *et al.*, 2008) 1-5 µM (Hwang and Lee, 2010) 5-10 µM (Wu *et al.*, 2011) 7.5 µM (Tang and Zhang, 2004)
Induction of apoptosis	Induction of both the intrinsic and the extrinsic pathways has been observed in a wide variety of cell lines derived from both solid and haematological malignancies.	20 µM (Satyan *et al.*, 2006) 5-10 µM (Huong *et al.*, 2011) 15 µM (Tang and Zhang, 2005) 5 µM (Trachootham *et al.*, 2008) 5 µM (Xiao and Singh, 2002)
In vivo tumour growth	PEITC inhibits tumour growth in a variety of xenograft models.	9-12 µM/d (Monday to Friday) by oral gavage for up to 31 days (Xiao *et al.*, 2006) 50 mg/kg (five days a week) given intraperitoneally for up to 60 days (Trachootham *et al.*, 2006) 50 mg/kg given intraperitoneally for 20 days (Gao *et al.*, 2011)
Angiogenesis	ITCs inhibit angiogenesis in *in vitro* and *in vivo* models.	1 µM (Xiao and Singh, 2007) 5 µg/ml AITC (Thejass and Kuttan, 2007a) 0.1-1 µM SFN (Bertl *et al.*, 2006)
Metastasis	ITCs inhibit cell adhesion, invasion and migration *in vitro*, and metastasis *in vivo*.	5 µM (Hwang and Lee, 2006) 2 µM (Xiao and Singh, 2007) 10 µM (Wu *et al.*, 2010a)

Table 1.6: Potential mechanisms contributing to the anticancer effects of PEITC.
In the interests of space, only selected references are provided. [1] Typical effective concentration. Note that differences in assay design preclude direct comparison between studies. Due to limited studies on the anti-angiogenic activity of PEITC, I have included data collected on additional ITCs, namely allyl isothiocyanate (AITC) and sulforaphane (SFN).
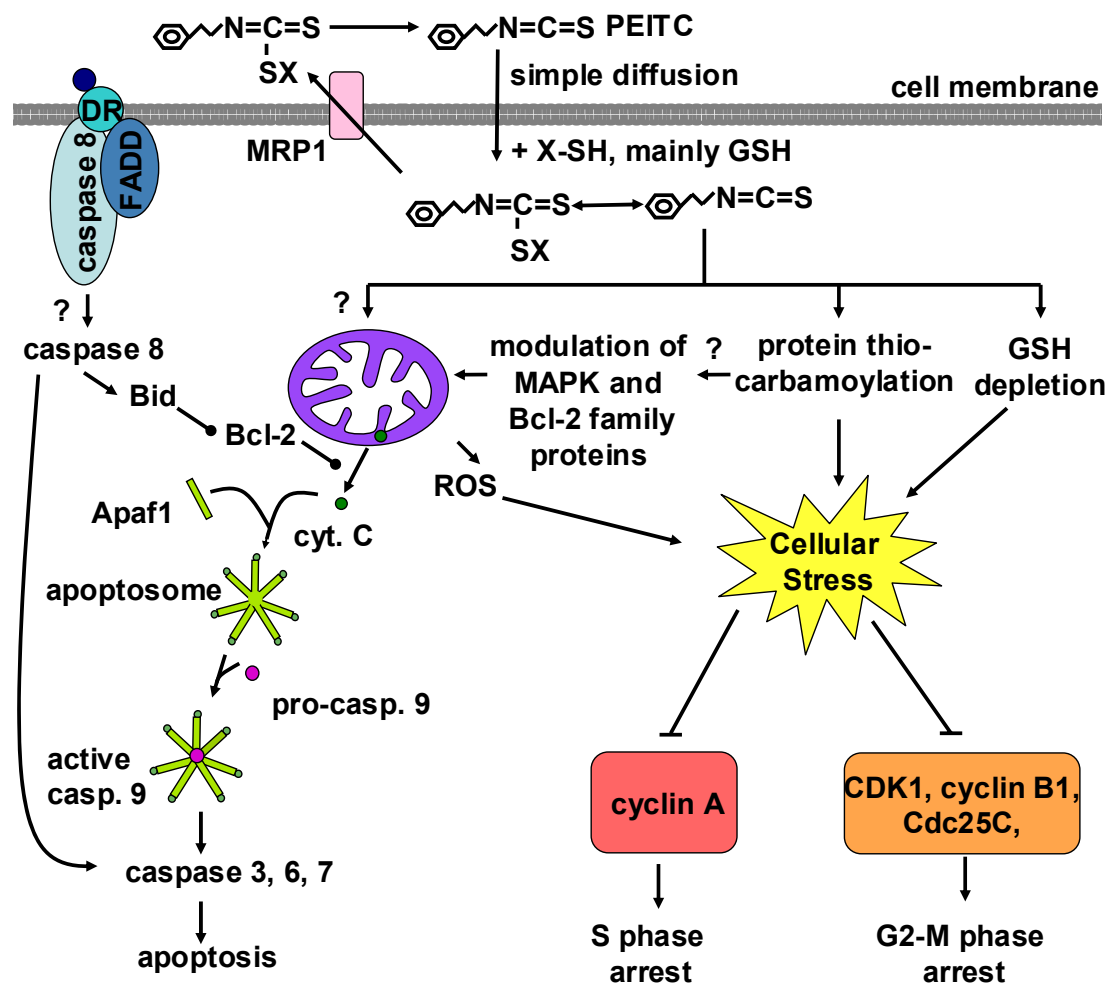
Figure 1.14: The various signalling pathways, known or suggested, in which PEITC can induce apoptosis and cell cycle arrest.

DR (death receptor), FADD (Fas-associated protein with death domain), GSH (glutathione), X (thiol compounds, such as GSH), Bid (BH3 interacting domain death agonist), Bcl-2 (B cell lymphoma 2), Apaf1 (apoptosis protease activation factor 1), Cyt. C (cytochrome C), MRP1 (multidrug resistance-associated protein 1), ROS (reactive oxygen species), MAPK (mitogen-activated protein kinase), HDAC (histone deacetylase), CDK1 (cyclin dependent kinase 1), Cdc25C (cell division cycle 25C). Adapted from Zhang *et al.* (2006).

1.8.2.1 Induction of cell cycle arrest

Cell cycle arrest as a result of PEITC treatment predominantly occurs in the G_2/M phase, this is associated with a downregulation of cyclin dependent kinase 1 (CDK1), cyclin B1 and/or cell division cycle 25C (cdc25C) (Hwang and Lee, 2010; Xiao *et al.*, 2004). Ordinarily CDK1 forms complexes with cyclin B and is then activated by cdc25C which targets it for dephosphorylation. The activated CDK1-cyclin B complex is required for transition into mitosis and cell cycle arrest occurs by decreasing levels of CDK1 or cyclin B1 or preventing CDK1 phosphorylation. PEITC has also been reported to cause mitotic arrest by disrupting tubulin polymerisation and spindle assembly (Mi *et al.*, 2008). Notably, Mi *et al.* (2008) demonstrated that PEITC disrupted tubulin polymerisation by directly conjugating to α-tubulin, later this was shown to induce proteasome-mediated degradation of tubulin (Mi *et al.*, 2009). In some cell lines, arrest in the S phase is also observed, and is associated with a reduction in protein levels of S phase regulator cyclin A (Wu *et al.*, 2011).

1.8.2.2 Influence on apoptotic machinery

Satyan *et al.* (2006) demonstrated that PEITC induces apoptosis exclusively via activation of the intrinsic pathway in the ovarian OVCAR3 cancer cell line. This was associated with a reduction in anti-apoptotic Bcl-2 protein levels and an increase in pro-apoptotic Bax levels, as well as activation of the MAPKs, JNK and p38. Furthermore, in UM-UC-3 bladder cancer cells, PEITC was found to increase Bcl-2 phosphorylation and promote translocation of Bak to the mitochondria (Tang and Zhang, 2005). Therefore it seems PEITC ultimately induces apoptosis through the transcriptional or post-translational modulation of multiple Bcl-2 family members. Although, PEITC-mediated apoptosis has also been shown to occur via the extrinsic pathway through increased expression of death receptors 4 and 5 (DR4, DR5) (Huong *et al.*, 2011).

1.8.2.3 Inhibition of angiogenesis

Studies on the anti-angiogenic and anti-metastatic activity of PEITC are limited. However, several groups have found that other isothiocyanates can also inhibit angiogenesis and metastasis. Both PEITC and SFN have independently been shown to inhibit the growth of cultured human umbilical vein endothelial (HUVEC) cells and prevent tube formation of these cells on matrigel, a well recognised technique for

determining *in vitro* angiogenesis (Asakage *et al.*, 2006; Xiao and Singh, 2007). Similar results have been shown for SFN using the immortalised human microvascular endothelial cell line, HMEC-1, whereby treatment prevented tube formation of these cells on basement membrane (Bertl *et al.*, 2006).

In *ex vivo* tissue culture models, SFN caused a dose dependent reduction of microcapillary density in a placental vessel fragment outgrowth assay (Bertl *et al.*, 2006) and PEITC decreased vascular density in chicken egg chorioallantoic membrane assays (Xiao and Singh, 2007). AITC demonstrated both *in vitro* and *in vivo* anti-angiogenic activity by decreasing outgrowth of microvessels from rat aortic rings, and inhibiting *in vivo* tumour directed capillary formation in the mouse B16F-10 melanoma model (Thejass and Kuttan, 2007a). Furthermore, intravenous administration of SFN decreased *in vivo* angiogenesis in VEGF-impregnated matrigel plugs in female Balb/C mice (Jackson *et al.*, 2007). SFN also reduced blood vessel density *in vivo* in MIA-PaCa2 xenografts (Kallifatidis *et al.*, 2009).

Inhibition of angiogenesis by ITCs *in vivo* has been associated with reduced production of multiple pro-angiogenic factors including VEGF, nitric oxide and tumour necrosis factor α (Thejass and Kuttan, 2007a; Thejass and Kuttan, 2007b). Bertl *et al.* (2006) also observed a decrease in HIF1α accumulation, and VEGF production in HUMEC-1 cells, an *in vitro* model of angiogenesis, following SFN treatment. HIF1α is a major regulator of angiogenesis which promotes transcription of VEGF. PEITC and SFN have been shown by both our lab and other groups to reduce HIF1α accumulation and VEGF expression in a variety of cancer cell lines (Wang *et al.*, 2009; Yao *et al.*, 2008).

1.8.2.4 Inhibition of metastasis

Xiao and Singh (2007) demonstrated that PEITC inhibited the *in vitro* migration of endothelial HUVEC cells and prostate cancer PC3 cells. PEITC has also been shown to inhibit adhesion, invasion and migration of human hepatoma SK-Hep1 cells and highly metastatic human lung cancer L9981 cells (Hwang and Lee, 2006; Wu *et al.*, 2010a). This activity was associated with a decrease in matrix metalloproteinase (MMP) 2 and 9, and membrane type 1 matrix metalloproteinase (MT-MMP) expression, and an increase in expression of tissue inhibitors of matrix metalloproteinase (TIMPs) 1 and 2, which inhibit the activity of the MMPs (Hwang and Lee, 2006). *In vivo*, oral administration of SFN reduced pulmonary metastasis in TRAMP mice, a transgenic mouse model of prostate cancer (Singh *et al.*, 2009b).

1.9   Anticancer effects of watercress

In addition to studies of isolated phytochemicals, a number of studies have investigated potential anti-cancer effects of watercress extracts (referred to as "watercress juice"), predominantly focusing on chemopreventive capabilities. Boyd *et al.* (2006) demonstrated that watercress extract protects colon cancer HT29 cells from oxidative DNA damage induced by various genotoxins, 4-Hydroxy Nonenal (4-HNE), hydrogen peroxide, and fecal water. Furthermore, the addition of watercress juice to human hepatoma Hep G2 cells has been shown to protect against benzo(a)pyrene (B(a)P) induced DNA damage. However, this effect appears not to be due to PEITC alone, as when it was added in concentrations similar to that present in the juice, it was unable to protect the Hep G2 cells against the DNA damage induced by B(a)P. The watercress juice also increased the activities of the drug metabolising enzymes, GST and CYP1A1, although this action alone was unlikely to be responsible for the protection against the genotoxic effect of B(a)P, as garden cress juice showed similar protection against B(a)P-induced DNA damage in Hep G2 cells but the activity of GST and CYP1A1 was not affected (Kassie *et al.*, 2003). Overall, this indicates that there are phytochemicals present in watercress, other than PEITC, that seem to be having an effect and that these effects are not limited to modifying drug metabolising enzymes.

Indeed, Rose *et al.* (2000) discovered that despite being present in watercress at concentrations approximately 3 times less than PEITC, the methylsulfinylalkyl isothiocyanates, 7-methylthioheptyl-ITC and 8-methylthiooctyl-ITC were far more potent at inducing NQO1 activity in murine heptatoma Hepa 1c1c7 cells. While only 0.2 µM of 7-methylthioheptyl-ITC and 0.5 µM of 8-methylthiooctyl-ITC were required to induce a two-fold increase in NQO1 activity, 5 µM of PEITC was needed to produce the same results. The PEITC glutathione conjugate, *S*-(*N*-β-phenylethylthiocarbomyl) glutathione (PECG) was also found to have a similar potency to PEITC in regards to increasing quinine reductase activity.

These chemopreventive actions have also been demonstrated in human consumption studies. Hecht *et al.* (1995) reported that the consumption of watercress, 56.8 g with every meal for 3 days, inhibited the metabolic activation of a key tobacco carcinogen NNK in smokers. The reduction in NNK carcinogenicity is thought to be the result of PEITC, as changes in the excretion of PEITC metabolite PEITC-NAC in the urine correlated with the increased urinary detoxification metabolites used to measure

oxidative metabolism of NNK. Furthermore consumption of 85 g of raw watercress once a day for eight weeks decreased several measures of DNA damage in lymphocytes, a cancer biomarker, and increased plasma levels of the antioxidants, β-carotene and lutein. There was a statistically significant decrease in basal DNA damage, basal plus oxidative purine DNA damage, and basal DNA damage in response to hydrogen peroxide challenge. In addition to this there was a greater and more significant change in those subjects who were smokers, indicating that their increased exposure to toxins resulted in their observed lower antioxidant status, and therefore they benefited more from a boost in antioxidants than non-smokers (Gill *et al.*, 2007).

Interestingly Gill *et al.* (2007) also examined the activity of the detoxifying enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPX), with little change being observed between the watercress and control phase. Similar to what was concluded from the *in vivo* studies, this suggests that there are other mechanisms at work, which may be attributed to PEITC or even to other compounds present in watercress. When these data were later subdivided according to the genetic profile of the subjects, an increase in the activity of SOD and GPX in individuals with a *GST Mu 1* (*GSTM1*) null genotype was identified (Hofmann *et al.*, 2009). GST is responsible for the conjugation of ITCs with GSH, and individuals with *GSTM1* or *GST theta 1* (*GSTT1*) null genotypes have been previously reported to benefit more than those that are GSTM1 or T1 positive from the protective effects of cruciferous vegetables (Seow *et al.*, 2005). As ITC-GSH conjugates are rapidly exported from the cell, it is proposed that loss of GST enzyme activity in these individuals slows the metabolism of ITCs prolonging their presence in the body. However, a recent study found that irrespective of the *GSTM1* or *T1* null genotype, there was no difference in excretion rates of PEITC-NAC in the urine of subjects who drank 200 ml watercress juice (Dyba *et al.*, 2010). While ITCs are known to initially form conjugates with GSH, one mechanism by which ITCs are thought to exert their anticancer effects is through conjugation with other cellular proteins once GSH has been depleted. It is possible that ITCs preferentially conjugate with GSH because of the activity of GST, and loss of that activity would allow ITCs to conjugate more readily with other cellular thiols, accounting for the enhanced protective effect seen in *GSTM1* or *T1* null individuals.

Some studies have begun to investigate the anti-proliferation and anti-metastatic activity of watercress, for the first time looking not only at a chemopreventive role for watercress but more wholly as an anti-cancer agent. Watercress extract was found to

inhibit cell cycle progression of colon cancer HT29 cells, specifically halting them in S phase, as well as inhibiting the invasion of HT115, a particularly invasive colon cancer cell line, through matrigel (Boyd *et al.*, 2006). Rose *et al.* (2005) demonstrated that watercress extract, although more specifically the non-volatile 7-methylsulphinylheptyl isothiocyanate component, reduced MMP9 activity in the human breast cancer MDA-MB-231 cell-line. MMP9 is a proteolytic enzyme whose activity is essential for cell invasion and is often upregulated in cancer cells. Subsequently treatment of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulated MDA-MB-231 cells with either watercress extract or simply a fraction containing methylsulfinylheptyl-ITC resulted in inhibition of their invasiveness. Due to the volatility of the other ITCs present in watercress they where not tested in this study but Rose *et al.* (2005) also looked at broccoli extract in which they identified 4-methylsulphinylbutyl (sulforaphane) which demonstrated similar anti-invasive and anti-metalloproteinase activity, suggesting that this activity is not specific to particular ITCs.

1.10 Aims

Epidemiological studies indicate that there is an inverse relationship between the consumption of cruciferous vegetables and the risk of cancer development (Verhoeven *et al.*, 1996; Voorrips *et al.*, 2000). As a result there is much interest in understanding the anticancer potential not only of cruciferous vegetables themselves but also of the key phytochemicals contained in them. I hypothesise that the *in vitro* anticancer activity of watercress can be enhanced by altering environmental factors. To address this, the following aims will be investigated:

i)   Select assays to assess the *in vitro* anticancer effects of watercress-derived compounds.

ii)   Explore the mechanisms by which PEITC exhibits its anticancer effects.

iii) Carry out proof-of-principle experiments to discover the suitability of the assays selected for the analysis of crude watercress extracts.

iv)   Investigate whether altering growth environment can increase the *in vitro* anticancer activity of watercress.

Chapter 2

Materials and methods

2.1   Cell culture

2.1.1   Cell lines

Human breast cancer cell lines, MCF7 and SK-BR-3 (Table 2.1) were purchased from American Type Culture Collection (ATCC; Manasses, USA). MCF7 cells stably transfected with antioxidant response element (ARE) plasmid (Wang *et al.*, 2006) were a generous gift of Professor Roland Wolf (University of Dundee, Scotland).

Mouse embryonic fibroblasts (MEFs) lacking tuberin (TSC2-/-TP53-/-) and wild type control MEFs (TSC2+/+TP53-/-) (Zhang *et al.*, 2003a) were a kind gift of Professor Christopher Proud (University of Southampton, UK) who obtained them from Dr David Kwiatkowski (Harvard Medical School, Boston, USA). MEFs that homozygously express the eukaryotic elongation factor 2α Ser51Ala mutation (eIF2α AA) and wild type control MEFs (eIF2α SS) (Scheuner *et al.*, 2001) were a generous gift of Professor Randal Kaufman (University of Michigan Medical Center, Ann Arbor, USA). MEFs both homozygous and heterozygous for knockout of the phosphatase and tensin homolog deleted on chromosome 10 gene (PTEN -/-; PTEN +/-) along with wild type control (PTEN +/+) (Stambolic *et al.*, 1998) were a kind gift of Dr. Vuk Stambolic (University of Toronto, Canada).

All cell lines were maintained in complete Dulbecco's modified Eagle's Medium (DMEM; Lonza Group Ltd, Basel, Switzerland) (i.e. supplemented with 10% (v/v) fetal bovine serum (FBS; PAA Laboratories, Yeovil, UK), 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (Lonza Group Ltd). Cells were grown in a 37ºC incubator supplied with 10% (v/v) CO_2 (Hera cell, Heraeus).

Cell Line	Characteristics	References
MCF7	Malignant pleural effusion secondary to breast carcinoma. Retains characteristics of differentiated mammary epithelium including oestrogen receptor expression.	Tong *et al.* (1999), ATCC
SK-BR-3	Malignant pleural effusion secondary to breast carcinoma. Overexpresses the HER2 gene product. Negative for oestrogen receptor expression.	Kallioniemi *et al.* (1992), Tong *et al.* (1999), ATCC

Table 2.1: Cell lines and characteristics.

2.1.2 Cell culture

Cells were maintained in 175 cm^2 tissue culture flasks (Greiner Bio-One Ltd., Gloucestershire, UK) and routinely passaged at 60-70% confluence. Media was aspirated from flasks and Trypsin-Versene® (EDTA) Mixture (3 ml; 0.05% (w/v) trypsin and 0.02% (w/v) versene (EDTA); Lonza Group Ltd.) was used to rinse away any remaining cell debris before being discarded. Trypsin-Versene® (EDTA) Mixture (4 ml) was added to the flask and incubated at 37°C/10% CO_2 for a minimum of 3 min to detach cells. Following this complete DMEM (8 ml) was added to the flask to inactivate the trypsin and collect the cells. The required volume of cell suspension to produce the desired dilution was removed and either discarded or transferred to a 15 ml centrifuge tube (Greiner Bio-One Ltd.) where the cells were then counted and used for an experiment. Complete DMEM (25 ml) was added to the remaining cells in the flask and incubated at 37°C/10% (v/v) CO_2.

2.1.3 Hypoxia

To expose cells to hypoxia, cells were sealed in a modulator incubator chamber (Billups-Rothenberg, Inc.; Del Mar, USA) which was flushed for 2 min with 1% (v/v) O_2, 5% (v/v) CO_2, 94% (v/v) N_2 (BOC, Surrey, UK) and placed in a 37°C/5% (v/v) CO_2 incubator (Galaxy S, RSBiotech) alongside the normoxia control. After 1 h the chamber was re-flushed for 2 min with 1% (v/v) O_2, 5% (v/v) CO_2, 94% (v/v) N_2 and placed back in the incubator for the desired length of time. For some experiments hypoxic responses were induced using the hypoxia mimetics cobalt chloride ($CoCl_2$; Sigma, Poole, UK) or desferrioxamine (DFO; Sigma). Both stabilise HIF1α by preventing binding of the pVHL; $CoCl_2$ by directly binding to HIF1α and blocking the pVHL binding site (Yuan *et al.*, 2003), and DFO by acting as an iron chelator and inhibiting the activity of the iron-dependent proyl hydroxylases which hydroxylate HIF1α required for pVHL recognition (Wang and Semenza, 1993). Cells were pretreated with 100 µM $CoCl_2$ or DFO immediately prior to treatment with the test compound.

2.1.4 Freezing cells for long term storage

Freezing media was prepared by adding 10% dimethyl sulphoxide (DMSO; Sigma) to 90% FBS. Cells were trypsinised as described in Section 2.1.2 and counted before being centrifuged at 1300 rpm for 3 min in a Sorvall® Legend RT bench top centrifuge

(360 g). The cell pellet was resuspended in freezing media to at a concentration of $2 \times 10^6 - 6 \times 10^6$ cells per ml. Aliquots (1 ml) were transferred to labelled Cryo.s™ 1.0 ml cyrovial (Greiner Bio-One Ltd.) and frozen in a NALGENE® Mr Frosty (Thermo Fisher Scientific) filled with isopropanol (250 ml; Thermo Fisher Scientific) at -80°C overnight and transferred to liquid nitrogen for long term storage.

2.1.5 Thawing cells for culture

Cells were quickly thawed by hand, to maintain cell viability, whilst the cryovial lid was loosened gently. Thawed cells were transferred to a 1.5 ml microcentrifuge tube and centrifuged at 2000 rpm for 3 min in a Heraeus® Biofuge® fresco microfuge (380 g) to remove DMSO. The supernatant was discarded and the cell pellet resuspended in complete DMEM (1 ml) that was then further added to more complete DMEM (9 ml) in a 25 cm^2 tissue culture flask (Greiner Bio-One Ltd.) and incubated at 37°C/10% (v/v) CO_2.

2.2 Compounds and extracts

2.2.1 Analytical grade compounds

Phenethyl isothiocyanate (PEITC), indole-3-carbinol (I3C) and quercetin dihydrate were all purchased from Sigma. 10 mM PEITC, 100 mM I3C and 100 mM quercetin stock solutions were made in DMSO fresh on the day of use.

2.2.2 Watercress extracts

Green and red watercress samples were obtained directly from Vitacress Salads Ltd. (Andover, UK). Samples were snap frozen in liquid nitrogen and stored at -80°C. 2 g of leaf and 2 g of stem were weighed and placed in a 20 ml syringe (BD Biosciences, Oxford, UK) that had had the plunger removed and a circular 25 mm glass microfibre filter (Whatman, Dassel, Germany) placed at the bottom. The syringe was then placed inside a 50 ml centrifuge tube without the lid and centrifuged at 2641 rpm for 30 min in a Sorvall® Legend RT bench top centrifuge (1500 g) to collect the extract. This crude watercress extract was then filtered through a 0.22 µm filter (Whatman) and used immediately.

2.3   Cell proliferation

2.3.1   Preparation of assays

MCF7 cells and MEFs were seeded at a density of 1000 cells per well of a 96 well plate (Greiner Bio-One Ltd.) in complete DMEM (50 µl). SK-BR-3 cells were seeded at a density of 5000 cells per well of a 96 well plate in complete DMEM (50 µl). The outside wells of the 96 well plate were not used and were instead filled with phosphate buffered saline (100 µl; PBS; see Section 2.11) to avoid the cells drying out and to allow for no cell blank controls. Cells were incubated at 37ºC/10% (v/v) CO_2 overnight. The following day cells were treated with watercress-derived compounds, watercress juice or solvent controls. The non-specific protein kinase inhibitor staurosporine (50 nM; Sigma) was used as a positive control (Jacobson *et al.*, 1996). For compounds, a DMSO solvent control was carried out equivalent to the largest volume of compound added. Dilutions were made in a 24 well plate (Greiner Bio-One Ltd.) at 2x final concentration and added to the cells in complete DMEM (50 µl) to give a total volume of 100 µl. Breast cancer cell lines were incubated at 37ºC/10% (v/v) CO_2 for a further six days, while the MEFs were only incubated for a further four days due to their faster rate of proliferation.

2.3.2   MTS assay

Metabolically active cells convert the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(-4-sulfophenyl-2H-tetrazolium inner salt (MTS) that is contained in the CellTiter 96® AQ$_{ueous}$ One Solution Reagent (Promega, Southampton, UK) to the coloured product formazan. The colour intensity of the formazan dye is widely used as a measure of the number of viable cells. However, it should be noted that whilst this assay provides a simple method to measure the effect of compounds on cells, it does not provide a direct measure of cell division. Apparent cell number will be influenced by both cell division and cell death, and changes in metabolic activity may also affect the amount of formazan generated.

At the end of the culture period, growth media was removed from the wells of the 96-well plate and discarded. Roswell Park Memorial Institute 1640 media (100 µl; RPMI; Lonza Group Ltd) and CellTiter 96® AQ$_{ueous}$ One Solution Reagent (5 µl) was added per well. The 96 well plate was placed in a 37ºC incubator supplied with 5% (v/v) CO_2 for

90 min to allow for the colorimetric reaction to occur. Changes in absorbance were measured by a Varioskan Flash plate reader (Thermo Fisher Scientific Inc, Rockford, USA) at 490 nm. An average value obtained from the blanks was subtracted from all other values to allow for any background absorbance of the RPMI and these normalised values were set as a percent of the average from the untreated wells.

2.3.3 Analysis

Data obtained from the simple growth inhibition experiments was analysed by the software program GraphPad Prism 4 (GraphPad Software Inc.) to produce survival curves and calculate the IC_{50} value.

2.4 SDS-PAGE and western blotting

2.4.1 Preparation of cell lysates

Cells were seeded at a density of 1×10^6 cells per 60 mm dish (Greiner Bio-One Ltd.) in complete DMEM (4 ml) and incubated overnight. The following day cells were treated with the watercress derived compounds, DMSO as a solvent control, or were left untreated and incubated for the desired length of time. For serum starvation cells were plated in complete DMEM overnight, washed (3 x 1 ml) with serum free DMEM and incubated for 24 h in serum free DMEM (4 ml). For those cells that were re-stimulated with serum, the serum free DMEM was replaced with complete DMEM for 2 h.

To prepare protein lysates, the growth media was decanted from the tissue culture dishes and the cells were rinsed with ice cold PBS (1 ml). Ice cold PBS (1 ml) was then added and the cells were detached using a plastic cell scraper (Greiner Bio-One Ltd.) and collected in a 1.5 ml microcentrifuge tube. This protocol was carried out for all cell lines with the exception of TSC2 null and wild type MEFs as PEITC seemed to interfere with the adherence of the former cells. It was observed that after 3 h PEITC at as low as 5 µM resulted in a large number of the cells detaching, however if left incubated overnight re-adhered. Therefore in the case of TSC2 null and wild type MEFs, these cells were scraped on ice in their media before the whole volume was transferred to a 15 ml centrifuge tube and centrifuged at 4ºC and 1500 rpm in a Sorvall® Legend RT bench top centrifuge (500 g) for 5 min. The supernatant was

discarded and the cell pellet resuspended in ice-cold PBS (1 ml) before being transferred to a 1.5 ml microcentrifuge tube.

Cells were centrifuged at 4ºC and 3000 rpm for 1 min in a Heraeus$^®$ Biofuge$^®$ microfuge (860 g) and the supernatant discarded. Cell pellets were resuspended in 1x RIPA buffer (see Section 2.11) plus 1:100 dilution of protease inhibitor cocktail (Sigma; see Section 2.11) and phosphatase inhibitor cocktail 1 (Sigma) as required. Cell lysates were left on ice for 30 min and centrifuged at 4ºC and 13000 rpm for 15 min in a Heraeus$^®$ Biofuge$^®$ fresco microfuge (16100 g). The lysate supernatant was removed, aliquoted into a fresh microcentrifuge tube and kept on ice. Protein lysates were quantified on a Varioskan Flash plate reader spectrophotometer at 595 nm using the BSA protein assay described below.

2.4.2 BSA protein assay

BioRad protein assay dye concentrate (BioRad Laboratories, Hercules, USA) was diluted 1:5 with PBS and aliquoted (250 µl) into 96 well plate. 1 µl of each sample or desired amount of bovine serum albumin (BSA; Promega) was added and mixed. BSA standards were made up in the following concentrations by adding various volumes of 1mg/ml BSA, adding the appropriate volume of distilled H$_2$O to give a total volume of 5 µl each time:

0: 0 mg/ml: 0 µl (Blank)

1: 0.2 mg/ml: 1 µl

2. 0.4 mg/ml: 2 µl

3. 0.6 mg/ml: 3 µl

4. 0.8 mg/ml: 4 µl

5. 1 mg/ml: 5 µl

Wells were mixed thoroughly, left for 5 min at room temperature and absorbance measured at 595 nm on a Varioskan Flash plate reader spectrophotometer.

2.4.3 Making Tris-HCl gels

Tris-HCl gels were prepared reusing the BioRad Mini-PROTEAN 3 system in accordance to the recipes detailed in Table 2.2 and Table 2.3. 10% gels were used in

the majority of cases except when probing for extremely large proteins (i.e. TSC2) where 6% gels were used.

Reagents	Resolving Gel	
	6% gel	**10% gel**
H_2O	5.3 ml	4 ml
30% (v/v) acrylamide	2 ml	3.3 ml
1.5 M Tris pH 8.8	2.5 ml	2.5 ml
10% (w/v) SDS	100 µl	100 µl
10% (w/v) ammonium persulphate (APS)	100 µl	100 µl
TEMED (N, N, N', N'-tetramethylenediamine)	10 µl	10 µl

Table 2.2: Recipe for 10 ml 6% and 10% Tris-HCl resolving gels.

Reagents	Stacking Gel
H_2O	2.7 ml
30% (v/v) acrylamide	670 µl
1.5 M Tris pH 8.8	500 µl
10% (w/v) SDS	40 µl
10% (w/v) APS	40 µl
TEMED	5 µl

Table 2.3: Recipe for 8 ml Tris-HCl stacking gel.

2.4.4 Western blotting

Tris-HCl gels were placed in a BioRad Mini-PROTEAN 3 Cell containing approximately 500 ml 1x running buffer (see Section 2.11). Equal amounts (15-30 µg) of protein from each sample was made up to 10 µl with distilled H_2O and mixed with 5 µl 3x SDS Sample Buffer Red (Cell Signaling Technology, Danvers, USA; see Section 2.11) supplemented with 0.1 M dithiothreitol (DTT; Cell Signaling Technology) to give a final volume of 15 µl. The samples along with the protein marker (New England BioLabs, Ipswich, USA) were heated at 95ºC for 5 min before being spun briefly up to 13000 rpm in a Heraeus® Biofuge® microfuge (16100 g). Protein marker (5 µl) was loaded into the first well and the samples (15 µl) loaded into subsequent wells. The gel was resolved at 150 V for a minimum of 60 min. The gel was removed and placed between transfer filter paper and membrane in a 'sandwich' consisting of 1 sponge (BioRad Laboratories), 1 filter paper, nitrocellulose blotting membrane (Whatman), a further 1

filter paper and 1 sponge in a BioRad Mini-Trans Blot Cell as per manufacturers instructions. Approximately 500 ml transfer buffer (see Section 2.11) was added to the BioRad Mini-Trans Blot Cell before being transferred at 100 V for 1 h. This is with the exception of the 6% gel used when looking at large proteins (i.e. tuberin) where the gel was transferred at 30 V overnight at 4°C followed by 70 V for 1 h at room temperature the following day.

Membranes were blocked with 5% (w/v) milk (dried non-fat Marvel) made in 0.1% (w/v) TBS-Tween (5 ml; TBS-TW; see Section 2.11) for 1 h at room temperature on an automatic roller before being stained with primary antibody (see Table 2.4) overnight at 4°C. The following day membranes were then washed (3x 5 min) with 0.1% TBS-TW (5 ml) and stained with the appropriate secondary horseradish peroxidase-conjugated (HRP) antibody (see Table 2.4) for 1 h at room temperature. Membranes were again washed (3x 5 min) with 0.1% TBS-TW (5 ml). 1:1 mixture of stable peroxide solution and luminol enhancer that form the Supersignal West Pico Chemilluminescent substrate reagent (Thermo Fisher Scientific) was applied to the membrane for 1 min. Chemilluminescence was detected on a ChemiDoc-It® Imaging System (Ultra-Violet Products Ltd., Cambridge, UK) using VisionWorks®LS Image Acquisition and Analysis Software (UVP Ltd). Protein band quantification was carried out using ImageJ software (http://rsb.info.nih.gov/ij/). Protein expression values were normalised to expression of β-actin and then to untreated control. For phospho-specific antibodies, expression values were expressed relative to total protein. For 4E-BP1, phosphorylation was detected as a decrease in the migration of 4E-BP1 detected using non-phospho-specific antibody and phosphorylated forms were quantified as a proportion of total 4E-BP1 expression.

2.4.5 Stripping of membranes

Membranes were washed for 5 min in stripping buffer (5 ml; see Section 2.11) to strip off the antibody complexes prior to re-probing. Blots were further washed (3x 5 min and 2x 10 min) with 0.1% TBS-TW (5 ml) to restore pH and finally re-blocked in 5% (w/v) milk in 0.1% TBS-TW (5 ml) for 1 h at room temperature before being re-probed.

Primary antibody	Species raised in	Blocking step	Supplier	Dilution
4E-BP1	rabbit	BSA	Cell Signaling Technology	1:1000
Akt	rabbit	BSA	Cell Signaling Technology	1:1000
AMPK	rabbit	BSA	Cell Signaling Technology	1:1000
β-actin	rabbit	milk	Sigma	1:1000
eEF2	rabbit	BSA	Cell Signaling Technology	1:1000
eIF2α	rabbit	BSA	Cell Signaling Technology	1:1000
ERK1/2	mouse	BSA	Cell Signaling Technology	1:1000
HIF1α	mouse	milk	BD Biosciences	1:250
Hsc70	mouse	milk	Santa Cruz Biotechnology Ltd., Heidelberg, Germany	1:1000
NDRG1	rabbit	BSA	Cell Signaling Technology	1:1000
p70 S6K	rabbit	BSA	Cell Signaling Technology	1:1000
P-Akt (Thr308)	rabbit	BSA	Cell Signaling Technology	1:1000
P-Akt (Ser473)	rabbit	BSA	Cell Signaling Technology	1:1000
P-AMPK (Thr172)	rabbit	BSA	Cell Signaling Technology	1:1000
P-eEF2 (Thr56)	rabbit	BSA	Cell Signaling Technology	1:1000
P-eIF2α (Ser51)	rabbit	BSA	Cell Signaling Technology	1:1000
P-ERK1/2 (Thr202/Tyr204)	rabbit	BSA	Cell Signaling Technology	1:1000
P-NDRG1 (Thr346)	rabbit	BSA	Cell Signaling Technology	1:1000
P-p70 S6K (Thr389)	rabbit	BSA	Cell Signaling Technology	1:1000
PTEN	goat	milk	Santa Cruz Biotechnology Ltd.	1:200
Tuberin	mouse	milk	Santa Cruz Biotechnology Ltd.	1:200

Secondary antibody	Species raised in	Blocking step	Supplier	Dilution
goat	rabbit	milk	Dako, Stockport, UK	1:5000
mouse	sheep	milk	GE Healthcare UK Ltd., Buckinghamshire, UK	1:5000
rabbit	donkey	milk	GE Healthcare UK Ltd.	1:5000

Table 2.4: List of antibodies used for western blotting.

2.5 HDAC inhibition assay

Cells were seeded at a density of 1000 cells per well of a 96 well plate in complete DMEM (81 µl) and incubated overnight at 37ºC/10% (v/v) CO_2. The following day cells were treated in the absence or presence of watercress-derived compounds. A DMSO solvent control was carried out and known HDAC inhibitors trichostatin A (TSA; Sigma) (Yoshida *et al.*, 1990) or suberoylanilide hydroxamic acid (SAHA; Enzo Life Sciences Ltd., Exeter, UK) (Richon *et al.*, 1998) were used as positive controls. A blank containing all reagents but no cells was also analysed. Stock solutions were diluted in DMSO to give 100x final concentrations before 4 µl of each was added to complete DMEM (36 µl) in a dilution plate. 9 µl of the serially diluted compound was added to each well. This way every well has the same concentration of DMSO. Cells were incubated for 16 h. 10 µl of either 2 mM BOC-lys(ac)-AMC (Bachem, Weil am Rhein, Germany) or 2 mM control, BOC-lys-AMC (Bachem), was added to each well and cells incubated for a further 1 h. Lysis buffer/developer mix (100 µl; see Section 2.11) was added to each well and incubated for 16 h before fluorescence (excitation 355 nm, emission 460 nm) was measured on Varioskan Flash plate reader. See Figure 2.1 for schematic of the mechanism.

2.6 Luciferase reporter assay

2.6.1 Plasmid transformation

0.5 µl of each plasmid was added to 50 µl competent JM109 bacterial cells (kind gift of Patrick Duriez, Cr-UK Protein Core Facility, Southampton, UK) including a negative control (no addition) and incubated on ice for 30 min. The bacteria were then heat shocked for 60 seconds at 42°C and immediately cooled on ice for 2 min. L Broth (450 µl; LB) warmed to 37ºC was added and the tubes were agitated at 200 rpm for 45 min in an incubating shaker (37ºC). 50 µl was spread on LB agar plates containing 50 ug/ml ampicillin (Sigma) and incubated at 37°C overnight. A single colony from each positive plate was picked using a sterile pipette tip and placed in a 50 ml centrifuge tube containing LB (5 ml) supplemented with 50 ug/ml ampicillin and placed in an incubating shaker overnight (200 rpm at 37°C). The following day 400 µl from the culture was removed and placed in 1 L conical flask along with 200 ml LB and 200 µl ampicillin, and returned to an incubating shaker overnight (200 rpm at 37°C). Maxipreps were prepared using a Hi Speed® Plasmid Maxi Kit (Qiagen Ltd, Sussex,

a)

BOC-lys(ac)-AMC

Step 1: HDACs remove acetyl
group from substrate.

HDAC

BOC-lys-AMC

Step 2: Bond between lysine and
AMC is cleaved by trypsin.

Trypsin

**AMC fluorophore is free to
fluoresce.**

BOC-lys + **AMC**

b)

BOC-lys(ac)-AMC

Step 1: Activity of HDACs is
inhibited and the substrate
remains acetylated.

HDAC ┤── HDACi

BOC-lys(ac)-AMC

Step 2: Bond between lysine and
AMC is protected by the
acetyl group and trypsin is
unable to cleave it.

Trypsin

**AMC fluorophore remains
attached and does not fluoresce.**

BOC-lys(ac)-AMC

Figure 2.1: Schematic of the mechanism behind the HDAC inhibition assay.
(a) In the absence of a HDAC inhibitor the BOC-lys(ac)-AMC compound is
deacetylated by HDACs in the cells. This allows the trypsin in the lysis buffer to cleave
the bond between the now unprotected lysine and the AMC fluorescent fluorophore,
releasing it. The fluorophore gives off a fluorescent signal. (b) In the presence of a
HDAC inhibitor the cells are prevented from deacetylating the BOC-lys(ac)-AMC
compound, so the lysine remains acetylated. The trypsin is unable to cleave the
fluorophore and subsequently there is a reduction in fluorescent signal. HDAC, histone
deacetylase; Lys, lysine; Ac, acetyl group; AMC, 7-amino-4-methylcoumarin; HDACi,
histone deacetylase inhibitor.

UK) as per manufacturer's instructions. Plasmid DNA was quantified on a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and stored at + 4°C.

2.6.2 Transfecting cell lines

Cells were seeded at a density of 3×10^6 cells per 10 cm culture dish (Greiner Bio-One Ltd.) in complete DMEM (15 ml) and incubated overnight at 37°C/10% (v/v) CO_2. The following day cells were transfected with either the HIF-dependent luciferase reporter construct pGL2-TK-HRE (Rapisarda *et al.*, 2002) (a kind gift of Giovanni Melillo, Tumour Hypoxia Laboratory, NCI, USA) or a control pGL3-promotor (Promega). Transfection mixtures containing 6 ml serum free DMEM, 4 µg desired plasmid DNA and 90 µl TransFast™ Transfection Reagent (Promega) were incubated for 12 min at room temperature and mixed intermittently. Media was removed from cells and replaced with the transfection mixture. Cells were incubated for a further 2 h at 37°C/10% (v/v) CO_2 after which the transfection mixtures were removed and replaced with complete DMEM (15 ml). Cells were incubated overnight at 37°C/10% (v/v) CO_2.

2.6.3 Treating the cells

Transfected cells were recovered and plated in white 96 well plates (PerkinElmer Ltd., Cambridge, UK) at a density of 2000 cells per well in complete DMEM (50 µl). Cells were incubated for 5 h to allow cells to adhere before being treated with increasing concentrations of crude watercress extract, and distilled water as a control. Cells were cultivated with or without hypoxia mimetic DFO for 24 h at 37°C/10% (v/v) CO_2.

When analysing the effect on antioxidant response element (ARE) activity, stably transfected MCF-7 derived reporter cells, AREc32, were used. In this instance cells were simply plated in white 96 well plates at a density of 5000 cells per well in complete DMEM (50 µl) and left overnight to adhere. The following day the cells were treated with increasing concentrations of PEITC or crude watercress extract, and either DMSO or distilled water as solvent controls respectively for 24 h at 37°C/10% (v/v) CO_2.

2.6.4 Bright Glo assay

Luciferase activity was measured after 24 h. Bright -Glo™ (100 µl; Promega) was added to each of the wells before the 96 well plate was placed in a Varioskan Flash

plate reader where the plates were shaken for 2 min and incubated at 37°C for 12 min. Luminescence was then measured.

2.7 Quantitative-reverse transcription-polymerase chain reaction (Q-RT-PCR)

2.7.1 Total RNA extraction

MCF7 cells were seeded at a density of 1×10^6 cells in 60 mm culture plates in complete DMEM (5 ml) and incubated at 37°C/10% (v/v) CO_2 overnight to adhere. The following day cells were treated in duplicate with PEITC or DMSO as a solvent control, or were left untreated. Cells were cultured in a hypoxia chamber for 16 h before being harvested. On ice, media was decanted and discarded and the cells were rinsed with ice cold PBS (1 ml). More ice cold PBS (1 ml) was added and the cells were scraped with a plastic cell scraper and collected in a 1.5 ml microcentrifuge tube.

Cells were centrifuged at 4°C and 3000 rpm for 1 min in a Heraeus® Biofuge® fresco microfuge (860 g) and the supernatant decanted and discarded. The cell pellet was resuspended in TRIZOL (1 ml; Invitrogen, Paisley, UK) and incubated at room temperature for 5 min. Samples were further centrifuged at 4°C and 13000 rpm for 15 min in a Heraeus® Biofuge® fresco microfuge (16100 g) and the supernatant transferred to a fresh 1.5 ml microcentrifuge tube. Phenol chloroform (200 µl; Applied Biosystems, CA, USA) was added to each sample before they were vortexed vigorously for 15 seconds and incubated at room temperature for 3 min. Samples were again centrifuged 4°C and 13000 rpm for 15 min in a Heraeus® Biofuge® fresco microfuge (16100 g) to separate the mixture into a lower phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was carefully transferred into a fresh 1.5 ml microcentrifuge tube without disturbing the interface. Isopropanol (500 µl) was added to precipitate the RNA and the samples were incubated at room temperature for 10 min before being centrifuged at 4°C and 13000 rpm for 15 min in a Heraeus® Biofuge® fresco microfuge (16100 g). The supernatant was decanted and discarded and the pellets washed with 75% (v/v) ethanol (1 ml) by vortexing samples and centrifuging at 4°C and 7500 rpm for 15 min in a Heraeus® Biofuge® fresco microfuge (5360 g) and again discarding the supernatant. The remaining RNA pellet was partially air-dried for 5-10 min and dissolved in nuclease free water (20 µl; Promega). RNA was quantified

on NanoDrop 1000 Spectrophotometer and all samples had an A_{260}/A_{280} ratio > 2.0 confirming the purity of the samples.

2.7.2 Generation of cDNA by reverse transcriptase

1 µg total RNA was added to 1 µl oligo (dT) primer (500 µg/ml; Promega) and made up to 15 µl with nuclease-free water (Promega). Samples were vortexed and spun briefly at 13000 rpm in a Heraeus® Biofuge® fresco microfuge (16100 g) before being incubated at 70ºC for 5 min. 5 µl M-MLV RT 5x buffer (see Section 2.11), 1.25 µl of 10 mM dNTP mix, 0.625 µl RNasin (40 U/µl), 1 µl M-MLV reverse transcriptase enzyme (200 U/µl) and 2.125 µl nuclease-free water were added to each sample. Samples were again vortexed and spun briefly at 13000 rpm in a Heraeus® Biofuge® fresco microfuge before being incubated at 42ºC for 60 min and 95ºC for 5 min. 75 µl nuclease-free water was added to make the final volume 100 µl and the samples stored at -20ºC.

2.7.3 Q-PCR

Q-PCR was performed in 20 µl reactions containing 10µl Taqman® Universal 2x PCR Master Mix (P/N 4304437 contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference, and optimized buffer components; Applied Biosystems), 1 µl of the Taqman® Gene Expression Assay of interest (Applied Biosystems), 5 µl cDNA and 4 µl nuclease free water. Expression assays used for this study were; BNIP3 (Hs00969293_mH), VEGF-A (Hs00173626_m1), CAIX (Hs00154208_m1), GLUT1 (Hs00892681_m1) and β-actin (Hs99999903_m1). All reactions were performed in duplicate. PCR plates were spun briefly up to 1000 rpm in a Sorvall® Legend RT bench top centrifuge (200 g) before PCR was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) according to the following thermal cycle protocol: 94°C 10 min followed by 40 cycles of denaturation at 94°C for 15 s and annealing/extension at 60°C for 1 min. Control reactions with no cDNA were run on each plate for each Taqman® Gene Expression Assay used and no amplification was detected in any control reaction. All expression values were normalised using expression of β-actin as a control.

2.8   Metabolic radiolabelling experiments

2.8.1 Metabolic radiolabelling

Cells were seeded at a density of 1×10^6 cells per well in a 6 well dish (Greiner Bio-One
Ltd.) in complete DMEM (2 ml) and incubated overnight to adhere. The following day
cells were washed three times in warm DMEM (1 ml) lacking L-glutamine, L-cysteine
and L-methionine (MP Biomedicals, Illkirch, France) before being incubated in DMEM
(1 ml) without L-glutamine, L-cysteine and L-methionine supplemented with 10% (v/v)
dialysed FBS and 2 mM glutamine for 1 h. Cells were pretreated for 1 h in the absence
or presence of crude watercress extract, PEITC, 0.3% DMSO (equivalent to the
highest volume of PEITC) or 10 µg/ml cycloheximide (CHX; Sigma) before the cells
were radioactively labelled. In one experiment cells were also treated with 25 nM of the
mTORC1 inhibitor rapamycin (Sigma). CHX is an inhibitor of general protein synthesis
(Baliga *et al.*, 1969) and acts as a positive control. In experiments where $CoCl_2$ was
used to mimic hypoxia, 100 µM was added at the same time as PEITC. 0.75 MBq per
ml TRAN35S - LABEL™ No - Thaw Metabolic Labelling Reagent (MP Biomedicals) with
specific activity >37.0 TBq/mmol was added to each well and the cells incubated for 2
h at 37°C/10% (v/v) CO_2. Future experiments were performed using the same amount
of [^{35}S]methionine and [^{35}S]cysteine.

2.8.2 Immunoprecipitation

Following S^{35} metabolic cell labelling (see Section 2.8.1), media was decanted and
discarded and the cells washed with ice cold PBS (1 ml) on ice. 1x RIPA buffer (800 µl)
with a 1:100 dilution of protease inhibitor cocktail and phosphatase inhibitor cocktail 1
was added and intermittently pipetted up and down to lyse all the cells. Lysates were
transferred to a 1.5 ml microcentrifuge tube and left on ice for 15 min. Samples were
centrifuged at 4°C and 13000 rpm in a Heraeus® Biofuge® fresco bench top centrifuge
(16100 g) for 10 min and the supernatant removed and aliquoted into a fresh
microcentrifuge tube. Samples were incubated with 1 µg anti-HIF1α (BD Biosciences)
overnight at 4°C on a suspension mixer. Meanwhile G-coupled Sepharose beads (GE
Healthcare UK Ltd.), were washed in 1x RIPA buffer (3x 1 ml) before being
resuspended in twice their volume of 1x RIPA buffer and stored at 4°C overnight. The
following day G-coupled Sepharose beads (100 µl of 2x slurry) were added to each
sample and incubated for 4 h at 4°C on a suspension mixer. Beads were washed in 1x

RIPA buffer (4x 500 µl) and resuspended in 30 µl 3x SDS Sample Buffer Red supplemented with 0.1 M DTT before being stored at -20ºC.

2.8.3 SDS-PAGE and phosphor imaging

See Section 2.4.3 for making Tris-HCl gels. Gels were placed in a BioRad Mini-PROTEAN 3 Cell and surrounded with approximately 500 ml 1x running buffer. The samples along with the protein marker were heated at 95ºC for 5 min before being spun briefly up to 13000 rpm in a Heraeus® Biofuge® fresco microfuge (16100 g). The protein marker (5 µl) was loaded into the first well and samples (15 µl) loaded into subsequent wells. The gel was resolved at 150 V for a minimum of 60 min before being removed and washed in 75% (v/v) ethanol (10 ml; Thermo Fisher Scientific) for 15 min at room temperature to fix it. The gel was dried for approximately 1 h in a BioRad GelAir Dryer according to the manufacturer's instructions with Saran™ wrap the size of the gel between the first layer of BioRad GelAir Cellophane Support and the gel for easy removal of the cellophane at the end of drying. The gel was placed in a BioRad Exposure Cassette-K for 24 h, face up against a Kodak storage phosphor screen (BioRad Laboratories). The Kodak storage phosphor screen was scanned by the BioRad Personal Molecular Imager® FX and analysed by BioRad Quantity One Software.

2.8.4 Trichloroacetic acid precipitation

Following S^{35} metabolic cell labelling (see Section 2.8.1), media was decanted and discarded and the cells washed with ice cold PBS (1 ml) on ice. Ice cold PBS (1 ml) was then added and the cells were detached with a plastic cell scrapper and collected in a 1.5 ml microcentrifuge tube. This protocol was carried out for all cell lines with the exception of TSC2+/+TP53-/- and TSC2-/-TP53-/- MEFs. In this case, cells were scraped on ice in their media before the whole volume was transferred to a 15 ml centrifuge tube and centrifuged at 4ºC and 1500 rpm in a Sorvall® Legend RT bench top centrifuge (500 g) for 5 min. The supernatant was discarded and the cell pellet resuspended in ice-cold PBS (1 ml) before being transferred to a 1.5 ml microcentrifuge tube. Cells were centrifuged at 4ºC and 1500 rpm in a Sorvall® Legend RT bench top centrifuge (500 g) for 5 min and were again washed in ice-cold PBS (1 ml).

Cell pellets were resuspended in distilled H_2O (100 µl) by vortexing. 50 µl of 3 mg/ml methionine (Sigma) and 3 mg/ml cysteine (Sigma) in 1 M NaOH (diluted from 10 M stock; Thermo Fisher Scientific) was added and the tubes incubated at 37°C for 15 min. 25% (w/v) trichloroacetic acid (100 µl; TCA; Sigma) was added to each tube before being cooled on ice. The resultant precipitate was collected on 25 mm glass microfibre filters (Whatman) using a sample manifold and the filters washed with 10% (w/v) TCA (3x 1 ml) and 95% (v/v) ethanol (2x 1 ml). The filter paper was air dried before being placed in a scintillation tube and covered with scintillation fluid (4 ml). Radioactivity was analysed on a WALLAC 1409 liquid scintillation counter (Perkin Elmer).

2.9   Statistical Analysis

All error bars represent ± the standard deviation of the mean for that data set. Data was analysed using the software package GraphPad Prism 4. The statistical significance of any differences was analysed using paired t-test. Asterisks on graphs are used to denote the following: *P < 0.05; **P < 0.01; ***P< 0.005.

2.10 Field trials

Three field trials were carried out at either Vitacress' Fobdown farm in Alresford or Vitacress' Doddings farm in Bere Regis in August 2009, September 2010 and April 2011. In general, watercress seeds were germinated in polytunnels on a thin layer of soil and transplanted into gravel lined concrete beds supplied with a constant flow of spring water from a bore hole. Seedling density and base application of fertiliser, containing 7.5% N ($CaNO_3$), 12% P_2O_4, 7.5% K_2O, 4.5% MgO + SO_3, followed by a second application once the plant roots had established themselves in the bed, were kept in accordance to Vitacress' standard practice. Growing period varied between the trials from 4-8 weeks, depending on weather, and the watercress was considered ready to harvest when leaf size reached approximately 3 cm in diameter. Samples of approximately 100 g were cut using scissors from each of the top, middle and bottom sections of the beds to allow for biological repeats and ensure any effect seen applied to the entire bed. Samples were collected in freezer bags and immediately put on ice, before being snap frozen in liquid nitrogen and stored at -80°C on return to the laboratory.

During the trials growing conditions were altered by cutting off spring water supply to the bed and the addition of sulfur-rich fertilisers. Microthiol® Special, which contains 80% w/w sulfur, was purchased from Fargro Ltd. (Littlehampton, UK) and was applied to the watercress as a foliar spray. Palm Brand sulfur powder, which is 99.8% sulfur, was also purchased from Fargro Ltd. and was added as a base application prior to transplanting the watercress seedlings. SoluPotasse, containing 55.8% SO_4, 50.9% K_2O and 0.6% Cl, and citric acid anhydrous (used to increase nutrient uptake) were purchased from Solufeed Ltd. (West Sussex, UK) and applied as a top dressing to the watercress.

For the second and third field trials, fresh samples from each of the beds were sent to Natural Resource Management Ltd. (NRM; Berkshire, UK) for chemical analysis of total sulfur content.

2.11 Media and solutions

Complete Dulbecco's modified Eagle's medium (DMEM)

500 ml DMEM

50 ml FBS

2 mM L-glutamine

50 U/ml Penicillin

50 U/ml Streptomycin

(For culture of all cell lines)

Serum free DMEM

500 ml DMEM

2 mM L-glutamine

50 U/ml Penicillin

50 U/ml Streptomycin

Roswell Park Memorial Institute 1640 (RPMI)

500 ml RPMI

Phosphate buffered saline (PBS)

137 mM NaCl

2.7 mM KCl

4.3 mM Na_2HPO_4

1.47 mM KH_2PO_4

Adjust to a final pH of 7.4.

5x Radioimmunoprecipitation assay (RIPA) buffer

250 mM Tris-HCl (pH 8.0)

750 mM NaCl

5% (v/v) Igepal CA-630

2.5% (w/v) Deoxycholate (DOC)

0.5% (w/v) Sodium dodecylsulfate (SDS)

Protease inhibitor cocktail (Sigma)

2 mM AEBSF

1 mM EDTA

130 µM Bestatin

14 µM E-64

1 µM Leupeptin

0.3 µM Aprotinin

3x SDS sample buffer red (Cell Signaling Technology)

187.5 mM Tris-HCl (pH 6.8 at 25°C)

6% (w/v) SDS

30% (v/v) glycerol

0.03% (w/v) phenol red

10x Running buffer

250 mM Tris-base

1.92 M glycine

1% (w/v) SDS

Transfer buffer

25 mM Tris-base

192 mM glycine

0.1% (w/v) SDS

25% (v/v) ethanol

10x TBS (Tris-buffered saline)

100 mM Tris-HCl (pH 8.0)

1.5 M NaCl

TBS-Tween

10 mM Tris-HCl (pH 8.0)

150 mM NaCl

0.1% (v/v) Tween-20

Stripping buffer

25 mM glycine (pH 2.0)

1% (w/v) SDS

Lysis buffer/developer mix

25 mM Tris-HCl (pH 8.0)

70 mM NaCl

1.3 mM KCl

1 mM $MgCl_2$

0.5% (v/v) Igepal CA-630

Just before use add:

2 mg/ml Trypsin (Sigma)

10 μM TSA

M-MLV RT 5x buffer (Promega)

250 mM Tris-HCl (pH 8.3 at 25°C)

375 mM KCl

15 mM $MgCl_2$

50 mM DTT

Chapter 3

Selection of assays to assess the *in vitro* anticancer effects of watercress-derived compounds

3.1 Introduction

The long term goal of this project was to determine whether it was possible to manipulate the growth environment of watercress to improve its potential anticancer activities. An important first step in this process was to identify approaches suitable for the accurate quantitation of anticancer effects. Therefore, the aim of the work described in this chapter was to select assays to assess the *in vitro* anticancer effects of watercress-derived compounds. Once assays have been identified, subsequent studies would then use these assays to assess the effects of watercress extracts from crops grown under different cultivation conditions.

This initial study focused on specific watercress-derived compounds, rather than watercress extracts. This approach was adopted before moving on to more complex proof-of-principle studies using extracts. Three specific watercress-derived compounds, PEITC, I3C and quercetin (see Figure 3.1 for compound structures) were selected for these studies. These compounds were selected because they have been shown to exert anticancer effects in a range of assays and were known to be present at high levels in watercress extracts. The assays selected for analysis included (i) inhibition of cell growth, (ii) inhibition of PI3K/Akt signalling, (iii) inhibition of HDAC activity and (iv) activation of Nrf2. Watercress compounds have been shown to inhibit the growth of various cancer cell lines (Chinni *et al.*, 2001; Lee *et al.*, 2009; Zhang *et al.*, 2003b) and PEITC has previously been shown to inhibit PI3K signalling and HDAC activity, and to activate Nrf2 (Bonnesen *et al.*, 2001; Myzak *et al.*, 2006; Satyan *et al.*, 2006). Human MCF7 breast cancer cells were used for the majority of experiments, although some studies were also performed in SK-BR-3 cells to determine potential effects within distinct subtypes of breast cancer.

3.2 Results

3.2.1 Effect of PEITC, I3C and quercetin on the growth inhibition of breast cancer cell lines

The MTS assay was used as a quick and simple way to measure growth inhibition by means of metabolic activity following treatment with PEITC, I3C or quercetin. In this context the term growth inhibition refers to the number of cells although it should be
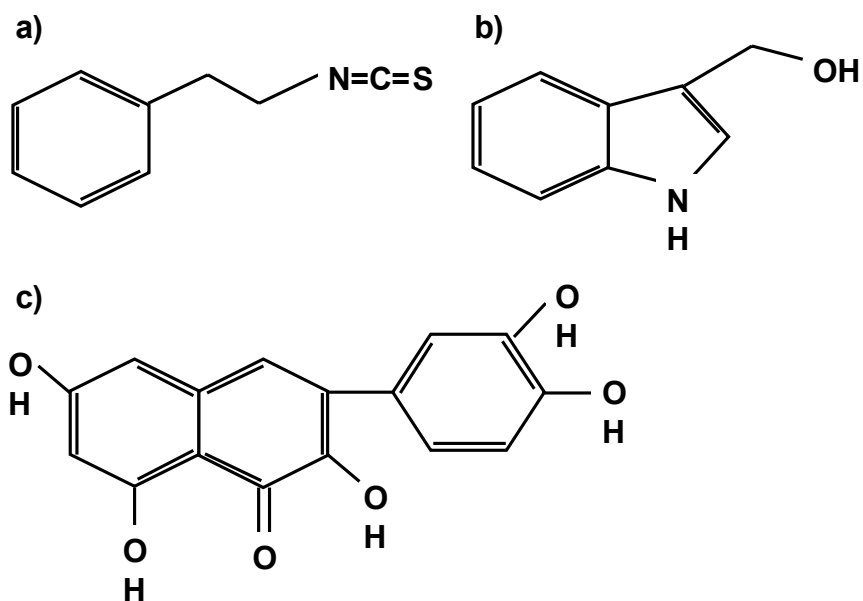
Figure 3.1: Structure of watercress-derived compounds.
(a) phenethyl isothiocyanate (PEITC), (b) indole-3-carbinol (I3C), (c) quercetin.

noted that the MTS assay in fact assesses metabolic activity and this is not an absolute measure of cell number. Changes to number of mitochondria can also alter metabolic activity. However, all plates were routinely checked under a microscope to confirm that the observed decrease in MTS reduction also correlated with reduced cell number. MCF7 and SK-BR-3 breast cancer cell lines were treated with the watercress-derived compounds for 6 days prior to the MTS assay. Representative growth inhibition data in the form of survival curves are shown for PEITC, I3C and quercetin in Figure 3.2, Figure 3.3, and Figure 3.4 respectively.

All compounds inhibited the growth of MCF7 and SK-BR-3 cells in a dose-dependent manner. Compounds provided simple sigmoidal dose response curves, allowing for robust curve-fitting and reproducible determination of relative IC_{50} values using the Prism software (Table 3.1). PEITC was found to be the most potent of the watercress-derived compounds in both MCF7 and SK-BR-3 cells, although MCF7 cells appear to be approximately 2.5-fold more sensitive to PEITC than SK-BR-3 cells. In contrast there was no statistically significant difference in IC_{50} values for either I3C or quercetin between the cell lines.

Watercress-derived compound	IC_{50}/µM Value (mean ± SD)	
	MCF7	SK-BR-3
PEITC	10.6 ± 1.4	27.0 ± 6.7***
I3C	104.7 ± 25.6	77.3 ± 25.0
Quercetin	51.1 ± 11.7	133.9 ± 96.7

Table 3.1: IC_{50} values of PEITC, I3C and quercetin in MCF7 and SK-BR-3 cells. IC_{50} values represent mean values ± standard deviation (SD) from at least five experiments performed in triplicate. Statistically significant differences in IC_{50} values for the compounds between the two cell lines are indicated (*** $p<0.005$).

3.2.2 Molecular assays

3.2.2.1 Effect of PEITC, I3C and quercetin on PI3K/Akt pathway

HER2 is a member of the epidermal growth factor receptor (EGFR) family that when activated initiates a signal via the PI3K/Akt pathway that results in cell proliferation. HER2 has been found to be overexpressed in many cancers, and this overexpression
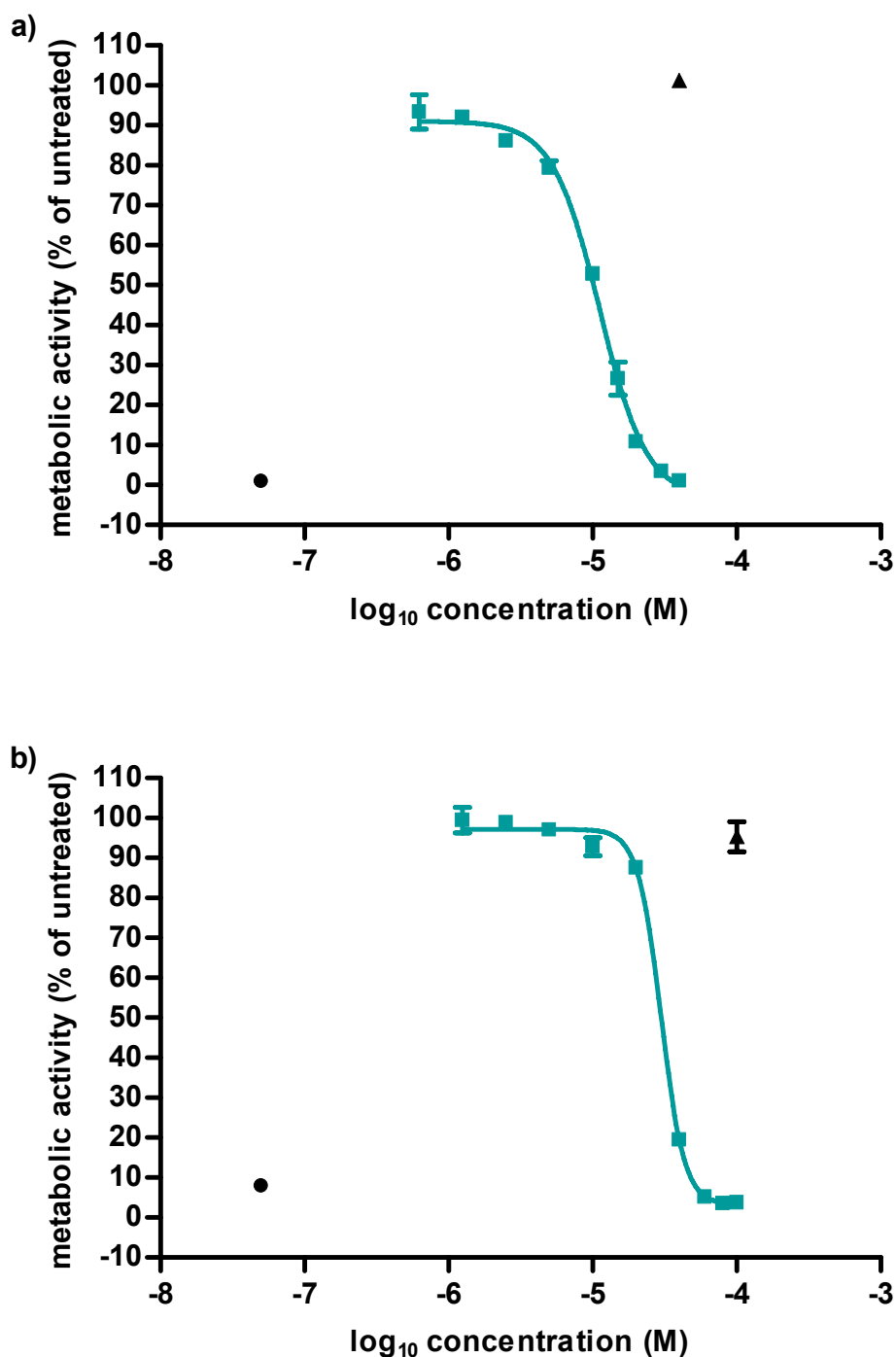
Figure 3.2: Effect of PEITC on growth inhibition of MCF7 and SK-BR-3 cells.
Representative growth inhibition experiments of (a) MCF7 and (b) SK-BR-3 cell lines.
Cells were treated with various concentrations of PEITC (■), DMSO (▲) equivalent to
the highest volume of PEITC and 0.5 µM STS as a positive control (●). After six days
metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One
Solution reagent. Data shown are derived from means of triplicate wells (± SD).
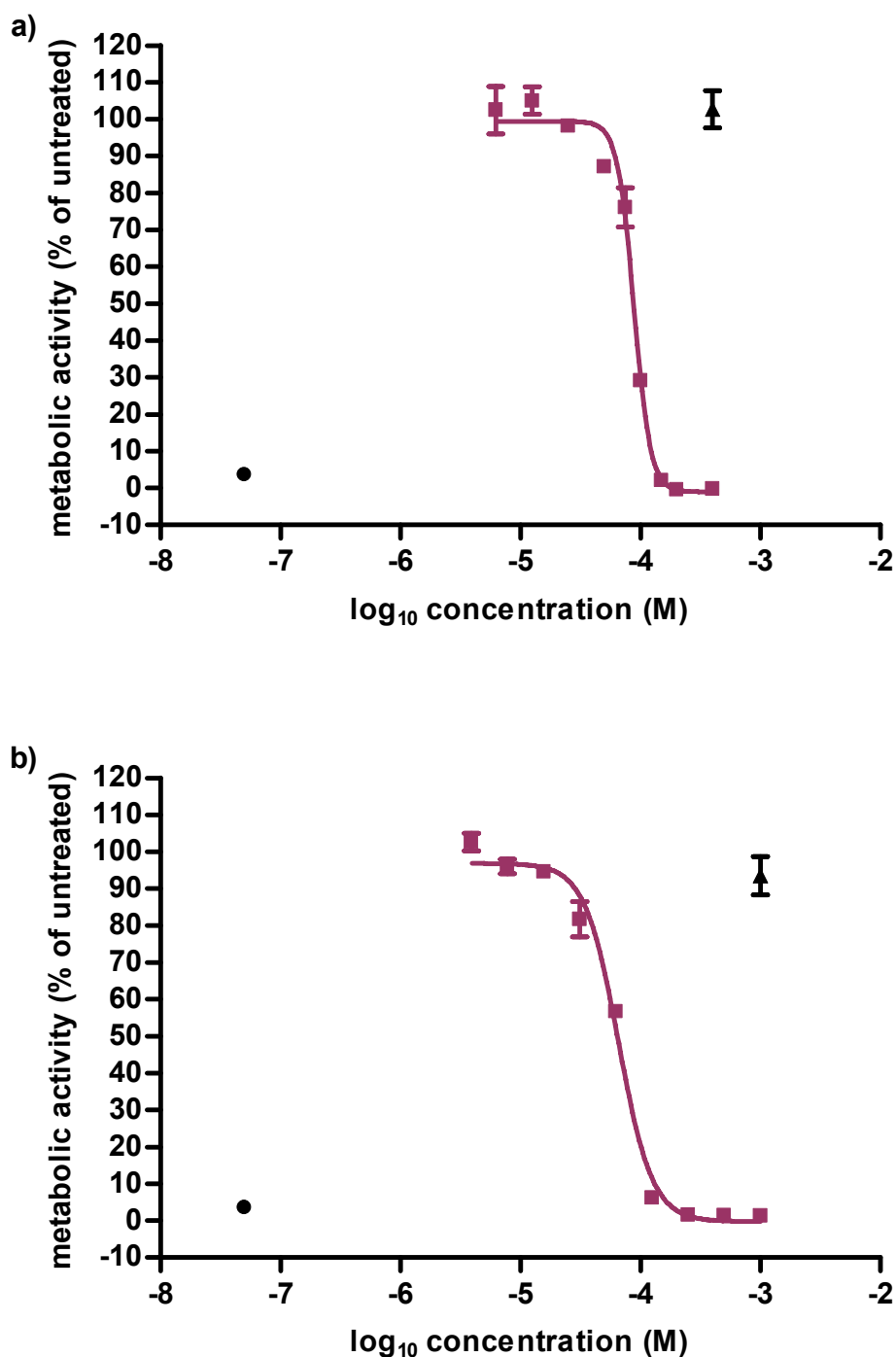
Figure 3.3: Effect of I3C on growth inhibition of MCF7 and SK-BR-3 cells.
Representative growth inhibition experiments of (a) MCF7 and (b) SK-BR-3 cell lines.
Cells were treated with various concentrations of I3C (■), DMSO (▲) equivalent to the
highest volume of I3C and 0.5 µM STS as a positive control (●). After six days
metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One
Solution reagent. Data shown are derived from means of triplicate wells (± SD).
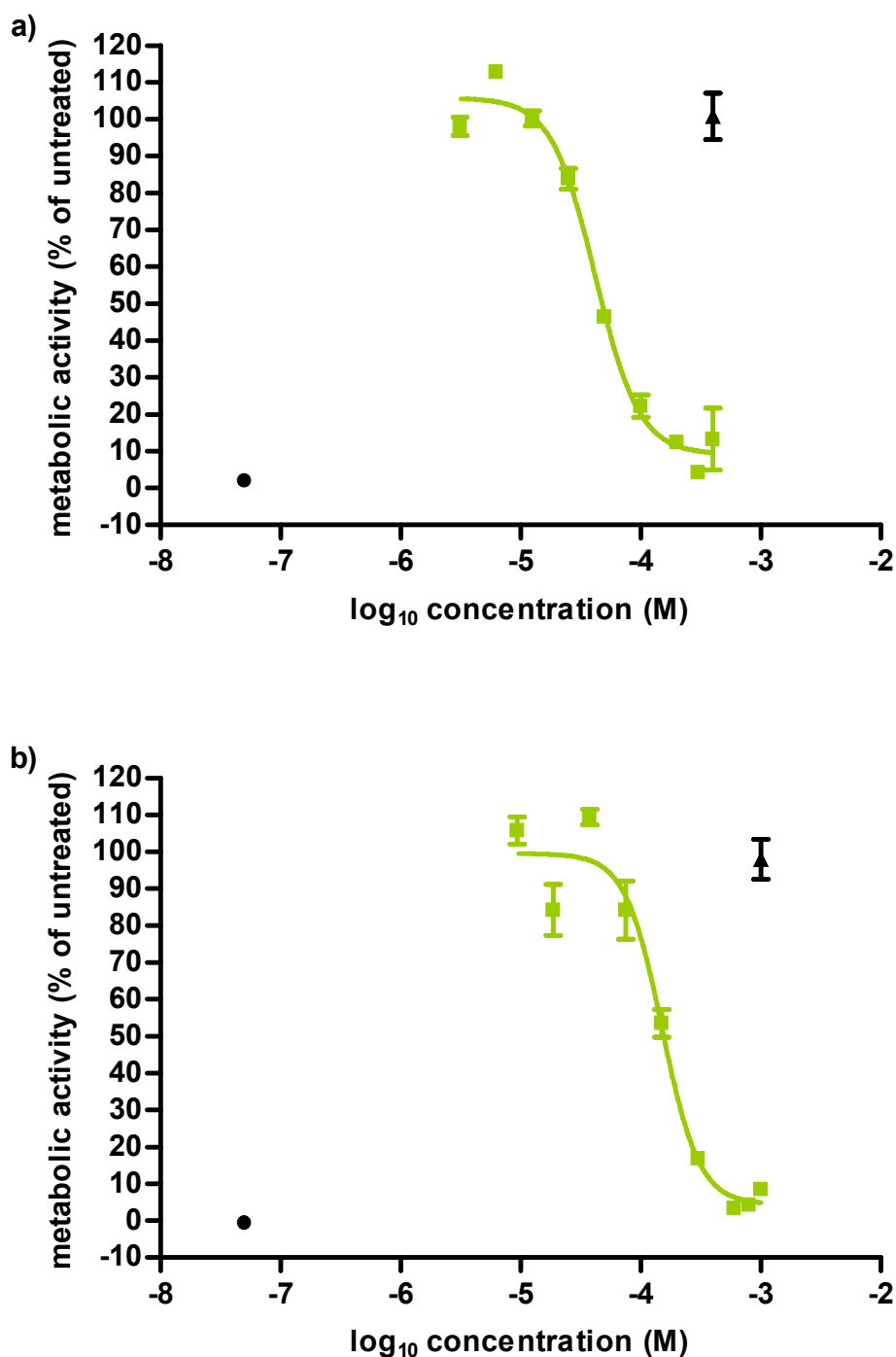
Figure 3.4: Effect of quercetin on growth inhibition of MCF7 and SK-BR-3 cells.
Representative growth inhibition experiments of (a) MCF7 and (b) SK-BR-3 cell lines.
Cells were treated with various concentrations of quercetin (■), DMSO (▲) equivalent to
the highest volume of quercetin and 0.5 µM STS as a positive control (●). After six days
metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One
Solution reagent. Data shown are derived from means of triplicate wells (± SD).

is consistent with poor clinical outcome (Slamon *et al.*, 1987; Slamon *et al.*, 1989). This pathway was chosen to be investigated as it represents a key cancer pathway, especially in certain breast cancer subtypes, and quercetin has been previously shown to reduce HER2 expression (Kim *et al.*, 2005).

Akt phosphorylation was used as a measure of the effect of watercress-derived compounds on HER2. The activation of HER2 triggers a signal cascade involving recruitment of Akt to the cell membrane and its subsequent phosphorylation. Akt is phosphorylated at multiple sites, at threonine 308 (Thr308) by PDK1 (Alessi *et al.*, 1997) and at serine 473 (Ser473) by mTORC2 (Sarbassov *et al.*, 2005). It has been shown that the monoclonal antibody Herceptin inhibits HER2 induced proliferation and this correlates with reduced Akt phosphorylation at Thr308 and Ser473 (Longva *et al.*, 2005; Tseng *et al.*, 2006). Herceptin is only effective in cancers where HER2 is overexpressed (Vogel *et al.*, 2002) and as a result the SK-BR-3 breast cancer cell line was used initially in this experiment as these cells overexpress HER2. Cells were incubated in the presence or absence of PEITC, I3C or quercetin for 2 h and Akt phosphorylation at both Thr308 and Ser473 analysed by western blot (Figure 3.5 and 3.6). Blots were stripped and reprobed for β-actin and total Akt to demonstrate equal loading and that changes in Akt phosphorylation occurred independent of changes to total Akt protein levels. It should be noted that sometimes there are two bands detected when probing for phospho-Akt (Thr308). The faster migrating band may be Akt 2 isoform that has a Mw of 56 kDa compared to 60 kDa of Akt 1 and 3.

Quercetin induced a statistically significant decrease in phosphorylation of Akt at Ser473 at both concentrations tested, but had no effect on phosphorylation at Thr308. There appeared to be a trend towards increased phosphorylation of both sites in PEITC-treated cells, although this did not reach statistical significance. There was no effect of I3C on either Akt phosphorylation site.

As receptors other than HER2 can lead to Akt phosphorylation the effect of watercress-derived compounds on Akt phosphorylation was also investigated in MCF7 cells (Figure 3.7 and 3.8). Similar to SK-BR-3 cells, quercetin has no effect on Thr308 phosphorylation but caused a statistically significant reduction in Akt Ser473 phosphorylation. There was no change observed following I3C treatment. Similar to SK-BR-3 cells, PEITC increased Akt phosphorylation at both Thr308 and Ser473 and, in MCF7 cells, this did reach statistical significant at all concentrations tested. Taken together the data demonstrate that watercress compounds do modulate Akt
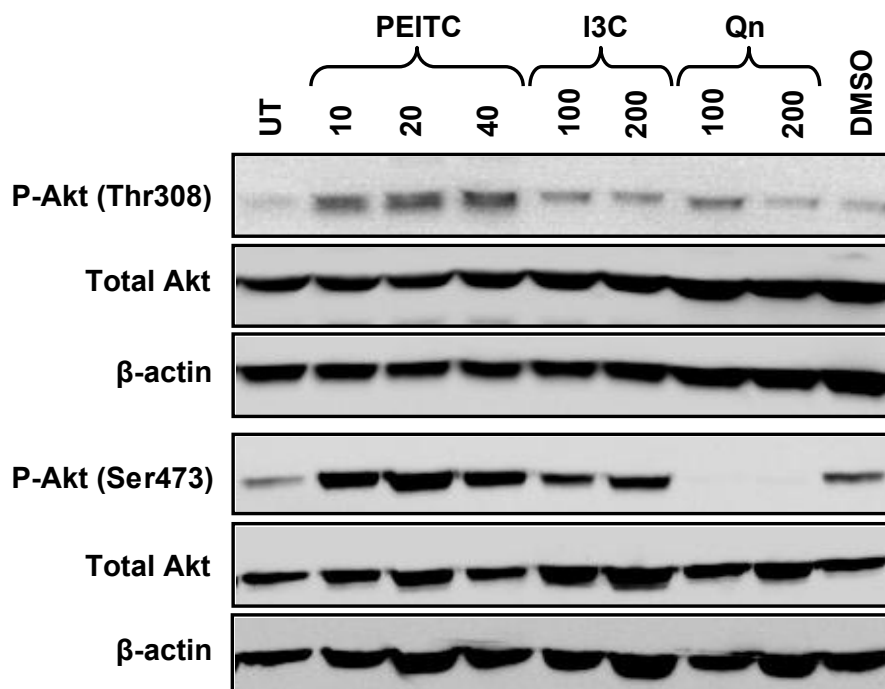
Figure 3.5: Effect of PEITC, I3C and quercetin on Akt phosphorylation in SK-BR-3 cells.
SK-BR-3 cells were treated with indicated concentrations (µM) of PEITC, I3C, or quercetin (Qn), DMSO (equivalent to the highest volume), or left untreated (UT) for 2 h. 20 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for either phospho-Akt (Thr308) or phospho-Akt (Ser473) then stripped and reprobed for total Akt (60 kDa) and β-actin (42 kDa). Western blots are representative of three independent experiments, the results of which are combined in the densitometry graph (Figure 3.6).
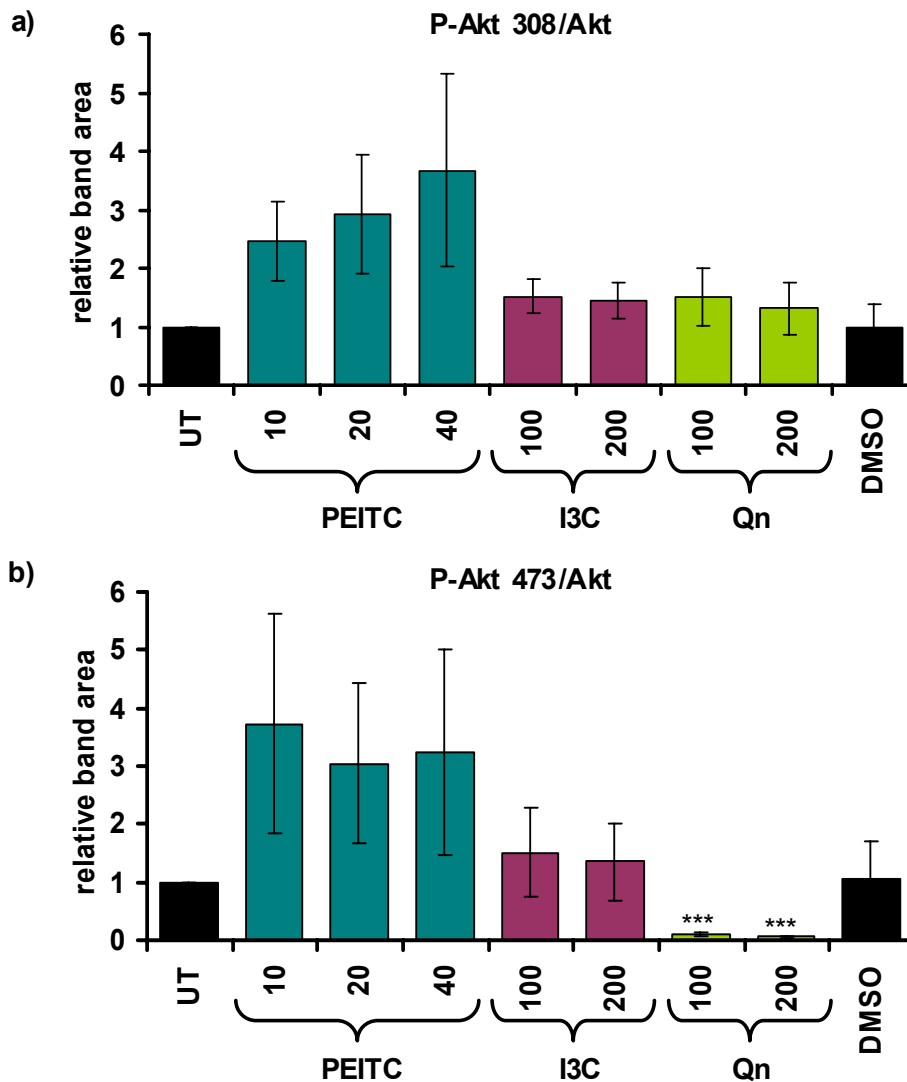
Figure 3.6: Densitometry of effect of PEITC, I3C and quercetin on Akt phosphorylation in SK-BR-3 cells western blot.
SK-BR-3 cells were treated with indicated concentrations (µM) of PEITC, I3C, or quercetin (Qn), DMSO (equivalent to the highest volume), or left untreated (UT) for 2 h. Whole cell lysate was subjected to SDS-page and analysed by western blot for either phospho-Akt (Thr308) or phospho-Akt (Ser473) then stripped and reprobed for total Akt. Band area from three independent experiments were analysed by ImageJ software and mean band area of (a) P-Akt (308) or (b) P-Akt (473) relative to total Akt are shown (± SD). Statistically significant differences between UT and the treatments are indicated (*** $p < 0.005$). All other comparisons were not statistically significant.
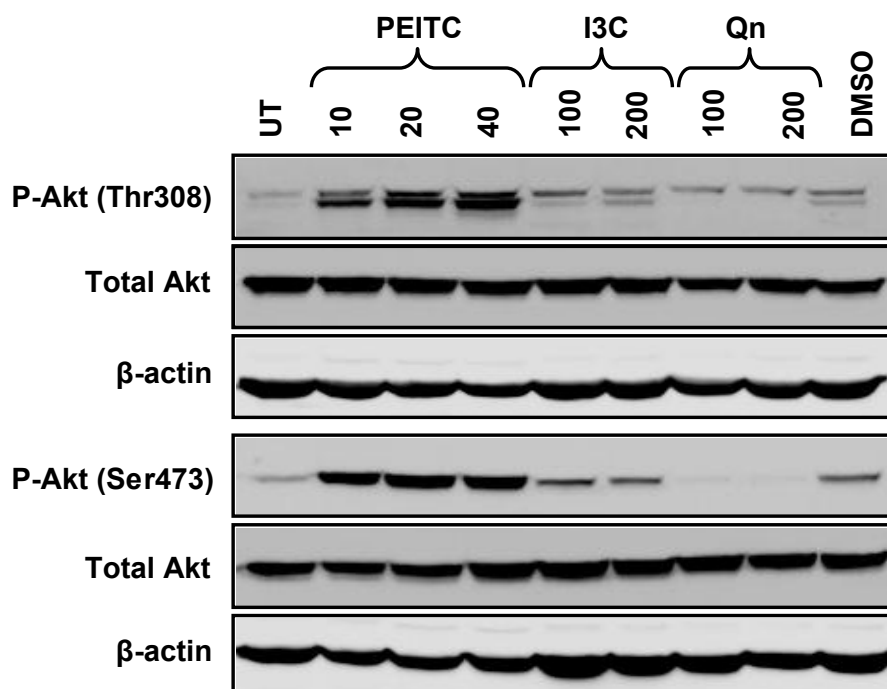
Figure 3.7: Effect of PEITC, I3C and quercetin on Akt phosphorylation in MCF7 cells.
MCF7 cells were treated with indicated concentrations (µM) of PEITC, I3C, or quercetin (Qn), DMSO (equivalent to the highest volume), or left untreated (UT) for 2 h. 20 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for either phospho-Akt (Thr308) or phospho-Akt (Ser473) then stripped and reprobed for total Akt (60 kDa) and β-actin (42 kDa). Western blots are representative of three independent experiments, the results of which are combined in the densitometry graph (Figure 3.8).
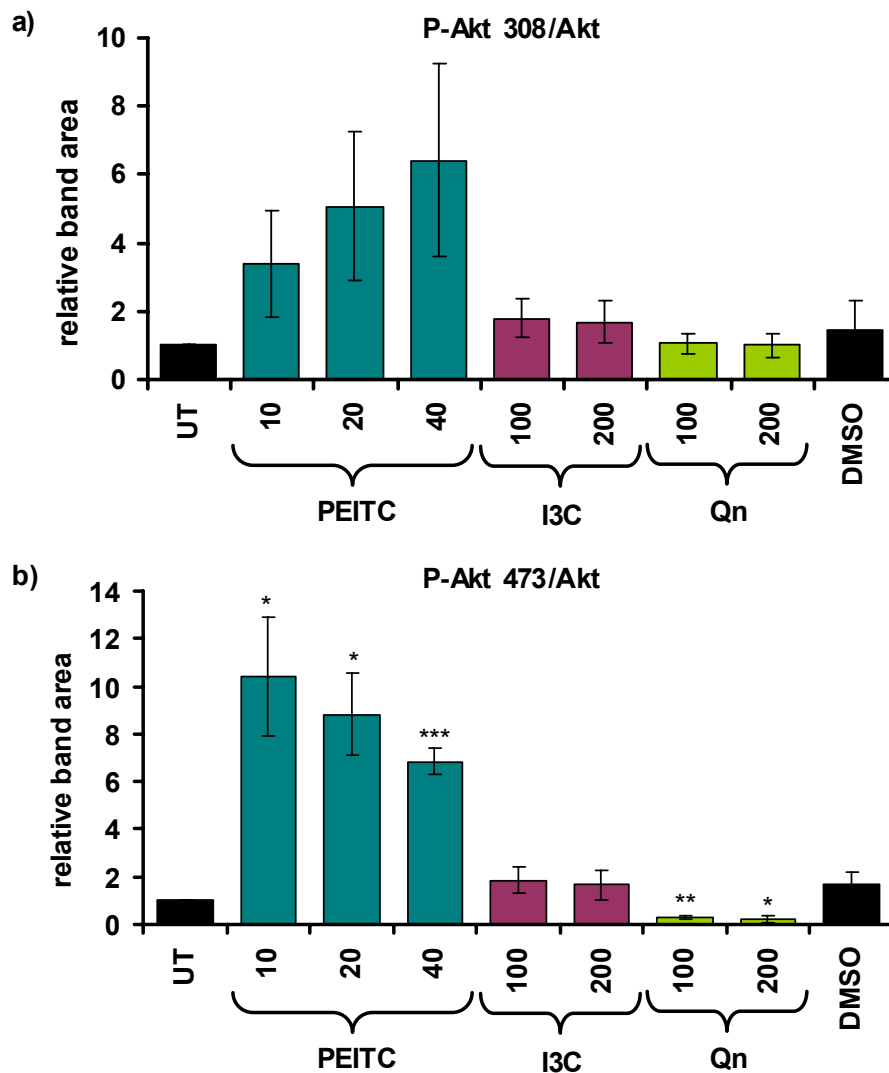
Figure 3.8: Densitometry of effect of PEITC, I3C and quercetin on Akt phosphorylation in MCF7 cells western blot.
MCF7 cells were treated with indicated concentrations (μM) of PEITC, I3C, or quercetin (Qn), DMSO (equivalent to the highest volume), or left untreated (UT) for 2 h. Whole cell lysate was subjected to SDS-page and analysed by western blot for either phospho-Akt (Thr308) or phospho-Akt (Ser473) then stripped and reprobed for total Akt. Band area from three independent experiments were analysed by ImageJ software and mean band area of (a) P-Akt (308) or (b) P-Akt (473) relative to total Akt are shown (± SD). Statistically significant differences between UT and the treatments are indicated (* $p<0.05$; ** $p<0.01$; *** $p<0.005$). All other comparisons were not statistically significant.

phosphorylation in breast cancer cell lines. However, different compounds either increase or decrease phosphorylation. Overall there appears to be little difference in the effect of watercress derived compounds between the two cell lines suggesting that these responses are independent of HER2. Although as only two cell lines were looked at this would require further validation.

3.2.2.2 Effect of PEITC, I3C and quercetin on HDAC inhibition

Altered gene expression patterns due to modified chromatin organisation are found in many cancers. Epigenetic modifications such as the level of histone acetylation, which is determined by the balance between the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs), are responsible for remodeling chromatin into a transcriptionally active or inactive state (Ropero and Esteller, 2007). In particular, HDAC inhibitors are emerging as a promising new tool in the treatment of cancer. They offer the possibility to reverse the repression of various genes that are involved with apoptosis and cell cycle regulation such as *Bax* and *p21*. The assay to determine HDAC inhibition activity in the watercress-derived compounds was selected as there is some evidence that conjugates of ITCs act as HDAC inhibitors (Myzak *et al.*, 2004). This HDAC inhibition coincides with increased global acetylation at histone H3 and H4, as well as specific acetylation at the *p21* promoter. To examine if PEITC, I3C and quercetin act as HDAC inhibitors MCF7 cells were treated in the absence or presence of each compound for 16 h before the addition of a HDAC substrate, BOC-lys(ac)-AMC, for a further hour.

Cells were subsequently lysed and incubated overnight before fluorescence was determined on a plate reader. If the BOC-lys(ac)-AMC compound is able to be deacetylated by the cells then when the lysis buffer is added the trypsin in it is able to cleave the bond between the now unprotected lysine and the AMC fluorescent fluorophore, thus releasing it and allowing it to give off a fluorescent signal. However, if the compounds act as HDAC inhibitors and prevent the cells from deacetylating the BOC-lys(ac)-AMC compound, then the lysine will remain acetylated. The trypsin will then be unable to cleave the fluorophore and subsequently there will be a reduction in fluorescent signal.

The known HDAC inhibitor Trichostatin A (TSA) (Yoshida *et al.*, 1990) was used as a positive control and shows complete loss of fluorescence at all concentrations tested

(Figure 3.9). With regards to the watercress-derived compounds there was very little change in fluorescence following either PEITC or I3C treatment, although there was a dose dependent decrease in fluorescence when the cells were treated with quercetin with an IC_{50} of 100.2 μM.

It was important to determine whether quercetin acted as a true HDAC inhibitor, or whether it interfered with trypsin cleavage in the second phase of the assay (i.e. was a false positive). To investigate this, non-acetylated BOC-lys-AMC was added to quercetin treated cells alongside the BOC-lys(ac)-AMC reagent. If quercetin was a *bona fide* HDAC inhibitor than it would have no effect on the fluorescence of the non-acetylated reagent as it is not a HDAC substrate. In this experiment another known HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) (Richon *et al.*, 1998) was used as a positive control and demonstrated a dose-dependent loss of fluorescence (Figure 3.10). While a decrease in fluorescence was again observed in cells treated with quercetin and BOC-lys(ac)-AMC, a similar reduction in fluorescence was detected in cells treated with the non-acetylated reagent. Therefore, quercetin is not a true HDAC inhibitor but produced a false positive result by interfering with trypsin's ability to cleave the bond between the deacetylated lysine and the AMC fluorescent fluorophore.

3.2.2.3 Effect of PEITC on Nrf2 activity

One of the key targets for xenobiotics and phytochemicals is the Nrf2 transcription factor. Activation of Nrf2 induces a battery of target genes involved in antioxidant protection. This is important not just for protecting cells from oxidative/electrophilic damage, but also the chemopreventive actions of compounds such as PEITC. I therefore analysed the effect of PEITC on Nrf2 activity. Nrf2 activity was analysed using AREc32 cells, a stable MCF-7 derived reporter cell-line containing a luciferase construct controlled by multiple copies of the antioxidant response element (ARE) (Wang *et al.*, 2006). AREc32 cells were incubated in the presence of PEITC for 24 h before luciferase was assayed. PEITC caused a dose-dependent increase in ARE-driven gene expression that was statistically significant at all doses tested (Figure 3.11). In previous studies, effective concentrations have been considered as the concentration of compound that doubled induction of luciferase gene activity (CD) (Wang *et al.*, 2006). In my hands, 2.5 μM PEITC resulted in over a 2-fold increase in Nrf2 activity.
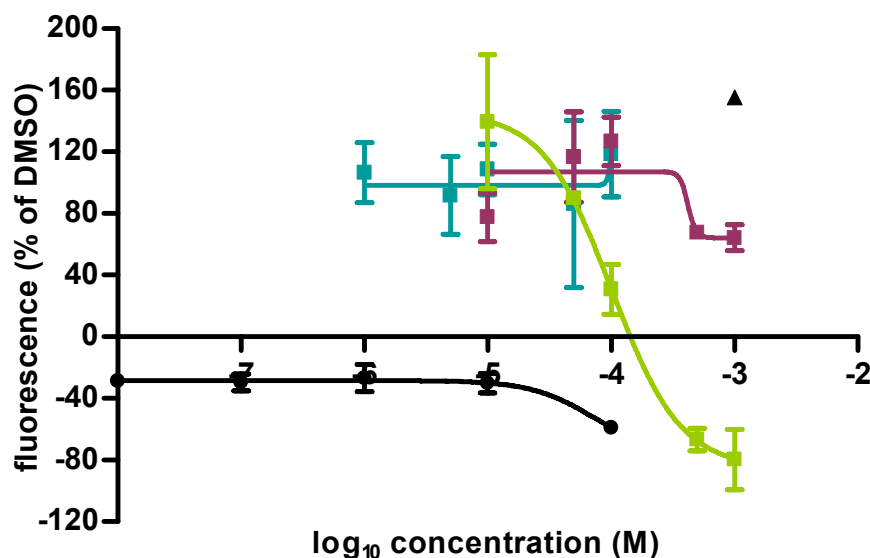
Figure 3.9: Effect of watercress-derived compounds on HDAC inhibition.
MCF7 cells were treated in the absence or presence of either PEITC (■), I3C (■), or quercetin (■) for 16 h before the BOC-lys(ac)-AMC reagent was added for 1 h. Lysis buffer was added and the cells were incubated overnight before fluorescence was read on a 96-well plate reader. Known HDAC inhibitor TSA (●) was used as a positive control and ▲ represents untreated. Data shown are derived from means of triplicate wells ± SD.
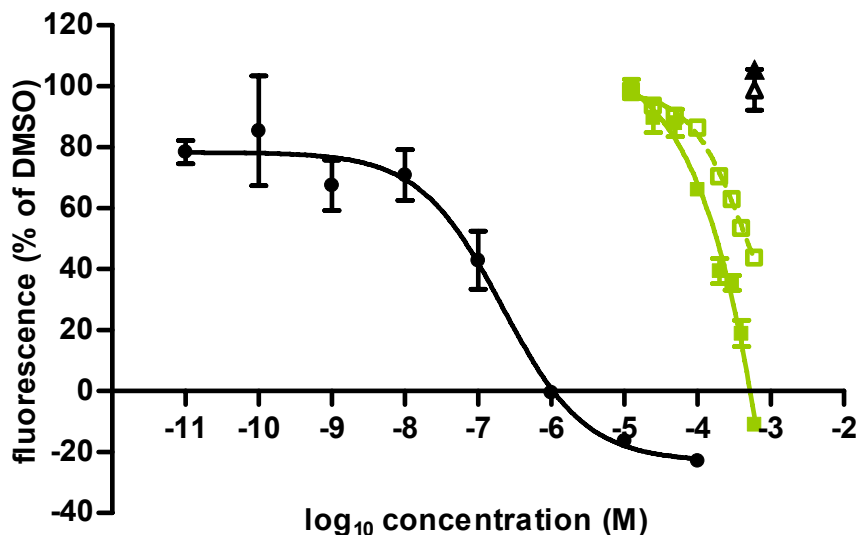
Figure 3.10 Effect of quercetin on HDAC inhibition.
MCF7 cells were treated in the absence or presence of quercetin for 16 h before either the BOC-lys(ac)-AMC reagent (■) or non-acetylated control BOC-lys-AMC (□) was added for 1 h. Lysis buffer was added and the cells were incubated overnight before fluorescence was read on a 96-well plate reader. Known HDAC inhibitor SAHA (●) was used as a positive control. ▲ and △ represent untreated with either the BOC-lys(ac)-AMC or BOC-lys-AMC respectively. Data shown are derived from means of triplicate wells ± SD.
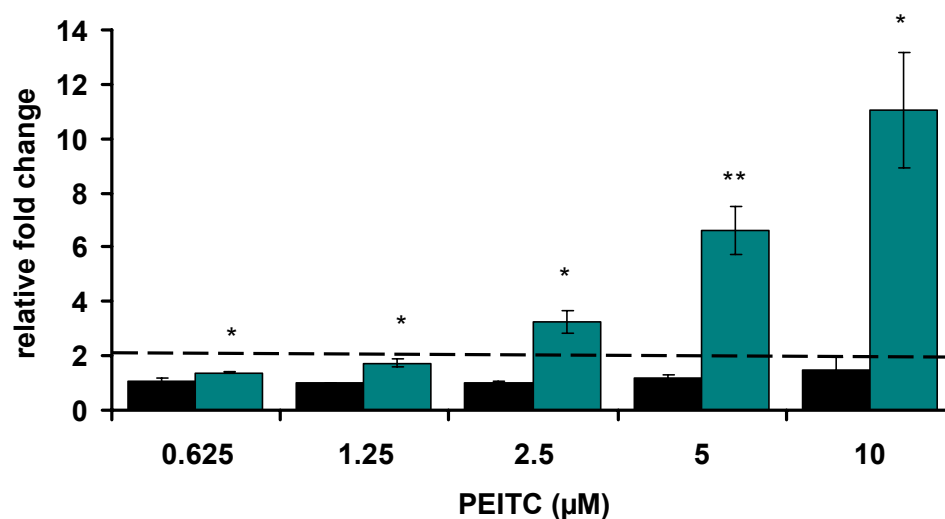
Figure 3.11: Effect of PEITC on ARE-driven expression.
MCF7 cells stably transfected with ARE were treated with the indicated concentrations of PEITC (■) or equivalent volume of DMSO as a control (■) for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments. Statistically significant differences between DMSO and PEITC treated cells are indicated (* $p<0.05$; ** $p<0.01$).

3.3   Discussion

The aim of the work described in this chapter was to identify assays that could be used for the subsequent analysis of the *in vitro* anticancer effects of watercress extracts. Based on published literature, I selected four assays to investigate, (i) growth inhibition, (ii) inhibition of PI3K/Akt signalling, (iii) HDAC activity and (iv) Nrf2 activity.

3.3.1   Growth inhibition

Growth inhibition appeared to be an appropriate assay to monitor the activity of watercress-derived compounds. The MTS assay is rapid and simple, and can be performed in 96 well plates allowing determination of IC_{50} values with good reproducibility. All compounds inhibited the growth of both MCF7 and SK-BR-3 cells in a dose-dependent manner. This is consistent with previous studies demonstrating that all three compounds inhibited the growth of a variety of human cancer cells grown in culture and within a similar concentration range (Chinni *et al.*, 2001; Lee *et al.*, 2009; Zhang *et al.*, 2003b). Overall, PEITC was the most potent compound tested. There was relatively little difference in the response of the two cell lines to the compounds, and only for PEITC were responses statistically different, with MCF7 cells being approximately 2.5-fold more sensitive.

It is not clear why MCF7 cells are more sensitive to growth inhibition by PEITC. MCF7 cells have wild type p53 whereas SK-BR-3 cells contain mutant p53. Some studies have suggested that PEITC has similar effects in wild type and mutant p53 containing colon cancer cells (Pappa *et al.*, 2006). However, other studies have shown that PEITC induced greater apoptosis in mouse epidermal JB6 p53 -/- cells compared to corresponding p53 +/+ cells (Huang *et al.*, 1998). Therefore, the role of p53 in the differential responses of MCF7 and SK-BR-3 cells is unclear. Recent studies in the laboratory suggest that MCF7 cells have relatively high basal levels of oxidative stress compared to other breast cancer cell lines (Sharaifah Syed Alwi, Breeze Cavell, Graham Packham, unpublished). Heightened basal ROS may explain why these cells are more susceptible to further PEITC-induced ROS accumulation.

The mechanisms of PEITC induced growth inhibition are not clear. Due to the indirect nature of the MTS assay, decreased signals may be due to decreased cell division and/or increased apoptosis. Decreased metabolism could also contribute to reduced

MTS reduction. However, visual inspection of the plates confirmed that reduced MTS signals were associated with reduced cell numbers. Further studies in the group have shown that PEITC induces a G2/M arrest and high levels of apoptosis in MCF7 cells, and this could be studied further in SK-BR-3 cells using PI/cell cycle analysis and annexin V staining, respectively.

The molecular mechanisms that mediate cell death/cell cycle arrest in response to watercress compounds are likely to be complex. As described in the introduction, PEITC has been shown to induce G2/M and S phase arrest via downregulation of CDK1, cyclin B and cyclin A (Hwang and Lee, 2010; Wu *et al.*, 2011; Xiao *et al.*, 2004), in addition to inducing apoptosis via both the intrinsic and extrinsic pathways (Satyan *et al.*, 2006; Tang and Zhang, 2005).

By contrast, I3C has consistently been shown to induce G1 cell cycle arrest in several different cancer cell lines, although the specific factors involved varied (Rogan, 2006). In MCF7 cells I3C has been reported to downregulate CDK6 expression by disrupting the binding of transcription factor SP1 to its promoter region, and inhibit CDK2 activity by altering the composition of the CDK2 protein complex to a larger less active form, both essential in the progression of G1 phase. I3C has also been recently shown to promote the degradation of phosphatase Cdc25A, required for dephosphorylation and activation of CDK2, in a panel of breast cancer cell lines (Wu *et al.*, 2010b). With regards to apoptosis, it appears I3C may act to suppress the activation of nuclear transcription factor (NF-κB), which regulates a wide range of genes involved in apoptosis including *Bcl-2* and *Bcl-xL* (Ahmad *et al.*, 2011). This could be the result of the inactivation of Akt, an upstream regulator of NF-κB, as I3C treatment has been associated with a reduction in Akt activity (Rahman *et al.*, 2004).

Quercetin has been shown to inhibit cell cycle progression through the G2/M or G1/S transition. In the SK-BR-3 breast cancer cell line quercetin was shown to cause cell cycle arrest at G1 and this was associated with an increase in *p21* expression and hypophosphorylation of retinoblastoma tumour suppressor protein (Rb). p21 is a CDK inhibitor and is proposed to inhibit cyclin-CDK activity required for phosphorylation of Rb. Unphosphorylated Rb sequesters transcription factor E2F1 preventing it from promoting expression of genes essential for cell cycle progression from G1 to S phase. However, in the same study quercetin was also shown to downregulate cyclin B and CDK1 in SK-BR-3 cells which are required for G2/M cell cycle progression (Jeong *et al.*, 2009). Quercetin can also inhibit phosphorylation of Akt which is thought to result in

apoptosis by causing dissociation of Bax from Bcl-xL (Lee *et al.*, 2008), and upregulate death receptor 5 (DR5) expression in prostate cancer DU-145 cells, sensitising them to TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis (Jung *et al.*, 2010).

3.3.2 PI3K/Akt signalling

Several previous studies have shown that PEITC, I3C and quercetin modulate Akt phosphorylation and for the most part this was confirmed in my experiments using HER2 overexpressing SK-BR-3 cells. Although HER2 is an important activator of Akt in SK-BR-3 cells, the effects of the compounds may be independent of any specific affect on HER2 signalling, since similar results were obtained in MCF7 cells which lack HER2 overexpression, and a wide range of signalling pathways have been shown to activate PI3K/Akt signalling. Despite the observed effects, analysis of Akt phosphorylation did not appear to be a suitable assay to monitor effects of watercress extracts. First, the assay was very time-consuming and quantitation limited by the semiquantitative nature of immunoblotting. Second, different compounds either increased or decreased Akt phosphorylation making it likely that effects of watercress extracts would be highly complex.

Loss of Akt phosphorylation in response to quercetin has been observed in multiple cell lines and is associated with quercetin-induced apoptosis (Kim and Lee, 2007; Lee *et al.*, 2008; Sun *et al.*, 2010). The majority of these studies look only at Akt phosphorylation at Ser473, and this is consistent with my findings that quercetin inhibits phosphorylation at this site. Kim and Lee (2007) did observe inhibition of Akt phosphorylation at both Thr308 and Ser473 sites following treatment with 200 μM quercetin for 4 h in human prostate adenocarcinoma DU-145 cells. However, in MCF7 and SK-BR-3 breast cancer cells I found quercetin had no effect on Thr308 Akt phosphorylation. Consequently, the effect of quercetin on Akt phosphorylation, particularly at Thr308, may be cell type specific.

It is widely reported that PEITC inhibits Akt activation and this is believed to contribute to its anticancer activity (Gao *et al.*, 2011; Satyan *et al.*, 2006; Xiao and Singh, 2007). In contrast, I found Akt phosphorylation at both Thr308 and Ser473 sites was enhanced in response to PEITC. However, it was recently shown that while PEITC did cause a decrease in Akt phosphorylation in multiple myeloma MM.1S cells at later time points (12 h), this followed an initial increase in Akt phosphorylation (2 h) (Jakubikova *et al.*, 2011). Therefore, the increase in Akt phosphorylation I observe after 2 h may

also only be transient. It has been previously reported that I3C can inhibit Akt phosphorylation in breast cancer and prostate cancer cell lines after 24-48 h treatments (Chinni and Sarkar, 2002; Rahman *et al.*, 2004). While I did not observe any effect of I3C on Akt phosphorylation this may be due to the shorter time point (2 h) selected for this study.

3.3.3 HDAC activity

PEITC, I3C and quercetin were examined for HDAC inhibition activity in a cell-based assay using MCF7 cells. Wang *et al.* (2007) have previously shown by both a cell-free assay and through the analysis of PEITC treated LNCap AD prostate cancer cell lysates, that as little as 5 µM PEITC is able to inhibit HDAC activity. In contrast to this, I observed no inhibition of HDAC activity following treatment with PEITC. Similar results were obtained with I3C. Quercetin did appear to be a potential HDAC inhibitor. However, subsequent control experiments demonstrated that this apparent activity was in fact likely to be due to inhibition of trypsin activity. Several flavonoids including quercetin have previously been shown to act as trypsin inhibitors through binding with the S^1 region of trypsin via various electrostatic interactions that include at least one hydrogen bond (Jedinak *et al.*, 2006; Maliar *et al.*, 2004; Zhang *et al.*, 2009). It is not clear why I did not observe HDAC inhibition with PEITC, but this may reflect the relatively short time point selected for these studies. Longer exposure may be required to allow accumulation of the PEITC-metabolites which are thought to be the active components for HDAC inhibition. Regardless, the HDAC assay was not considered to be suitable for analysis of watercress extracts. It was expensive and time-consuming to perform and clearly susceptible to false positive results.

3.3.4 Nrf2 activity

The effect of PEITC on Nrf2 activity was determined using a stable MCF7 derived reporter cell line containing a luciferase construct controlled by multiple copies of the ARE. As discussed in the introduction, PEITC has been shown to induce the expression of phase II drug metabolising enzymes via the ARE present in the promoter regions of these genes, and this is believed to be responsible for inhibition of carcinogen-induced tumourigenesis in rodents by PEITC. I confirmed that PEITC increased Nrf2-dependent transcription in a dose-dependent manner, with a concentration of 2.5 µM required to double the induction of gene expression. This is in agreement with other studies that demonstrated 1.7 µM and 2 µM PEITC caused 2-fold

increase in the induction of Nrf2-dependent transcription using a chloramphenicol acetyltransferase (CAT) and luciferase reporter construct respectively (Bonnesen *et al.*, 2001; Xu *et al.*, 2006b). Overall, the Nrf2 activity assay was considered suitable for subsequent analysis of watercress extracts since it was straightforward to perform and the assay produced quantitative data. Moreover, in contrast to Akt and HDAC, where the significance of any regulation remains poorly understood, it is clear that Nrf2 activation is a key mediator of chemopreventive activities.

I did not perform any studies with I3C as previous studies have shown it to be a relatively weak inducer of ARE-driven gene expression, instead inducing expression of phase I and phase II drug metabolising enzymes via xenobiotic response elements (XREs) (Bonnesen *et al.*, 2001). In this case, transcriptional regulation is mediated by the aryl hydrocarbon receptor (AhR). Similarly, quercetin has also been reported to be a ligand for the AhR (Ciolino *et al.*, 1999).

Chapter 4

The effect of PEITC on hypoxia inducible factor

4.1   Introduction

In addition to identifying assays suitable for analysis of watercress extracts, it is also important to discover more about how watercress-derived phytochemicals interfere with the growth of cancer cells. In this chapter I investigated the effects of PEITC on hypoxia inducible factors (HIFs), oxygen sensitive transcription factors that activate genes involved in the regulation of angiogenesis and cell survival. Overexpression of HIF has been found in many human cancers including breast cancer (Zhong *et al.*, 1999) and is often associated with resistance to cancer therapies such as chemotherapy. This work focused on PEITC since several isothiocyanates, including PEITC, have already been shown to inhibit angiogenesis (Xiao and Singh, 2007) which is linked to inhibition of HIF1α expression (Yao *et al.*, 2008). Furthermore, work in this laboratory has demonstrated that PEITC can inhibit HIF transcriptional activity (Wang *et al.*, 2009).

4.2   Results

4.2.1 Effect of PEITC on the expression of HIF target genes

It has been previously found in this laboratory that PEITC inhibits the activity of HIF as shown by using a hypoxia-dependent construct in a luciferase reporter assay (Wang *et al.*, 2009). As HIFs are transcription factors it would be expected that a decrease in activity would result in decreased expression of HIF target genes. Therefore, the effect of PEITC on the expression of various target genes was examined. Four well known target genes were chosen that represent a range of processes effected by HIF; BNIP3 (Bcl2/adenovirus E1B 19kDa interacting protein 3) involved in cell survival (Sowter *et al.*, 2001), CAIX (carbonic anhydrase IX) involved in pH regulation (Wykoff *et al.*, 2000), GLUT1 (glucose transporter 1) involved in glucose metabolism (Airley *et al.*, 2001), and VEGF-A (vascular endothelial growth factor A) involved in angiogenesis (Forsythe *et al.*, 1996).

MCF7 cells were exposed to hypoxia for 16 h in the absence or presence of PEITC and the expression of the target genes analysed by Q-RT-PCR. All the target genes investigated were induced by hypoxia and demonstrated dose-dependent inhibition by PEITC that was statistically significant (Figure 4.1). CAIX was most strongly induced by hypoxia, showing a 50-fold increase in mRNA expression, and displayed statistically

significant inhibition by PEITC at all concentrations tested (Figure 4.1b). In comparison, mRNA expression of BNIP3, GLUT-1 and VEGFA was induced only 3-6 fold, and higher concentrations of PEITC were required to inhibit this expression. It has previously been reported that the promoter activity of CAIX is highly dependent on hypoxia and expression is very low under normoxic conditions giving a possible explanation for the increased sensitivity observed (Wykoff *et al.*, 2000). Therefore, in addition to effects in artificial reporter assays, PEITC also decreases the expression of endogenous HIF-regulated target genes.

4.2.2 Effect of PEITC on HIF1α accumulation

To explore the mechanism by which PEITC inhibits HIF-dependent transcription the effect of PEITC on HIF1α accumulation in cells cultured under hypoxic conditions was investigated. MCF7 cells were placed in a hypoxia chamber for 5 h in the presence or absence of PEITC before being harvested and analysed by western blot (Figure 4.2). As expected, increased HIF1α expression was observed in cells exposed to hypoxia. However, treatment with 10 μM PEITC greatly reduced the levels of HIF1α, and at 15 μM and 20 μM PEITC this reduction was statistically significant. Therefore, PEITC prevents the accumulation of HIF1α in hypoxia-treated cells.

4.2.3 Effect of PEITC on mTOR

Previous work in this laboratory has shown that inhibition of HIF activity by PEITC occurs independently of changes in the level of HIF1α mRNA as well as the activity of prolyl hydroxylases, the pVHL and the proteasome, which are all required for the rapid degradation of HIF1α in normoxia (Wang *et al.*, 2009). It was therefore possible that PEITC inhibited the translation of HIF1α mRNA. Inhibition of HIF1α mRNA translation has previously been linked to impaired activity of the mammalian target of rapamycin complex 1 (mTORC1) (Bernardi *et al.*, 2006). This forms part of a negative feedback loop to decrease HIF1α accumulation during prolonged hypoxia, as mTORC1 activity is suppressed in response to hypoxia through both HIF dependent and independent mechanisms (Arsham *et al.*, 2003). Mouse embryonic fibroblasts (MEFs) lacking TSC2, which forms half of the heterodimeric negative regulator of mTORC1, TSC1/2, were unable to suppress mTORC1 activity in response to hypoxia and this was associated with increased HIF1α accumulation (Brugarolas *et al.*, 2004). Furthermore, it has already been reported that HIF1α translation is reduced following treatment with
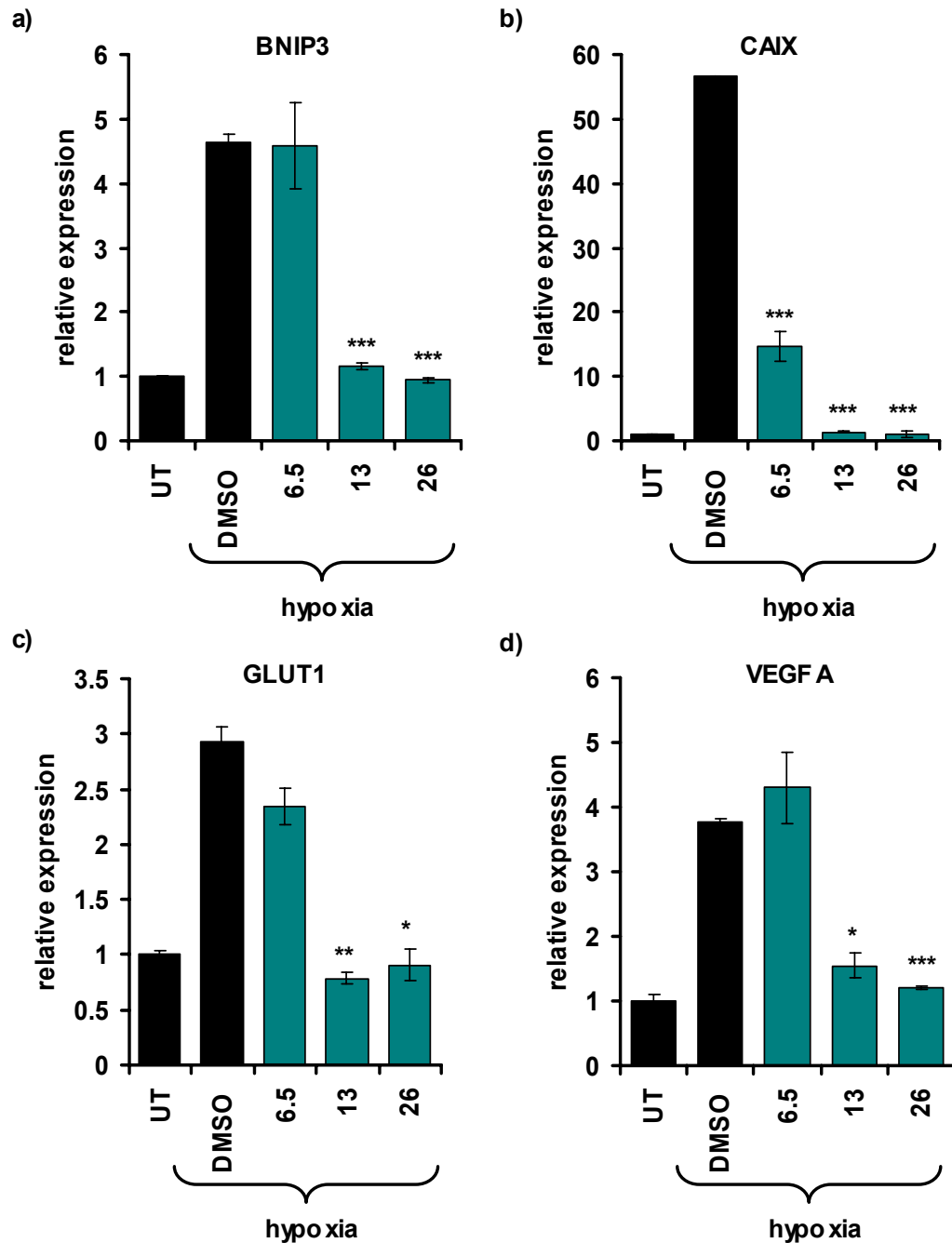
Figure 4.1: Effect of PEITC on expression of endogenous HIF target genes.
MCF7 cells were left untreated as a control or incubated in hypoxic conditions for 16 h in the presence or absence of the indicated concentrations of PEITC (μM), or DMSO (equivalent volume to 26 μM PEITC). Expression of (a) BNIP3, (b) CAIX, (c) GLUT1 and (d) VEGFA were analysed by Q-RT-PCR. Data are mean of duplicate determinations, normalised to expression of β-actin. Relative expression in untreated cells was set to 1.0. Statistically significant differences between DMSO and PEITC treated cells are indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$). All other comparisons were not statistically significant different.
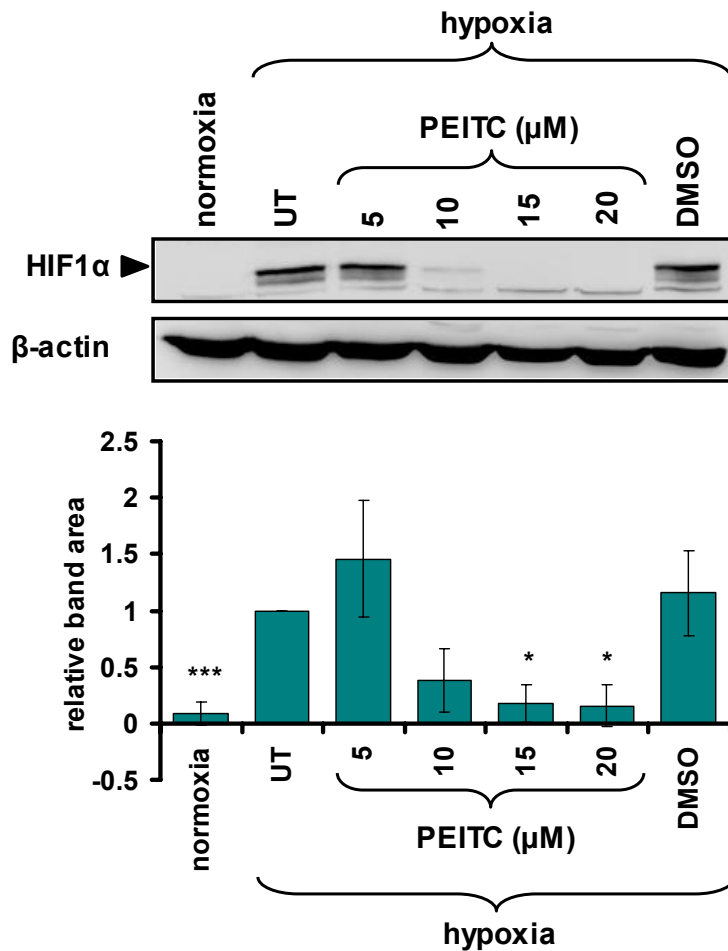
Figure 4.2: Effect of PEITC on HIF1α accumulation.
MCF7 cells were incubated in hypoxic conditions for 5 h in the absence (UT) or
presence of the indicated concentrations of PEITC (µM), or DMSO (equivalent to 20
µM PEITC). 20 µg of whole cell lysate was subjected to SDS-page and analysed by
western blot for HIF1α (120 kDa). Equal loading was determined by probing for β-actin
(42 kDa). Western blot is representative of three independent experiments and all
results are combined in the densitometry graph (± SD). Statistically significant
differences between UT and the treatments are indicated (* $p<0.05$; *** $p<0.005$). All
other comparisons were not statistically significant.

the mTORC1 antagonist, rapamycin (Thomas *et al.*, 2006).

I analysed the phosphorylation status of downstream substrates to investigate the effects of PEITC on mTORC1 kinase activity. The best known substrates of mTORC1 are the eIF4E binding proteins (4E-BPs) and 70 kDa protein kinases (p70 S6K), both of which are involved in the control of mRNA translation. The 4E-BP family, of which 4E-BP1 is the most prominent, are key negative regulators of the eukaryotic initiation factor 4F (eIF4F) complex required for cap-dependent translation. Hypophosphorylated 4E-BP1 binds strongly to eIF4E, the cap-binding protein of eIF4F, thus preventing the assembly of this complex (Cormier *et al.*, 2003). It was discovered that mRNAs with complex secondary structures within their 5'-untranslated region (5'UTR) were preferentially upregulated following overexpression of eIF4E (Koromilas *et al.*, 1992). This subset of mRNAs may be more sensitive to changes in eIF4E, the limiting eIF4F factor, as they are particularly dependent on mRNA unwinding by the eIF4A component. Interestingly, HIF1α possess a highly structured 5'UTR and therefore could be particularly sensitive to 4E-BP1 sequestering eIF4E (Iyer *et al.*, 1998).

Phosphorylation of the other main mTORC1 substrate, p70 S6K, at Thr389 provides an excellent indicator of mTORC1 activity and was therefore looked at alongside 4E-BP1. Targets of p70 S6K, include the S6 ribosomal protein, which forms part of the 40S ribosomal subunit, eukaryotic initiation factor 4B (eIF4B) (Raught *et al.*, 2004) and programmed cell death protein 4 (Pdcd4) (Dorrello *et al.*, 2006), which respectively enhance and impair mRNA helicase eIF4A, and the eukaryotic elongation factor 2 kinase (eEF2K), which inhibits eEF2 (Wang *et al.*, 2001).

MCF7 cells were treated with PEITC for 3 h and analysed by western blot (Figure 4.3). Consistent with the idea that PEITC inhibits mTORC1 activity, treatment of cells with PEITC resulted in reduced phosphorylation of p70 S6K. Phosphorylation of 4E-BP1 is a complex process involving multiple sites of modification (Thr37, Thr46, Thr70, and Ser65). This is consistent with the multiple slower-migrating isoforms of 4E-BP1 that were detected on a total 4E-BP1 blot. Similar to p70 S6K, treatment with PEITC reduced the levels of 4E-BP1 phosphorylation, as demonstrated by greater electroporetic mobility. The reduction in p70 S6K and 4E-BP1 phosphorylation was statistically significant in cells treated with 15 or 20 µM PEITC. Therefore, PEITC inhibits mTORC1 activity.
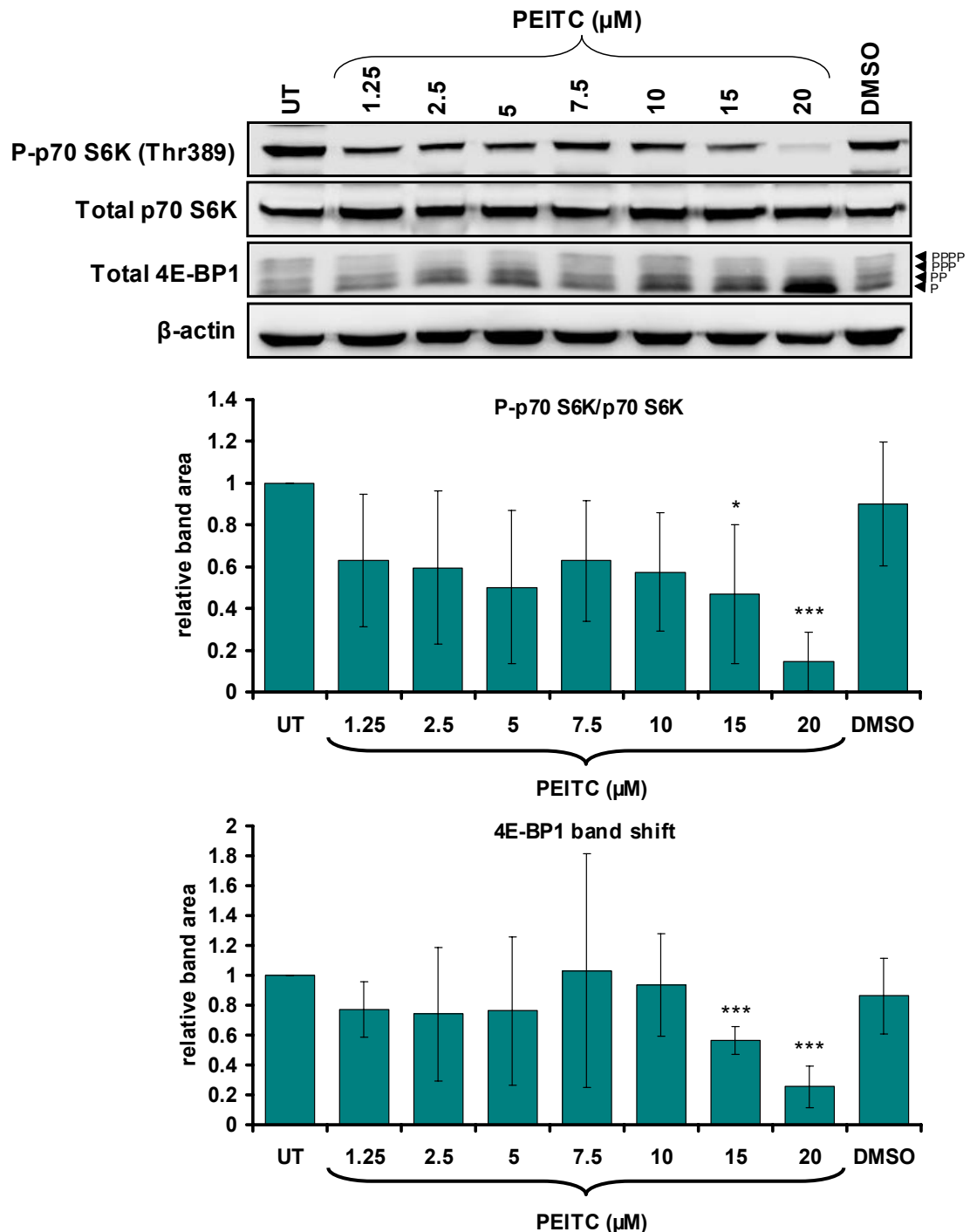
Figure 4.3: Effect of PEITC on mTORC1 signalling in MCF7 cells.

MCF7 cells were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 µM PEITC) for 3 h. 25 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-p70 S6K (Thr389; 70 kDa) and total 4E-BP1 (15-20 kDa; as indicated, multiple bands represent different phospho-forms) then stripped and re-probed for total p70 S6K. Equal loading was determined by probing for β-actin (42 kDa). The blot is representative of three independent experiments and the results from all three are combined in the densitometry graphs (± SD). Statistically significant differences between UT and the treatments are indicated (* p<0.05; *** p<0.005). All other comparisons were not statistically significant.

To further investigate the effect of PEITC on mTORC1 activity a time course was carried out (Figure 4.4). mTORC1 activity was assessed by loss of p70 S6K phosphorylation as this seemed to be a clearer readout than 4E-BP1 phosphorylation. A noticeable reduction of p70 S6K phosphorylation was observed after only 30 min PEITC treatment, with almost total dephosphorylation seen at 1 h. This decrease in phospho-p70 S6K is maintained for at least 8 h indicating prolonged inhibition of mTORC1 by PEITC.

4.2.4 Where in the mTORC1 pathway does PEITC act?

It is clear PEITC inhibits the activity of mTORC1 as demonstrated by the reduced phosphorylation of p70 S6K and 4E-BP1, but how is this achieved? mTORC1 is regulated by multiple pathways that sense whether or not conditions are favorable for cell growth and proliferation, such as nutrient availability, cellular energy levels, oxygen levels and the presence growth factors, which then activate or inhibit mTORC1 accordingly. In this section I investigated the effects of PEITC on various modulators of mTORC1 activity, including the PI3K/Akt pathway, ERK pathway, and 5' adenosine monophosphate-activated kinase (AMPK).

4.2.4.1 Effect of PEITC on PI3K/Akt pathway

One of the ways growth factors can stimulate mTORC1 is through activation of the PI3K/Akt pathway. Akt has been shown to phosphorylate TSC2 at multiple sites although two in particular, Ser939 and Ser981, seem to be important in the binding of 14-3-3 proteins which are proposed to sequester TSC2 to the cytosol and away from membrane associated TSC1 (Cai *et al.*, 2006). In addition, Akt can phosphorylate PRAS40, which is known to associate with the mTORC1 complex (Haar *et al.*, 2007). Although, as discussed in the introduction, the functional role of this is not fully understood (Rapley *et al.*, 2011).

PTEN reverses the action of PI3K by dephosphorylating PIP_3, negatively regulating the PI3K/Akt pathway. As previously discussed one potential mechanism of action by PEITC is through protein thiocarbamoylation. The active site of PTEN contains several key cysteine residues that when oxidised form a disulphide bond, resulting in the inactivation of PTEN (Lee *et al.*, 2002). Consequently, PTEN is a potential candidate for direct covalent modification by PEITC. Heterozygous and homozygous null PTEN
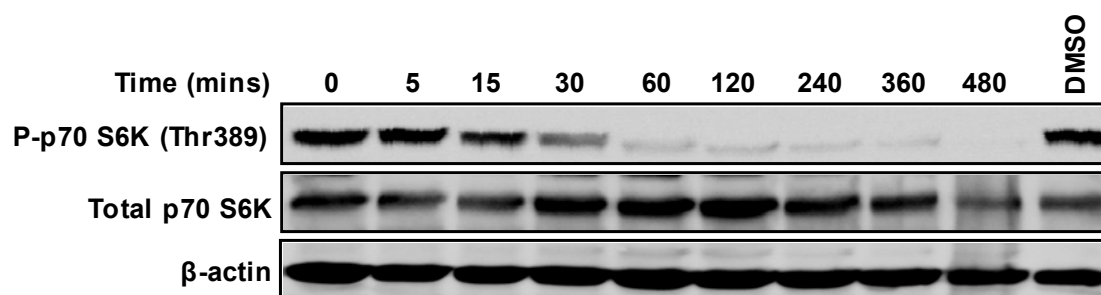
Figure 4.4: Time course of PEITC-induced inhibition of p70 S6K phosphorylation.
MCF7 cells were treated with 20 µM of PEITC or DMSO (equivalent to 20 µM PEITC)
for the indicated time points. 25 µg of whole cell lysate was subjected to SDS-page
and analysed by western blot for phospho-p70 S6K (Thr389; 70 kDa) then stripped and
re-probed for total p70 S6K. Equal loading was determined by probing for β-actin (42
kDa).

MEFs along with wild type control were used to determine whether the effects of PEITC were dependent on PTEN. After PTEN status in each of the MEFs was confirmed (Figure 4.5), the homozygous null PTEN and wild type MEFs were treated with PEITC for 3 h and analysed by western blot to determine whether inhibition of mTORC1 activity by PEITC was reversed in the PTEN null cells (Figure 4.6). Treatment of PEITC caused a dose-dependent decrease of phospho-p70 S6K and band shift of 4E-BP1 to less phosphorylated forms in the wild type cells, confirming what was seen in the MCF7 cell line. However, PEITC also inhibited phosphorylation of these mTORC1 substrates in the PTEN null MEFs. Thus, PTEN status does not appear to influence effects of PEITC on mTORC1 activity.

Furthermore, I have previously shown in Chapter 3 that PEITC treatment (2 h) did not inhibit Akt phosphorylation in MCF7 and SK-BR-3 cells (Figure 3.5 and 3.7), both of which have wild type PTEN (Li *et al.*, 1998). In fact Akt phosphorylation appears to be enhanced by PEITC at both of its phosphorylation sites, suggesting that PEITC does not act through the PI3K/Akt pathway to inhibit mTORC1 activity.

4.2.4.2 Effect of PEITC on ERK1/2

In addition to the PI3K/Akt pathway, growth factors relay signals to TSC1/2 to positively regulate mTORC1 via activation of the classical MAPK pathway. Both ERK1/2 and downstream target p90RSK have been shown to phosphorylate TSC2 (Ma *et al.*, 2005; Roux *et al.*, 2004). These ERK1/2 and p90RSK directed phospho-sites are distinct from each other, but those linked to p90RSK overlap with some of the proposed Akt phosphorylation sites (Huang and Manning, 2008). Furthermore, it has been demonstrated that p90RSK may regulate mTORC1 more directly through phosphorylation of Raptor (Carrière *et al.*, 2008).

In order to determine whether PEITC acts via the classical MAPK cascade to inhibit mTORC1, MCF7 cells were treated with PEITC for 3 h before being analysed by western blot for phospho-ERK1/2 (Figure 4.7). Although decreased ERK1/2 phosphorylation has been associated with reduced mTORC1 activity, ERK1/2 phosphorylation actually increased in PEITC treated cells. This increase was reproducible but did not reach statistical significance due to variation between individual experiments. Thus, it is unlikely that PEITC acts on mTORC1 via ERK1/2.
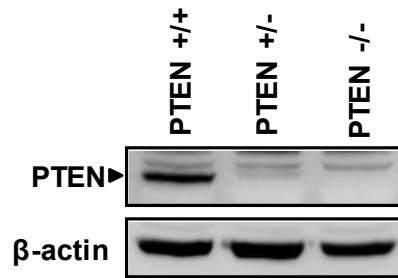
Figure 4.5: Confirmation of homozygous and heterozygous PTEN deficient MEFs.
30 μg of whole cell lysate from each of the MEF genotypes was subjected to SDS-page and analysed by western blot for PTEN (55 kDa). Equal loading was determined by probing for β-actin (42 kDa).
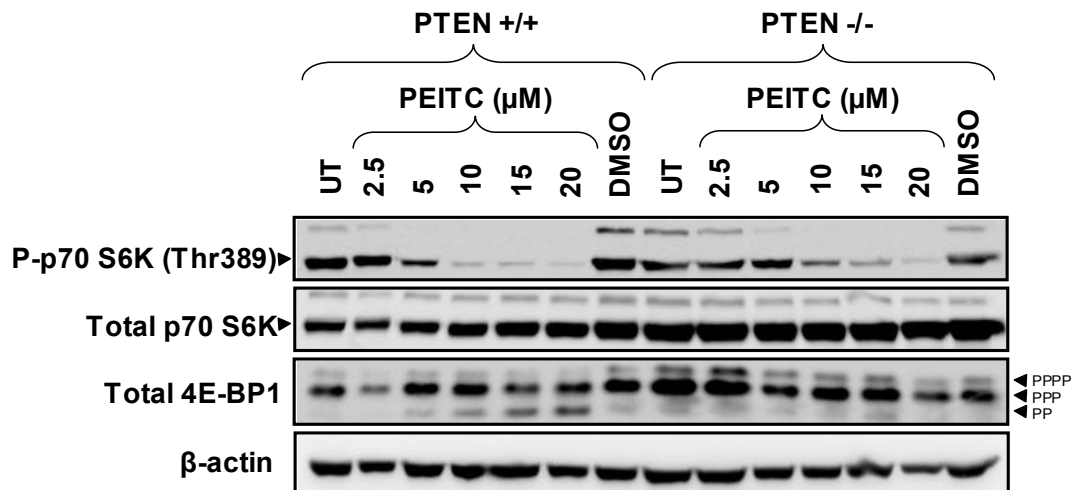
Figure 4.6: Effect of PEITC on mTORC1 signalling in PTEN null and wild type MEFs.
PTEN +/+ and -/- MEFs were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 μM PEITC) for 3 h. 25 μg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-p70 S6K (Thr389; 70 kDa) and total 4E-BP1 (15-20 kDa; as indicated, multiple bands represent different phospho-forms) then stripped and re-probed for total p70 S6K. Equal loading was determined by probing for β-actin (42 kDa).
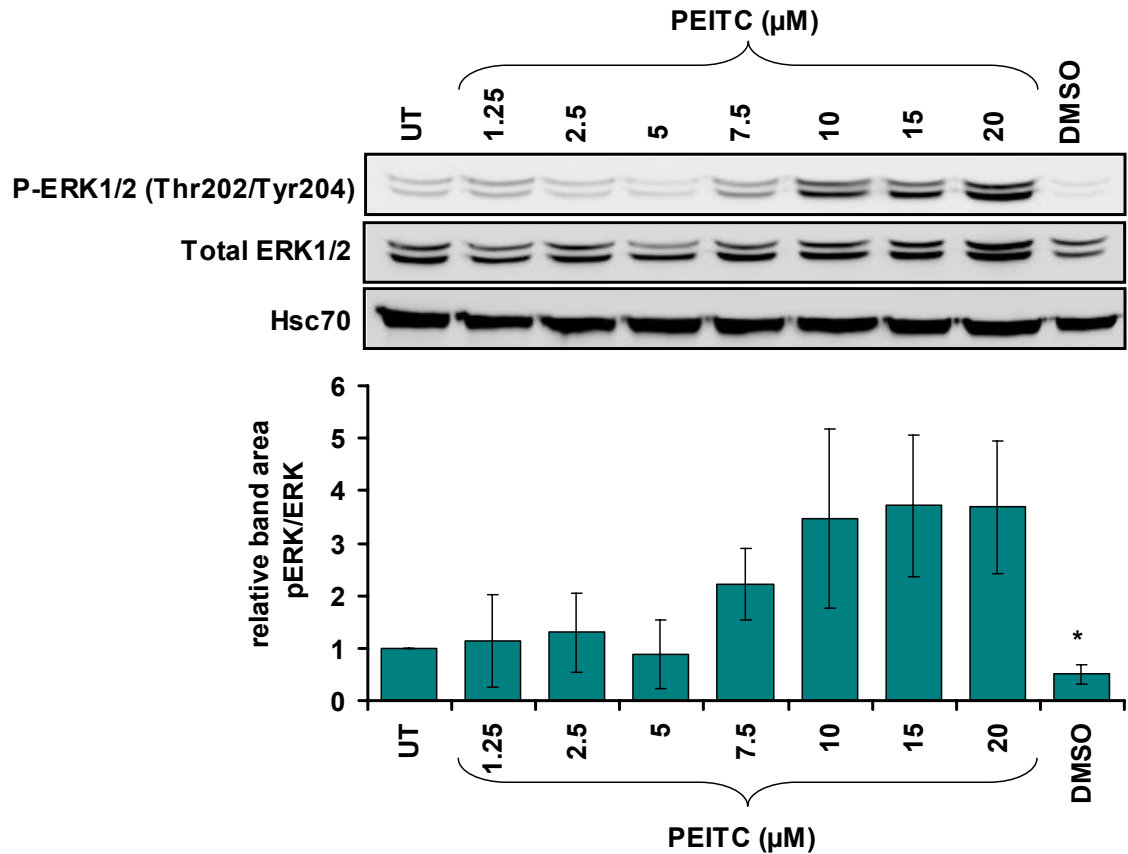
Figure 4.7: Effect of PEITC on phosphorylation of ERK1/2.
MCF7 cells were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 µM PEITC) for 3 h. 25 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-ERK1/2 (Thr202/Tyr204; 42/44 kDa) and then stripped and re-probed for total ERK1/2. Equal loading was determined by probing for Hsc70 (70 kDa). The blot is representative of three independent experiments and the results from all three are combined in the densitometry graphs (± SD). Statistically significant differences between UT and the treatments are indicated (* $p<0.05$). All other comparisons were not statistically significant.

4.2.4.3 Effect of PEITC on AMPK

5' adenosine monophosphate-activated kinase (AMPK) acts as an energy sensor by responding to changes in 5' adenosine monophosphate (AMP)/ 5' adenosine triphosphate (ATP) ratio. Phosphorylation of Thr172 by serine/threonine kinase 11 (STK11, also known as LKB1) is required for AMPK activation. It is thought that when ATP levels decline and AMP levels rise, the binding of AMP to AMPK facilitates this phosphorylation by LKB1 (Hardie *et al.*, 2006). Activated AMPK has been shown to phosphorylate residues Thr1227 and Ser1345 on TSC2, however unlike phosphorylation by Akt, ERK1/2 and p90RSK, this is associated with enhanced TSC1/2 activity and subsequent inhibition of mTORC1 (Inoki *et al.*, 2003b). Phosphorylation of Ser1345 by AMPK has been proposed to prime TSC2 for further phosphorylation and activation by glycogen synthase kinase 3 (GSK3), which is negatively regulated by the Wnt pathway (Inoki *et al.*, 2006). Furthermore, AMPK has also been shown to phosphorylate Raptor, which is believed to inactivate mTORC1 by stimulating the binding of 14-3-3 proteins (Gwinn *et al.*, 2008).

To establish if PEITC impairs mTORC1 activity by activating AMPK, MCF7 cells were treated with PEITC for 3 h before being analysed by western blot for phospho-AMPK (Figure 4.8). There was no effect of PEITC treatment on Thr172 phosphorylation of AMPK. Therefore, it does not seem PEITC acts through AMPK to inhibit mTORC1.

4.2.4.4 Does mTORC1 inactivation by PEITC require TSC1/2?

TSC2 null and wild type MEFs (also deficient for p53) were used to determine whether PEITC-induced inhibition of mTORC1 activity was dependent on TSC1/2 (Zhang *et al.*, 2003a). Following verification of TSC2 status in each of the MEFs (Figure 4.9), the response of the two cell lines to serum starvation was also investigated. Serum contains growth factors that stimulate mTORC1, and serum withdrawal has been shown to impair mTORC1 activity in a TSC1/2-dependent manner (Zhang *et al.*, 2003a). TSC2 null and wild type MEFs were cultured in the absence of serum for 24 h before serum was reintroduced to control cells for 2 h. Phosphorylation of mTORC1 substrates were analysed by western blot (Figure 4.10). As discussed, growth factors in the serum are believed to activate mTORC1 activity via phosphorylation and inhibition of TSC1/2, resulting in loss of its inhibitory influence on mTORC1. Serum
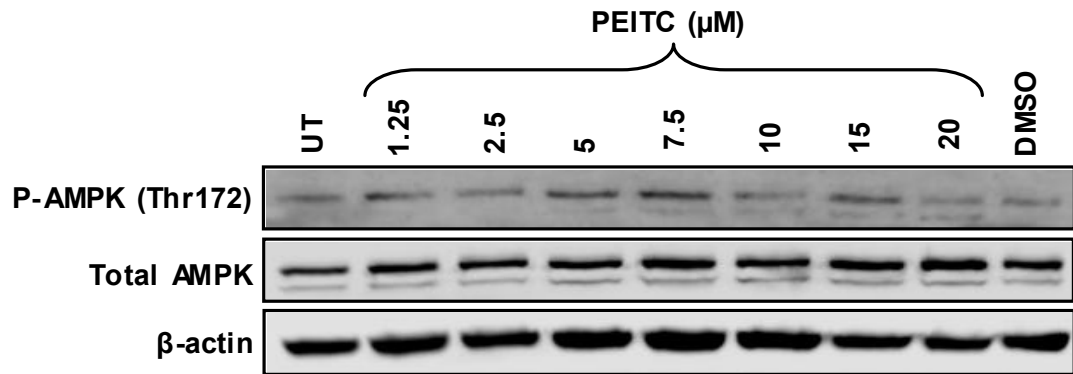
Figure 4.8: The effect of PEITC on phosphorylation of AMPK.
MCF7 cells were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 μM PEITC) for 3 h. 25 μg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-AMPK (Thr172; 62 kDa) and then stripped and re-probed for total AMPK. Equal loading was determined by probing for β-actin (42 kDa). Lack of positive data meant the experiment was only performed once.
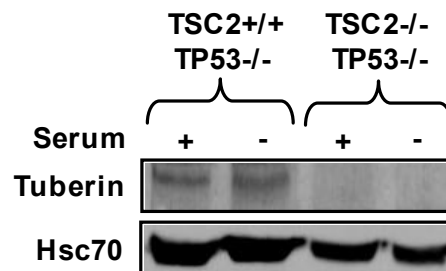

Figure 4.9: Confirmation of TSC2+/+TP53-/- and TSC2-/-TP53-/- MEFs.
TSC2+/+TP53-/- and TSC2-/-TP53-/- MEFs were serum starved for 24 h before media with the absence or presence of serum was added for 2 h. 40 μg of whole cell lysate was subjected to SDS-page and analysed by western blot for tuberin (TSC2; 200 kDa). Equal loading was determined by probing for Hsc70 (70 kDa).
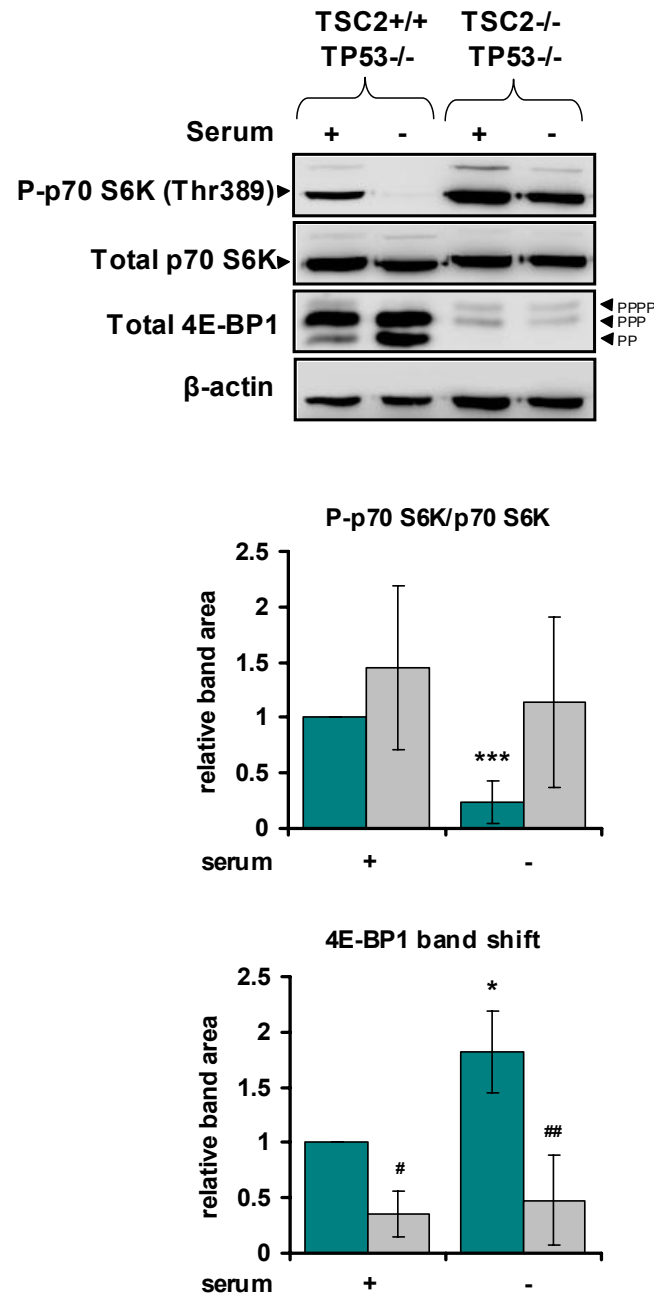
Figure 4.10: Effect of serum on mTORC1 signalling in TSC2 null and wild type MEFs.

TSC2+/+TP53-/- (▪) and TSC2-/-TP53-/- (▫) MEFs were serum starved for 24 h before media with the absence or presence of serum was added for 2 h. 25 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-p70 S6K (Thr389; 70 kDa) and total 4E-BP1 (15-20 kDa; as indicated, multiple bands represent different phospho-forms) then stripped and re-probed for total p70 S6K. Equal loading was determined by probing for β-actin (42 kDa). The blot is representative of three independent experiments and the results from all three are combined in the densitometry graphs (± SD). Statistically significant differences between UT and the treatments are indicated (* p<0.05; ** p<0.05; *** p<0.005), and between the same treatment in the different cell lines (# p<0.05). All other comparisons were not statistically significant.

starvation should therefore restore TSC1/2 activity which in turn will inhibit mTORC1. As expected, wild type MEFs were sensitive to serum starvation with a decrease in phospho-p70 S6K and increase in 4E-BP1 electroporetic mobility observed. In MEFs deficient for TSC2, mTORC1 activity should be unrestrained and therefore resistant to inhibition by serum starvation. Indeed, in the TSC2 null MEFs there was no change in p70 S6K or 4E-BP1 phosphorylation following serum starvation. The TSC2 null MEFs also had higher basal phosphorylation of p70 S6K and 4EBP1, which would be expected in cells were mTORC1 is uninhibited by TSC2. However, I also observed lower overall levels of 4E-BP1 protein in the TSC2 null MEFs compared to the wild type MEFs. It has previously been reported that both the cell lines have similar levels of total 4E-BP1, so the reason for this difference is unclear (Smith *et al.*, 2005).

Subsequent experiments with the TSC2 null and wild type MEFs were carried out in the presence of serum. MEFs of either genotype were treated with PEITC for 3 h and analysed by western blot to determine whether TSC1/2 is required for mTORC1 inhibition by PEITC (Figure 4.11). PEITC caused a statistically significantly dose-dependent decrease in the phosphorylation of p70 S6K and 4E-BP1 (as indicated by the band shift) in the wild type MEFs which was not seen in the TSC2 null cells. Therefore, PEITC-induced inhibition of mTORC1 activity is dependent on TSC1/2.

4.2.5 Effect of PEITC on mTORC2

mTORC1 can negatively regulate mTORC2 activity via phosphorylation of Rictor by p70 S6K (Julien *et al.*, 2010). Furthermore, mTORC1 inhibition can cause an increase in phosphorylation of Akt on Ser473, a target for mTORC2. Supposedly as a consequence of the lost inhibitory influence of p70 S6K (Breuleux *et al.*, 2009). Therefore, the effect of PEITC on mTORC2 was also investigated. One of the main functions of mTORC2 is to phosphorylate Akt on Ser473, which along with phosphorylation on Thr308 by PDK1 is required for full Akt activation (Sarbassov *et al.*, 2005). Another substrate for mTORC2 is the serum- and glucocorticoid-induced protein kinase 1 (SGK1). N-myc downstream regulated gene 1 (NDRG1) is a downstream target of SGK1 and while its function is not well understood it has been shown to be a good indicator of mTORC2 activity (García-martínez and Alessi, 2008).

The effect of PEITC on the phosphorylation of downstream targets of mTORC2, Ser473 on Akt, and SGK1 substrate, NDRG1 were analysed in the TSC2 null and
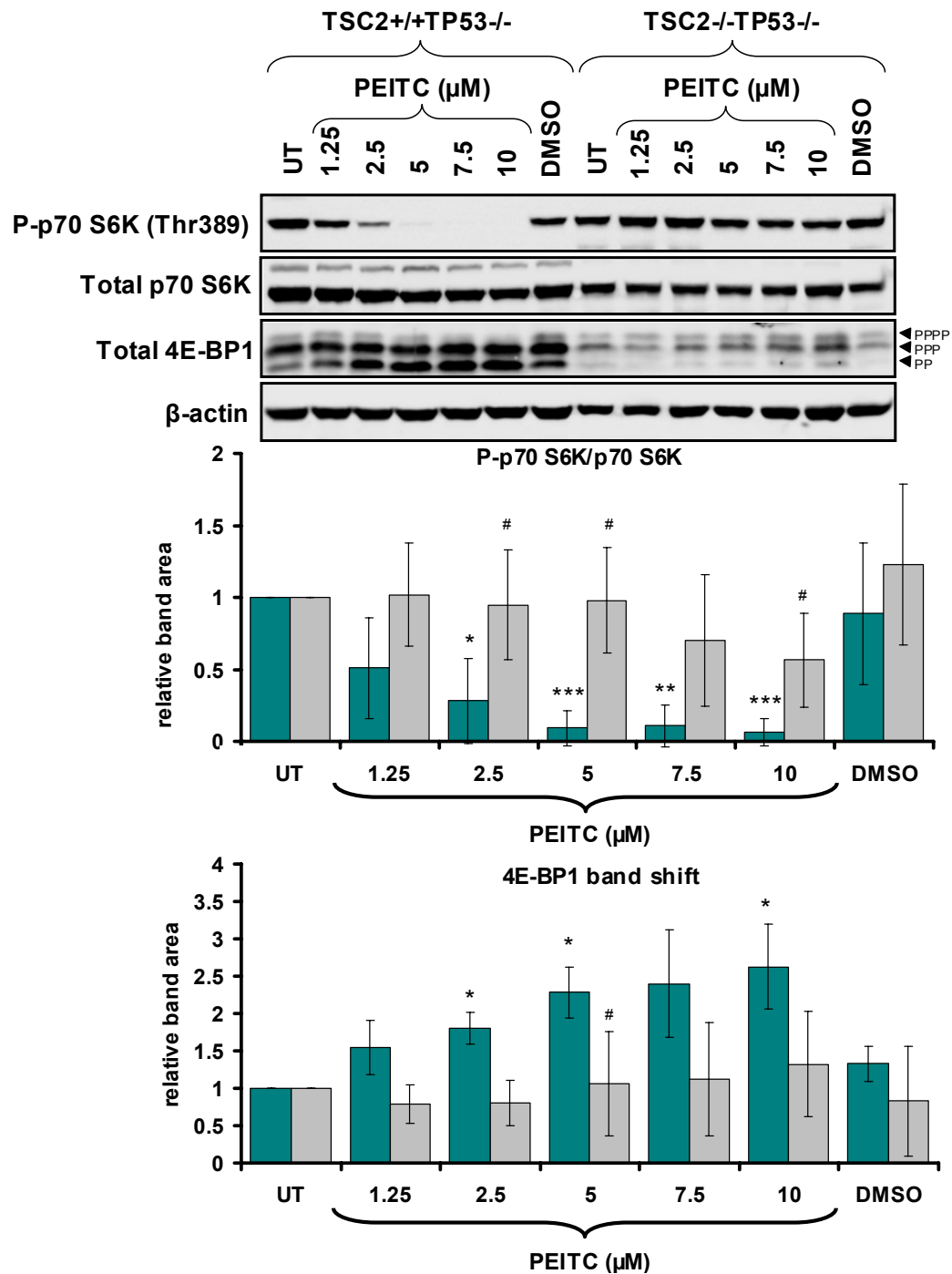
Figure 4.11: Effect of PEITC on mTORC1 signalling in TSC2 null and wild type MEFs.
TSC2+/+TP53-/- (■) and TSC2-/-TP53-/- (■) MEFs were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 µM PEITC) for 3 h. 25 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-p70 S6K (Thr389; 70 kDa) and total 4E-BP1 (15-20 kDa; as indicated, multiple bands represent different phospho-forms) then stripped and re-probed for total p70 S6K. Equal loading was determined by probing for β-actin (42 kDa). The blot is representative of three independent experiments and the results from all three are combined in the densitometry graphs (± SD). Statistically significant differences between UT and the treatments are indicated (* p<0.05; ** p<0.05; *** p<0.005), and between the same treatment in the different cell lines (# p<0.05). All other comparisons were not statistically significant.

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wild type MEFs (Figure 4.12). As previously reported by Huang *et al.* (2009), I found TSC2 null MEFs have low basal levels of phosphorylated Akt (Ser473) and NDRG1 in comparison to their wild type counterparts. In response to PEITC treatment a dose-dependent increase in Akt Ser473 and NDRG1 phosphorylation is observed in the wild type MEFs. This corresponds with the decrease in mTORC1 activity I have seen in previous experiments. However, PEITC also seems to induce mTORC2 activity in the TSC2 null MEFs (albeit from a lower basal level) even though mTORC1 activity is unaffected by PEITC in these cells.

4.2.6 Effect of PEITC on growth inhibition in the MEF cell lines

Since PEITC-dependent regulation of mTORC1 was dependent on the presence of TSC2, I investigated whether TSC2 also influenced the growth inhibitory response to PEITC. To assess the role of TSC2 in PEITC-induced growth inhibition, TSC2 null and wild type MEFs were treated with PEITC for four days prior to cell number being determined by the MTS assay (Figure 4.13). TSC2 null cells were less sensitive to PEITC-mediated growth inhibition relative to control cells. Although the difference in sensitivity of the two lines was small (IC_{50} 4.7 µM versus 7.9 µM), this difference was statistically significant. Therefore, similar to mTORC1 regulation, TSC2 is required for optimal growth inhibition in response to PEITC.

Similar experiments were performed using PTEN-deficient cells (Figure 4.14). There is a trend for heterozygous and homozygous null PTEN MEFs to be more resistant to PEITC-induced growth inhibition then the wild type cells, and in all experiments IC_{50} values were higher in the former two cell lines compared to the latter. However, the difference between the IC_{50} values of the wild type and PTEN-deficient cell lines varied considerably from 0.5-fold higher to 3-fold higher. As a result of this high standard deviation differences in IC_{50} values, they were not statistically significant. There was no discernable trend between the heterozygous and homozygous null MEFs.

4.2.7 Effect of PEITC on translation of HIF1α mRNA

The observation that PEITC decreased mTORC1 activity is consistent with the idea that PEITC inhibits the translation of HIF1α mRNA. To directly test whether PEITC effects HIF1α mRNA translation, MCF7 cells were metabolically labelled with [^{35}S]Met/Cys and HIF1α immunoprecipitated (Figure 4.15). To enhance the sensitivity
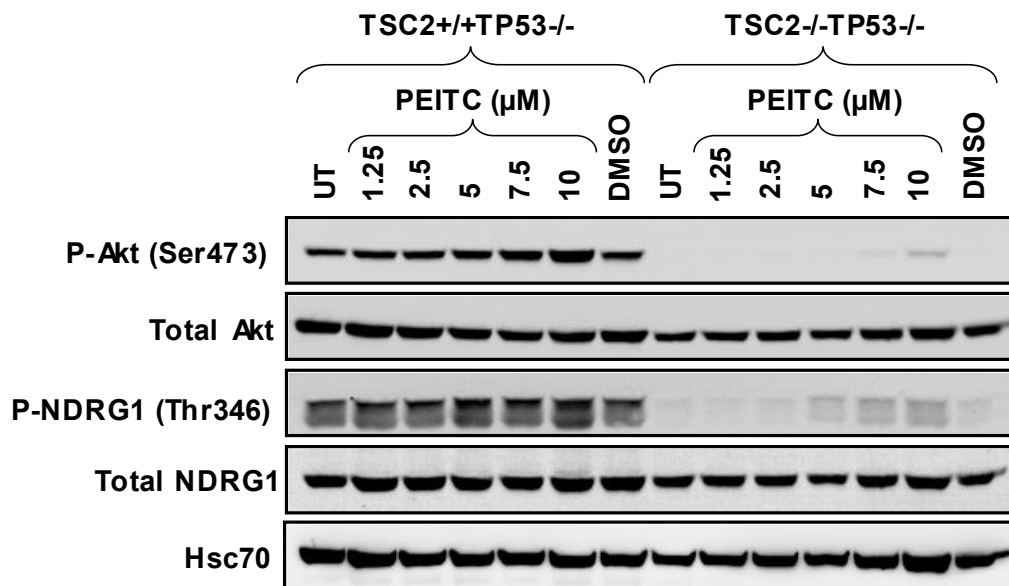
Figure 4.12: Effect of PEITC on mTORC2 signalling in TSC2 null and wild type MEFs.

TSC2+/+TP53-/-and TSC2-/-TP53-/- MEFs were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 µM PEITC) for 3 h. 25 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-Akt (Ser473; 60 kDa) as well as phospho-NDRG1 (Thr346; 46, 48 kDa) then stripped and re-probed for total equivalents. Equal loading was determined by probing for Hsc70 (70 kDa).
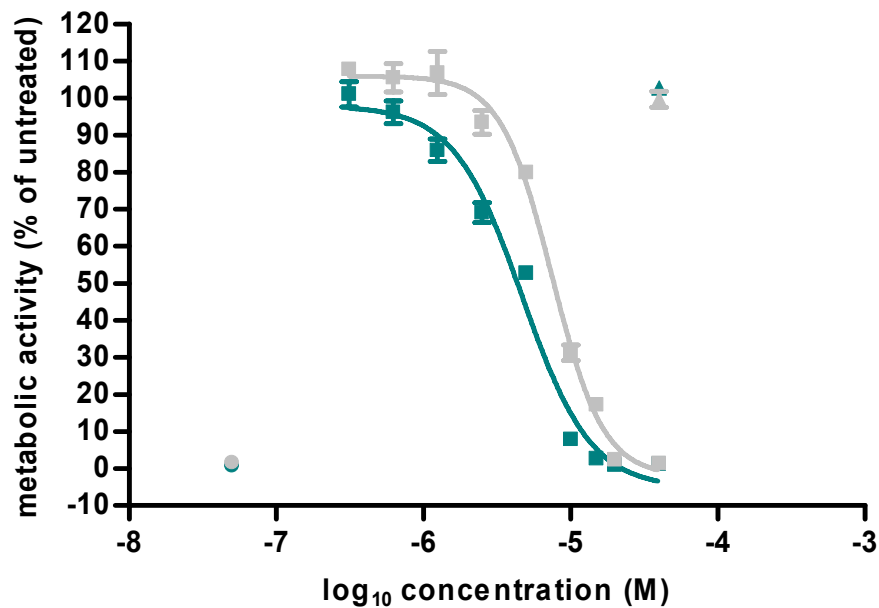
	IC$_{50}$/µM Value (mean ± SD)	
	TSC2+/+TP53-/-	**TSC2-/-TP53-/-**
PEITC	4.7 ± 1.3	7.9 ± 0.8*

Figure 4.13: Effect of PEITC on growth inhibition of TSC2 null and wild type MEFs.

Representative growth inhibition experiments. TSC2+/+TP53-/- (■) or TSC2-/-TP53-/- (■) MEFs were treated with various concentrations of PEITC, DMSO (▲) equivalent to the highest dose of PEITC and 0.5 µM STS as a positive control (●). After four days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of triplicate wells (± SD). Table showing IC$_{50}$ values represent mean values ± SD from three experiments performed in triplicate. A statistically significant difference between IC$_{50}$ values for TSC2 null and wild type MEFs is indicated (* p<0.05).
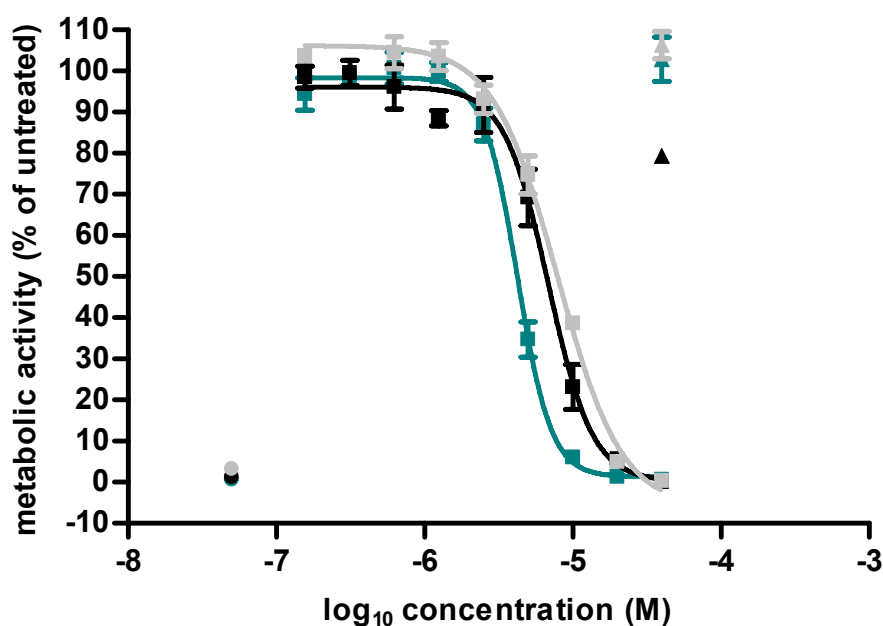
	IC$_{50}$/µM Value (mean ± SD)		
	PTEN +/+	**PTEN +/-**	**PTEN -/-**
PEITC	5.4 ± 1.3	13.0 ± 4.9	10.9 ± 5.4

Figure 4.14: Effect of PEITC on growth inhibition of PTEN knockout and wild type MEFs.

Representative growth inhibition experiments. PTEN +/+ (■), PTEN +/- (■) and PTEN -/- (■) MEFs were treated with various concentrations of PEITC, DMSO (▲) equivalent to the highest dose of PEITC and 0.5 µM STS as a positive control (●). After four days metabolic activity was determined by MTS assay using the CellTiter 96$^{®}$ AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of triplicate wells (± SD). Table showing IC$_{50}$ values represent mean values ± SD from five experiments performed in triplicate.
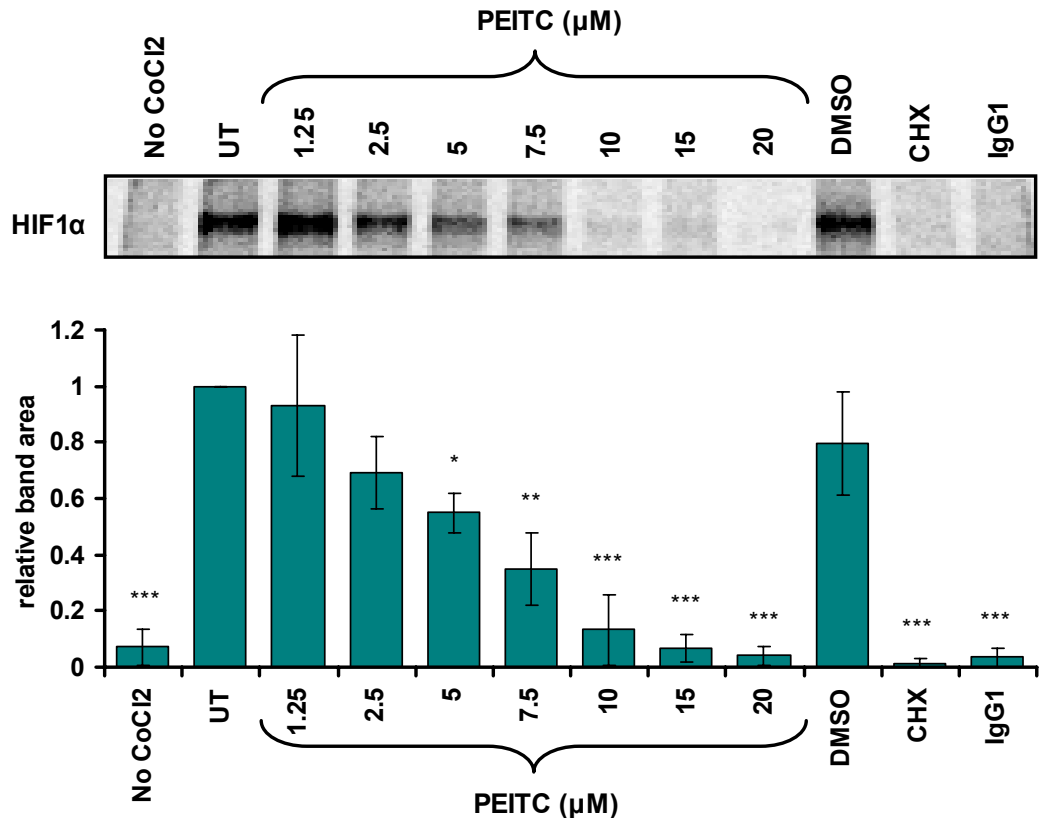
Figure 4.15: Effect of PEITC on the translation of HIF1α mRNA.
MCF7 cells were pretreated with 100 μM cobalt chloride (CoCl$_2$), a hypoxia mimetic, and then incubated in the absence (UT) or presence of indicated concentrations of PEITC, DMSO (equivalent to 20 μM PEITC), or 10 μg/ml cycloheximide (CHX) as a positive control for 1 h. 100 μCi/ml of [^{35}S]Met/Cys protein labelling mix was added for 2 h. Cells were lysed and immunoprecipitated for HIF1α protein (120 kDa). Immunoprecipitated proteins were separated on SDS-PAGE, gel dried and phosphor imaging used to detect radioactive bands. Gel image is representative of three independent experiments with all results combined in the densitometry graph (± SD). Statistically significant differences between UT and the treatments are indicated (* p<0.05; ** p<0.01; *** p<0.005). All other comparisons were not statistically significant.

of the assay, cells were pretreated with hypoxia mimetic cobalt chloride ($CoCl_2$), which acts to prevent binding of pVHL to HIF1α and its subsequent degradation. Consistent with this, addition of $CoCl_2$ increased the apparent metabolic labelling of HIF1α. Translation of HIF1α was inhibited by PEITC in a dose-dependent manner and was statistically significant at concentrations of 5 µM and above. Concentrations of 10 µM PEITC and above resulted in almost total loss of radioactive HIF1α, similar to the control translation inhibitor cycloheximide.

4.3   Discussion

It has been previously found in this laboratory that PEITC inhibited the activity of HIF as shown by using a hypoxia-dependent construct in a luciferase reporter assay (Wang *et al.*, 2009). I have further demonstrated that PEITC is able to inhibit the expression of a variety of endogenous HIF target genes, as well as inhibit HIF1α accumulation in hypoxia treated cells. Therefore, I conclude that PEITC is a potent inhibitor of HIF expression.

Work in this laboratory has also shown PEITC can inhibit HIF1α accumulation in the pVHL-deficient RCC4 renal carcinoma cell line, which is unable to degrade HIFα proteins via the canonical pVHL pathway and as a result constituently expresses HIF1α in normoxia. Furthermore, it was demonstrated that inhibition of HIF accumulation by PEITC occurred independently of the proteasome, and treatment with PEITC had no effect on the expression of HIF1α mRNA (Wang *et al.*, 2009). This suggests that PEITC-induced inhibition of HIF expression does not occur via suppression of HIF transcription or by interfering with the degradation pathway. It is therefore proposed that PEITC acts at the level of mRNA translation. Inhibition of HIF1α mRNA translation has previously been linked to impaired activity of mTORC1 (Bernardi *et al.*, 2006). Phosphorylation of 4E-BP1 by mTORC1 decreases its affinity to eIF4E, the rate limiting component of the eIF4F complex, enabling cap-dependent translation. Possibly due to a greater need for mRNA unwinding by the eIF4A component, mRNAs with complex secondary structures within their 5'UTR are more sensitive to changes in eIF4E (Koromilas *et al.*, 1992). HIF1α possess a highly structured 5'UTR (Iyer *et al.*, 1998) and therefore the effect of PEITC on mTORC1 activity was investigated. It was discovered that PEITC did inhibit mTORC1 activity as assessed by loss of substrate phosphorylation, p70 S6K and 4E-BP1. Consistent with my findings, PEITC has also been shown to inhibit 4E-BP1 phosphorylation at both

Thr70 and Ser65 sites in colorectal cancer HCT-116 cells and prostate cancer PC3 cells (Hu *et al.*, 2007). Hu *et al.* (2007) also demonstrated that PEITC preferentially inhibited cap-dependent translation as assessed by luciferase reporter constructs with and without a stem-loop secondary structure introduced into the 5' UTR.

In trying to unravel where in the mTORC1 pathway PEITC acts, it was found PEITC was unable to impair mTORC1 activity in TSC2 null MEFs, indicating that PEITC-induced inhibition of mTORC1 is dependent on TSC1/2 activity. TSC1/2 integrates several regulatory pathways to control mTOR in response to various cellular stresses and growth factors, and these were also investigated. Akt phosphorylates and inhibits TSC2 in response to growth factors via PI3K pathway leading to enhanced mTORC1 activity. This pathway is negatively regulated by PTEN. PTEN is subject to redox regulation and therefore presents an attractive target for direct protein modification by PEITC. However, using PTEN null MEFs it was found PEITC was able to suppress mTORC1 activity regardless of PTEN status. Furthermore, PEITC treatment did not inhibit Akt phosphorylation at either Thr308 or Ser473 sites, and was in fact found to be increased in both MCF7 and SK-BR-3 breast cancer cell lines. Activation of mTORC1 via phosphorylation and inhibition of TSC2 in response to growth factors also occurs via the classical MAPK pathway involving ERK and p90RSK. Phosphorylation of ERK at Tyr202/Thr204 that corresponds to its activity is not inhibited by PEITC, and as with Akt, actually results in an increase in ERK phosphorylation at these sites. Phosphorylated AMPK, which activates TSC1/2 and consequently inhibits mTORC1 in response to ATP depletion, was also unaffected by PEITC treatment.

mTORC1 activity can also be inhibited by regulated in development and DNA damage response 1 (REDD1; also known as RTP801), which is transcriptionally upregulated in response to a variety of cellular stresses including hypoxia (Shoshani *et al.*, 2002) and ER stress (Jin *et al.*, 2009; Whitney *et al.*, 2009). How exactly REDD1 suppresses mTORC1 has not been fully elucidated but studies carried out with TSC2 null MEFs suggest it is upstream of TSC1/2 (Brugarolas *et al.*, 2004). One theory is that REDD1 functions to bind to and sequester inhibitory 14-3-3 proteins that interact with TSC2 following its phosphorylation by Akt. When bound, the 14-3-3 proteins are thought to destabilise the interaction between TSC1 and 2 removing its inhibition on mTORC1 (DeYoung *et al.*, 2008). However, the rapid inhibition of p70 S6K phosphorylation by PEITC (30 min) likely discounts REDD1 as the target for PEITC-induced inhibition of

mTORC1, as per Balgi *et al.* (2011), considering *de novo* transcription of REDD1 takes considerably longer.

These findings suggest that PEITC either acts on TSC1/2 itself or upstream of TSC1/2 on an as of yet unknown regulator of mTORC1. A recent study has demonstrated that mTORC1 activity can also be impaired in response to acidic pH and that this is mediated via TSC1/2. Inhibition of mTORC1 activity was associated with a decrease in MEK and downstream ERK activity however, this had if anything only a modest effect on mTORC1 inhibition by acidification (Balgi *et al.*, 2011). Similar to my study, Balgi *et al.* (2011) found no evidence that any of the other pathways known to regulate mTORC1 through TSC1/2 were involved. Therefore, it is likely that there is at least one unidentified regulator of mTORC1 that acts as a pH sensor.

The anticancer effects of PEITC and other isothiocyanates are thought to target multiple aspects of tumour progression, including chemoprevention, induction of apoptosis and suppression of angiogenesis. However, knowledge of the upstream triggering events is limited. It is likely that generation of ROS and direct covalent modification of target proteins, particularly those with thiol containing cysteines, play a role.

Sarbassov and Sabatini (2005) demonstrated that cysteine oxidants, diamide and phenylarsine oxide (PAO), stimulated mTORC1 activity even during nutrient withdrawal, and this was associated with an instability of the mTOR-Raptor interaction. Interestingly, the mTOR-Rictor interaction was unaffected by cysteine oxidants. Furthermore, the reducing agent BAL (British anti-Lewisite, or 2,3-dimercapto-1-propanol) was found to inhibit mTORC1 activity. As already mentioned mTOR has several conserved cysteine residues that make it a potential target for redox regulation (Dames *et al.*, 2005). However, as PEITC-induced inhibition of mTORC1 is dependent on TSC2 it does not seem that direct modification of mTOR is involved. Recently it was discovered that cysteine oxidants increased levels of Rheb-GTP and that redox regulation of mTORC1 by both cysteine oxidants and reducing agents was also dependent on TSC1/2. Similar to PEITC, mTORC1 activity, as assessed by phosphorylation of p70 S6K, was unaffected by treatment with either PAO or BAL in TSC2 null MEFs (Yoshida *et al.*, 2011).

There is clearly a role for redox in the regulation of mTORC1, therefore the ability of PEITC to conjugate with protein cysteinyl thiols may be important in its effect on mTORC1 activity. However, as ROS acts like PAO to oxidise cysteines it is unlikely that ROS generation contributes to the observed inhibition of mTORC1 activity following PEITC treatment. Although, it should be noted despite initially acting as a pro-oxidant, PEITC can also act indirectly as an anti-oxidant, by inducing Nrf2-mediated antioxidant gene expression.

Furthermore, while it is known Akt, ERK/p90RSK and AMPK phosphorylate TSC2 at diverse sites, it is unclear how this can result in either the inhibition or stimulation of TSC1/2. It has been suggested that 14-3-3 proteins recognise Akt-directed phosphorylation sites on TSC2, and cause translocation of TSC2 to the cytosol and away from TSC1, disrupting the complex (Cai *et al.*, 2006). Indeed, it is thought that REDD1 impairs mTORC1 by removing these 14-3-3 proteins and restoring the inhibitory influence of TSC1/2 (DeYoung *et al.*, 2008).

There are seven mammalian isoforms of 14-3-3 proteins that form hetero- or homodimers and bind to phospho-serine/phospho-threonine residues on a wide range of proteins. Binding can obstruct interaction of the target protein with essential partner proteins or substrates, or induce a conformational change to affect its stability or activity (Morrison, 2009). REDD1 does contain a serine residue (Ser137) within a consensus 14-3-3 binding motif, however this motif is not conserved and mutations to several key residues including the Ser137 did not affect REDD1-induced inhibition of mTORC1 (Vega-Rubin-de-Celis *et al.*, 2010). As it is unclear exactly if or how REDD1 binds 14-3-3 proteins, another protein may well exist that interacts with REDD1 and relays a signal to activate TSC1/2 that could be influenced by PEITC. It should be noted that 14-3-3 proteins can bind phospho-serine/phospho-threonine residues outside of recognised motifs and even in a phosphorylation-independent manner. However, another reservation on the proposed mechanism of 14-3-3 sequestration by REDD1 is that considering the abundance of 14-3-3 proteins and they're interaction with over 100 different proteins how is it possible REDD1 achieves specificity and sequesters enough to be effective (Vega-Rubin-de-Celis *et al.*, 2010; Wang and Proud, 2010). It has been found that binding of 14-3-3 proteins to one partner, CD81, is prevented by redox sensitive palmitoylation of several of its cysteine residues (Clark *et al.*, 2004). Perhaps binding of 14-3-3 proteins to TSC2 can also be redox regulated.

As recently reviewed by Wang and Proud (2010) there is much about mTORC1 signalling that is still unknown and it may be that PEITC could be used as a probe to explore this pathway further.

I have previously discussed the available literature on the effect of PEITC on Akt phosphorylation in Chapter 3. However, now in the context of mTOR, it is possible an increase in Akt phosphorylation is a consequence of PEITC-induced inhibition of mTORC1. In a negative feedback loop, mTORC1 and its substrate p70 S6K can phosphorylate insulin receptor substrate 1 (IRS1), reducing its stability (Shah and Hunter, 2006; Tzatsos and Kandror, 2006). As IRS1 is required for insulin and insulin-growth factor 1 (IGF1) mediated activation of PI3K, stimulation by these growth factors no longer leads to recruitment of Akt to the cell membrane and phosphorylation by PDK1 and mTORC2. mTORC1 has also been shown to impair Akt phosphorylation by decreasing transcription of platelet derived growth factor receptor (PDGFR), that activates PI3K in response to a number of different growth factors (Zhang et al., 2007). The recent discovery that growth factor receptor-bound protein 10 (Grb10) is a substrate for mTORC1 highlights that the mTORC1-mediated negative feedback mechanism on growth factor induced stimulation is multifaceted. Grb10 binds to and negatively regulates the insulin and IGF1 receptors, it is proposed phosphorylation of Grb10 by mTORC1 increases its activity by promoting protein stability (Hsu et al., 2011). Furthermore, p70 S6K has been found to directly phosphorylate mTORC2 specific component Rictor, an action that seemingly impairs mTORC2-mediated phosphorylation of Akt (Julien et al., 2010). Thus, suppression of mTORC1 and removal of these inhibitory influences may result in the increased Akt phosphorylation seen following PEITC treatment.

In addition, mTORC1 inhibition has been shown to activate ERK in a mechanism that is dependent on PI3K and involves activation of the classical MAPK cascade (Carracedo et al., 2008). Therefore, the increase in ERK phosphorylation I see in MCF7 cells is possibly also a consequence of PEITC-induced inhibition of mTORC1. An increase in ERK phosphorylation following PEITC treatment has been previously reported in human prostate and bladder carcinoma cell lines and is associated with PEITC-induced apoptosis and Nrf2 mediated ARE activity (Pullar et al., 2004; Xiao and Singh, 2002; Xu et al., 2006a; Xu et al., 2006b). However, the effect of PEITC on ERK phosphorylation seems to be cell type specific as PEITC has been shown to inhibit ERK phosphorylation in human cervical and ovarian cancer cell lines (Huong et al.,

2011; Satyan *et al.*, 2006). Specifically Huong *et al.* (2011) reported loss of ERK phosphorylation resulted in upregulation of death receptors 4 and 5 (DR4 and DR5) causing PEITC-induced apoptosis.

Consistent with the view mTORC1 inhibition enhances upstream signalling to mTORC2 due to the loss of p70 S6K-IRS1 negative feedback, I observed a dose-dependent increase in Akt Ser473 and NDRG1 phosphorylation in wild type MEFs. This corresponded with the decrease in p70 S6K phosphorylation I have seen in previous experiments. Furthermore, I found that basal phosphorylation of mTORC2 downstream effectors, Akt and NDRG1, was greatly impaired in TSC2 null MEFs where mTORC1 activity is unrestrained. Indeed, loss of mTORC2 activity in TSC2 null MEFs has been reported previously (Huang *et al.*, 2008; Huang *et al.*, 2009). Although, the authors attributed this observation to a direct effect of TSC2 on mTORC2, independent of its Rheb GAP activity. Despite this, I found PEITC treatment still caused a dose-dependent increase in mTORC2 activity in these cells. As I have previously shown, PEITC has a limited effect on mTORC1 activity in the TSC2 null MEFs, this increase in mTORC2 activity can not be attributed to a relief in p70 S6K-mediated inhibition. Little is known about the regulation of mTORC2. However, it seems that while PEITC inhibits mTORC1 activity in a TSC2-dependent manner, it can also activate mTORC2 in a way that is independent of its effect on mTORC1 and does not require TSC2. This also indicates that elevated Akt phosphorylation in response to PEITC can not simply be attributed to loss of mTORC1 negative feedback loops as discussed earlier.

The growth inhibitory action of PEITC has been well documented and I have demonstrated it myself in Chapter 3. mTORC1 inhibitor rapamycin has been shown to inhibit cell proliferation by arresting cell cycle progression at the G1/S boundary (Terada *et al.*, 1993). In particular, inhibition of cell proliferation due to loss of mTORC1 activity is dependent on 4E-BPs which when hypophosphorylated sequesters eIF4E, required for cap-dependent translation (Dowling *et al.*, 2010). Overexpression of eIF4E has been shown to protect HCT-116 cells from PEITC-induced growth inhibition, suggesting that growth inhibition by PEITC may in part be due to impaired protein synthesis (Hu *et al.*, 2007). These effects are unlikely to be attributed to reduced HIF activity as these experiments were performed in normoxia. However, other studies have demonstrated overexpression of eIF4E preferentially increased translation of proteins involved in cell survival (Bcl-XL) and proliferation (cyclin D1), which may confer some protection from PEITC-mediated growth inhibition

(Li *et al.*, 2003; Rosenwald *et al.*, 1995). I found that TSC2 null MEFs which were resistant to mTORC1 inhibition by PEITC were slightly less sensitive to PEITC-induced growth inhibition compared to their wild type counterparts. Although, as this effect was only modest there are likely multiple mechanisms by which PEITC inhibits growth inhibition. Indeed, loss of one or both PTEN alleles also seemed to have a modest effect on PEITC-mediated growth inhibition, despite there being no effect of PTEN status on the ability of PEITC to suppress mTORC1 activity.

I have proposed that PEITC-induced inhibition of HIF1α protein accumulation is the result of impaired translation of HIF1α mRNA and that this is a consequence of reduced mTORC1 activity. Through ^{35}S-metabolic cell labelling I was able to directly look at translation and found that PEITC does appear to inhibit protein synthesis of HIF1α. Indeed, a downregulation in hypoxia induced HIF1α expression and VEGF production in human prostate cancer and tongue squamous cancer cell lines by the isothiocyanate SFN also appeared to be due to inhibition of HIF1α/ HIF2α mRNA translation (Yao *et al.*, 2008). Although, there may also be a role for PEITC in modulating transcription, as while SFN was also shown to decrease HIF1α accumulation and VEGF production in HUMEC-1 cells this was associated with a reduction of HIF1α mRNA (Bertl *et al.*, 2006). The effects of PEITC on translation are likely to be complex. Previous work in my laboratory has shown that PEITC also inhibits HIF2α protein accumulation in MCF7 cells (Wang *et al.*, 2009), despite the absence of complex secondary structures within the 5'UTR of HIF2α mRNA. Furthermore, expression of HIF2α is unaffected by mTORC1 inhibitor rapamycin (Bhatt *et al.*, 2008). It is possible PEITC affects global protein synthesis via inhibition of cap-dependent translation, but that a small subset of mRNAs with highly structured 5'-UTRs are particularly sensitive to decreased efficiency of translation.

Chapter 5

The effect of PEITC on total mRNA translation

5.1 Introduction

Following on from the discovery described in Chapter 4 that PEITC decreases HIF1α mRNA translation the effect of PEITC on total mRNA translation was investigated. Increased protein synthesis is an inevitable consequence of carcinogenesis, with uncontrolled cell growth and proliferation being a defining hallmark of cancer (Hanahan and Weinberg, 2000). Multiple factors involved in the regulation of translation including eukaryotic initiation factor 4E (eIF4E) and 2 (eIF2) have been implicated in downstream oncogenic signalling and as a result the translational machinery represents a potential target for cancer therapy (Rajasekhar and Holland, 2004). Work described in this chapter demonstrates that PEITC acts as a general inhibitor of protein synthesis, and reveals that in addition to mTORC1, PEITC influences other key translational regulators.

5.2 Results

5.2.1 Effect of PEITC on total mRNA translation

To determine the effect of PEITC on total mRNA translation, global protein synthesis was analysed following PEITC treatment. Treated MCF7 cells were metabolically labelled with [^{35}S]Met/Cys and trichloroacetic acid (TCA) was used to precipitate the protein prior to quantitation of incorporation using a scintillation counter (Figure 5.1). Similar to HIF1α synthesis, PEITC resulted in a dose-dependent inhibition of total protein synthesis with 7.5 μM PEITC resulting in a statistically significant reduction. Furthermore, 10 μM PEITC decreased mRNA translation to the same extent as CHX, a general inhibitor of protein synthesis, suggesting that PEITC has an equivalent universal effect on translation.

5.2.2 The role of mTORC1 pathway in PEITC-induced inhibition of total mRNA translation

In Chapter 4 I established that PEITC inhibited the activity of mTORC1 as assessed by a decrease in 4E-BP1 and p70 S6K phosphorylation. As discussed, the exact role of p70 S6K in translation is not well defined, but 4E-BP1 is known to sequester cap-binding protein eIF4E, which is required to form the eIF4F complex in cap-dependent translation. However, it seems that only a small subset of proteins are sensitive to
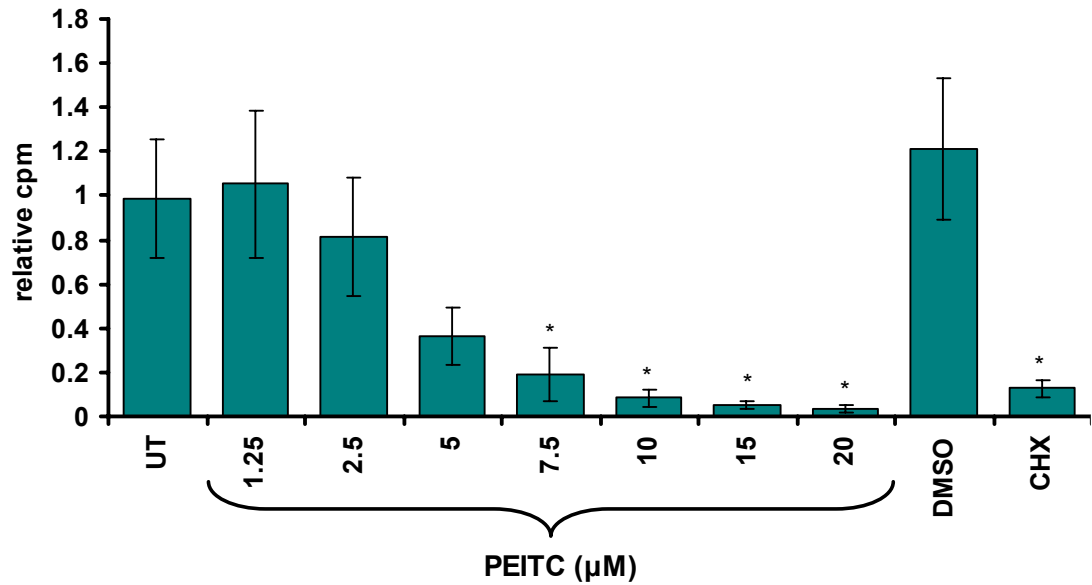
Figure 5.1: The effect of PEITC on total mRNA translation.
MCF7 cells were incubated in the absence (UT) or presence of indicated
concentrations of PEITC, DMSO (equivalent to 20 µM PEITC), or 10 µg/ml
cycloheximide (CHX) as a positive control for 1 h. 100 µCi/ml of [^{35}S]Met/Cys protein
labelling mix was added for 2 h. Cells were harvested and protein extracted using TCA
precipitation. Radioactivity was measured on a scintillation counter. Experiments were
performed in duplicate and normalised to UT, graph shows means from three
independent experiments ± SD. Statistically significant differences between UT and the
treatments are indicated (* p<0.05). All other comparisons were not statistically
significant.

changes in eIF4E, therefore it is unlikely that loss of mTORC1 activity can account for the almost complete inhibition of global protein synthesis by PEITC (Koromilas *et al.*, 1992). Indeed, treatment of wild type MEFs with mTORC1 inhibitor rapamycin only suppressed global protein synthesis by approximately 50% (Figure 5.2). Although, it should be noted that while not all functions of mTORC1 are inhibited by rapamycin, phosphorylation of p70 S6K and 4E-BP1 at site Ser65 are rapamycin-sensitive (Wang *et al.*, 2005).

To further explore the potential role of impaired mTORC1 activity in PEITC-induced inhibition of total mRNA translation, global protein synthesis was analysed in the TSC2 null and wild type MEFs (Figure 5.3). I have previously shown that a statistically significant decrease in 4E-BP1 and p70 S6K phosphorylation is observed following PEITC treatment in the wild type TSC2 MEFs, but that this inhibition is lost in the TSC2 deficient MEFs when mTORC1 is no longer under the regulatory influence of TSC1/2 (Figure 4.6). If the effect of PEITC on total mRNA translation was a result of this inhibition of mTORC1 activity I would expect the TSC2 null MEFs to be less sensitive. However, global protein synthesis is equally inhibited by PEITC in both the MEF genotypes.

5.2.3 Effect of PEITC on key translational proteins

As inhibition of total mRNA translation by PEITC occurs regardless of a decrease in mTORC1 activity, I investigated the effect of PEITC on several other key proteins involved in general protein synthesis, eukaryotic initiation factor 2 (eIF2) and eukaryotic elongation factor 2 (eEF2). Phosphorylation of eIF2α subunit at Ser51, or eEF2 at Thr56 impairs the function of these proteins, inhibiting translation.

MCF7 cells were treated with increasing concentrations of PEITC for 3 h and analysed by western blot (Figure 5.4). PEITC caused a statistically significant dose-dependent increase in eIF2α phosphorylation. However, there was no change in eEF2 phosphorylation.

To further investigate the effect of PEITC on eIF2α I used MEFs that were homozygous for a mutation of eIF2α at amino acid reside 51. This serine to alanine conversion renders eIF2α unphosphorylatable at this critical regulatory residue. Mutant (eIF2α AA) and wild type (eIF2α SS) MEFs were treated with PEITC for 3 h and analysed by western blot for phosphorylation of the translational modulators eIF2α and
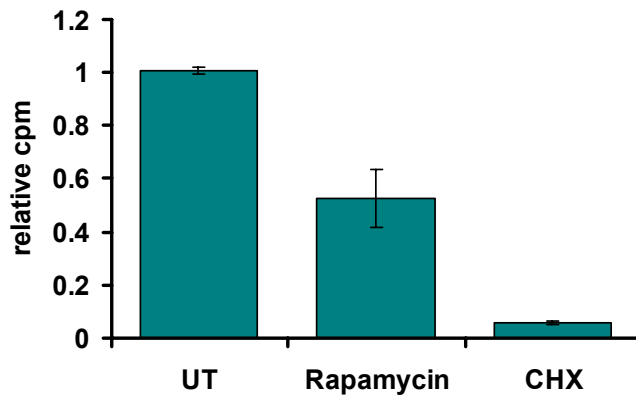
Figure 5.2: The effect of rapamycin on global mRNA translation in wild type MEFs.
Wild type MEFs were incubated in the absence (UT) or presence of 25 nM rapamycin or 10 µg/ml cycloheximide (CHX) as a positive control for 1 h. 100 µCi/ml of [^{35}S]Met/Cys protein labelling mix was added for 2 h. Cells were harvested and protein extracted using TCA precipitation. Radioactivity was measured on a scintillation counter. Experiment was performed once in duplicate and normalised to UT.
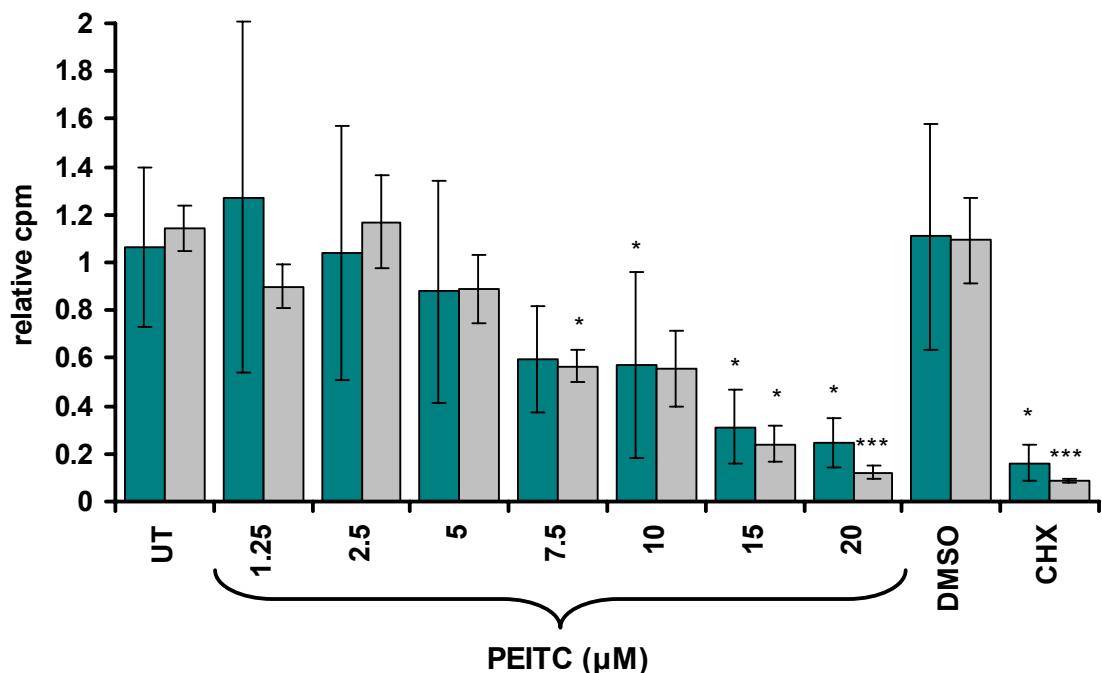
Figure 5.3: The effect of PEITC on global mRNA translation in TSC2 null and wild type MEFs.
TSC2+/+TP53-/- (■) and TSC2-/-TP53-/- (■) MEFs were incubated in the absence (UT) or presence of indicated concentrations of PEITC, DMSO (equivalent to 20 µM PEITC), or 10 µg/ml cycloheximide (CHX) as a positive control for 1 h. 100 µCi/ml of [^{35}S]Met/Cys protein labelling mix was added for 2 h. Cells were harvested and protein extracted using TCA precipitation. Radioactivity was measured on a scintillation counter. Experiments were performed in duplicate and normalised to UT, graph shows means from three independent experiments ± SD. Statistically significant differences between UT and the treatments are indicated (* $p<0.05$; *** $p<0.005$). All other comparisons were not statistically significant.

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Figure 5.4: The effect of PEITC on key translational proteins in MCF7 cells.
MCF7 cells were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 µM PEITC) for 3 h. 25 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-eEF2 (Thr56; 95 kDa) and phospho-eIF2α (Ser51; 38 kDa) then stripped and re-probed for total equivalents. Equal loading was determined by probing for β-actin (42 kDa). The blot is representative of three independent experiments and the results from all three are combined in the densitometry graphs (± SD). Statistically significant differences between UT and the treatments are indicated (* $p<0.05$; *** $p<0.005$). All other comparisons were not statistically significant.

eEF2, as well as indicators of mTORC1 activity, p70 S6K and 4E-BP1 phosphorylation (Figure 5.5). Confirming observations from MCF7 cells, PEITC caused a dose-dependent increase in eIF2α phosphorylation in the eIF2α SS wild type MEFs, but as expected not in the unphosphorylatable eIF2α AA MEFs. This phosphorylation occurred independently of changes in mTORC1 activity as loss of 4E-BP1 and p70 S6K phosphorylation is seen in both cell lines. In contrast to MCF7 cells, the basal levels of eEF2 phosphorylation were extremely low but eEF2 phosphorylation increased in a dose-dependent manner in PEITC treated cells.

5.2.4 Role of eIF2α phosphorylation in PEITC-induced inhibition of total mRNA translation

In order to establish the importance of eIF2α phosphorylation in PEITC-induced inhibition of total mRNA translation, global protein synthesis was analysed in the wild type eIF2α SS and eIF2α AA MEFs (Figure 5.6). The inability of eIF2α to become phosphorylated did render eIF2 AA MEFs less sensitive than the wild type MEFs to PEITC-induced inhibition of total mRNA translation. However, at higher concentrations of PEITC global protein synthesis was statistically significantly inhibited in both cell lines.

5.2.5 Role of eIF2α Phosphorylation in PEITC-Induced Growth Inhibition

To examine the role of eIF2α phosphorylation in PEITC-induced growth inhibition, the sensitivity of wild type and eIF2α AA MEFs to various concentrations of PEITC was examined. MEFs were treated with PEITC for four days before relative cell number was measured by the MTS assay. Representative growth inhibition data in the form of survival curves and mean IC_{50} values are shown in Figure 5.7. MEFs of both genotypes demonstrated dose-dependent growth inhibition following PEITC treatment, but interestingly the wild type MEFs retained significantly higher cell numbers, giving rise to an IC_{50} value two-fold higher, than the MEFs with unphosphorylatable eIF2α.

5.2.6 Effect of PEITC on eEF2 phosphorylation

I have observed that while PEITC has no effect on phosphorylation of eEF2 in human breast cancer MCF7 cells (Figure 5.4), PEITC caused a dose-dependent increase in phospho-eEF2 in both the eIF2α SS and AA MEFs (Figure 5.5). To determine whether
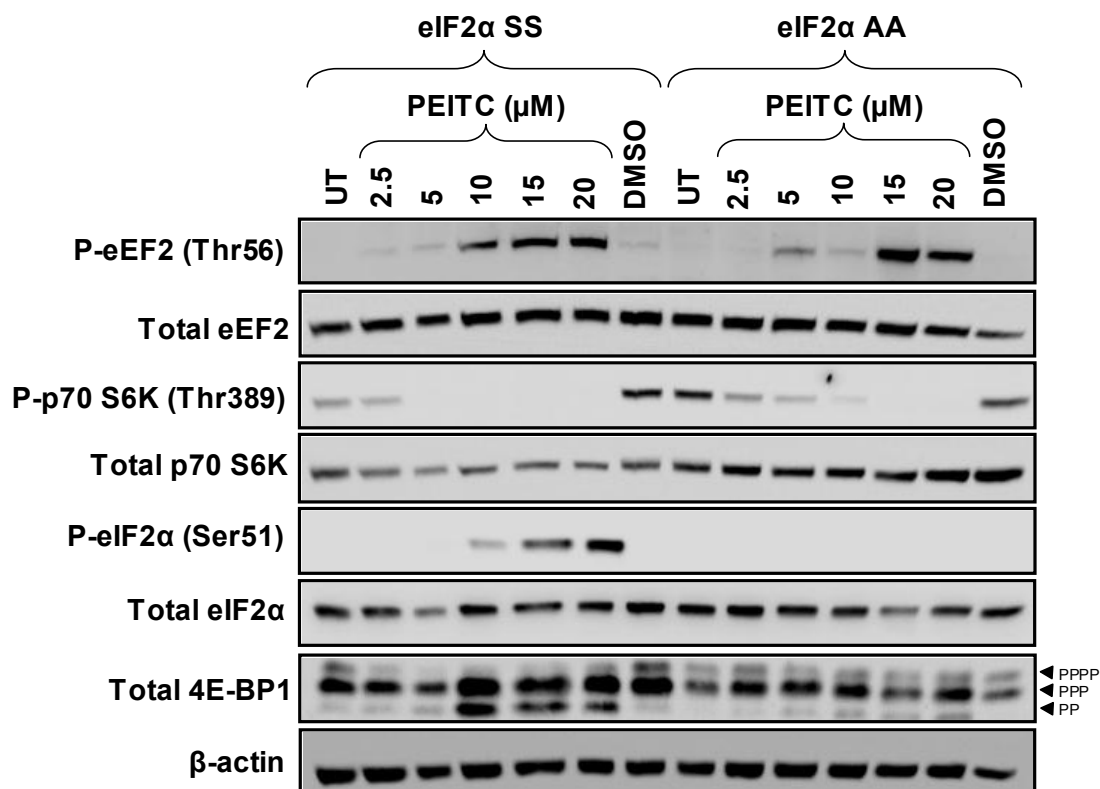
Figure 5.5: The effect of PEITC on key translational proteins in eIF2α SS and eIF2α AA MEFs.
MEFs were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 µM PEITC) for 3 h. 25 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-eEF2 (Thr56; 95 kDa), phospho-p70 S6K (Thr389; 70 kDa), phospho-eIF2α (Ser51; 38 kDa) and total 4E-BP1 (15-20 kDa; as indicated, multiple bands represent different phospho-forms) then stripped and re-probed for total equivalents. Equal loading was determined by probing for β-actin (42 kDa).
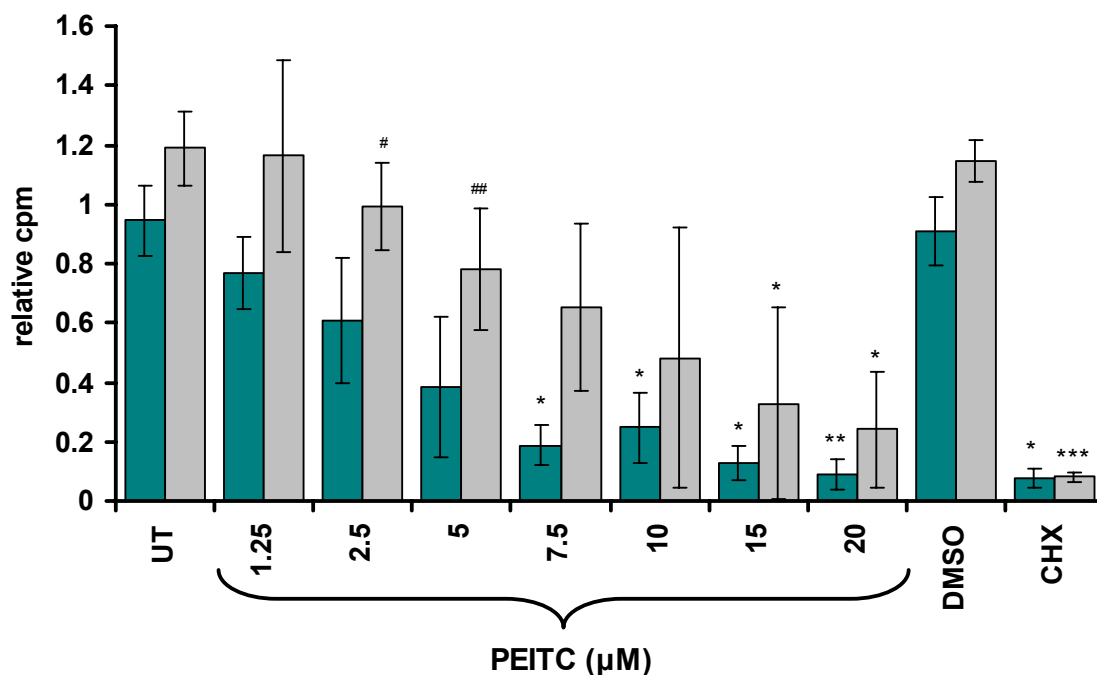
Figure 5.6: The effect of PEITC on global mRNA translation in eIF2α SS and eIF2α AA MEFs.

eIF2α SS (■) and eIF2α AA (■) MEFs were incubated in the absence (UT) or presence of indicated concentrations of PEITC, DMSO (equivalent to 20 μM PEITC), or 10 μg/ml cycloheximide (CHX) as a positive control for 1 h. 100 μCi/ml of [^{35}S]Met/Cys protein labelling mix was added for 2 h. Cells were harvested and protein extracted using TCA precipitation. Radioactivity was measured on a scintillation counter. Experiments were performed in duplicate and normalised to UT, graph shows means from three independent experiments ± SD. Statistically significant differences between UT and the treatments are indicated (* $p<0.05$; ** $p<0.01$; *** $p<0.005$), and between the same treatment in the different cell lines (# $p<0.05$; ## $p<0.01$). All other comparisons were not statistically significant.
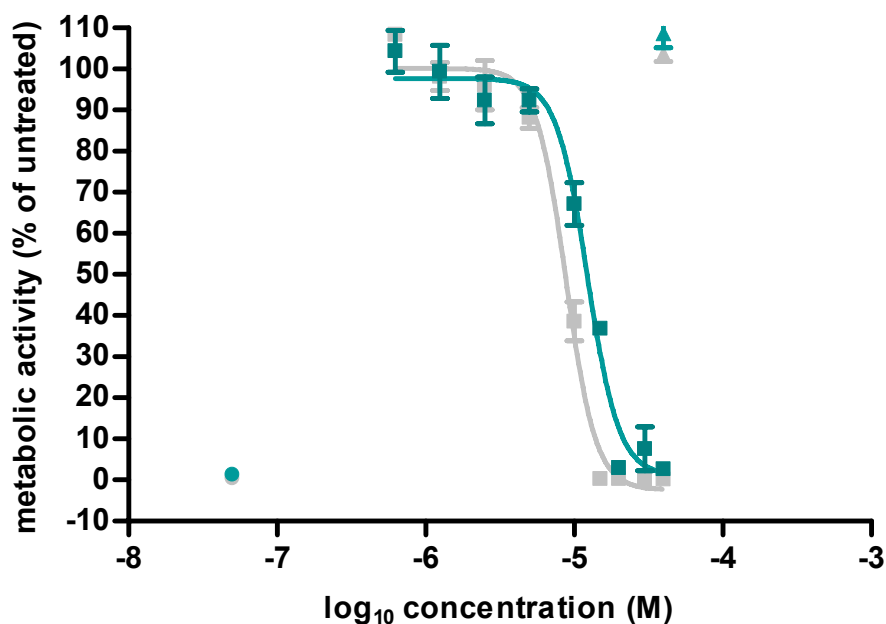
	IC$_{50}$/µM Value (mean ± SD)	
	eIF2α SS	**eIF2α AA**
PEITC	9.3 ± 1.4	4.8 ± 0.7**

Figure 5.7: PEITC-induced growth inhibition of eIF2α SS and eIF2α AA MEFs.
eIF2α SS (■) and eIF2α AA (▫) MEFs were treated with various concentrations of PEITC, DMSO (▲) equivalent to the highest dose of PEITC and 0.5 µM STS as a positive control (●). After four days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Graph is representative and shows data derived from means of triplicate wells (± SD). Table showing IC$_{50}$ values represent mean values ± SD from four experiments performed in triplicate. A statistically significant difference between IC$_{50}$ values for eIF2α SS and AA MEFs is indicated (** $p < 0.01$).

PEITC could also induce phosphorylation of eEF2 in the other MEFs used within this study, they were also treated with PEITC for 3 h and analysed by western blot for phospho-eEF2 (Figure 5.8). Similar to the eIF2α MEFs, the PTEN MEFs of both genotypes seem to have low basal levels of eEF2 phosphorylation which increases in response to treatment with PEITC. On the other hand, basal eEF2 phosphorylation appears to be higher in both genotypes of the TSC2 MEFs and PEITC does not have any effect.

Furthermore, as the response to PEITC is consistent between the wild type and genetically modified counterpart of each of the MEFs, the effect of PEITC on eEF2 phosphorylation must be independent of PTEN, TSC2 and eIF2α phosphorylation. It should be noted that the concentrations of PEITC treatments between the PTEN and TSC2 MEFs are slightly different as to correlate with the range of concentrations previously tested in each of the cell lines. However, in both the PTEN and eIF2α MEFs, an increase in eEF2 phosphorylation is clearly evident at 10 µM PEITC treatment, while no change is observed at this concentration in the TSC2 MEFs.

5.3   Discussion

Following on from the discovery in Chapter 4 that PEITC inhibits translation of HIF1α mRNA in MCF7 cells, I investigated the effect of PEITC on global inhibition of protein synthesis in these cells and found that PEITC seems to have a general inhibitory effect on mRNA translation. However, it seemed unlikely that PEITC-induced inhibition of mTORC1 activity was responsible, as mTORC1 inhibitor rapamycin only suppressed global protein synthesis by approximately 50% in wild type MEFs. This is consistent with previous studies, which using various mTOR inhibitors demonstrated mTORC1 only has a modest effect on total mRNA translation (Jefferies *et al.*, 1994; Thoreen *et al.*, 2009). Indeed, global protein synthesis in TSC null MEFs, which I have shown in Chapter 4 to be resistant to PEITC-induced suppression of mTORC1 activity, was inhibited by PEITC to a similar extent as their wild type counterparts. In an attempt to elucidate how PEITC was inhibiting total mRNA translation, I investigated the effect of PEITC on two other key regulators of protein synthesis, eIF2α and eEF2, in MCF7 cells. While there was no effect of PEITC on eEF2 phosphorylation, eIF2α phosphorylation was significantly increased. Using MEFs that were homozygous for a mutation of eIF2α Ser51 to unphosphorylatable alanine (eIF2α AA), I confirmed that while PEITC caused a dose-dependent increase in eIF2α phosphorylation in the eIF2α SS wild type MEFs, it was unable to in the eIF2α AA MEFs. Furthermore, this

a)

b)
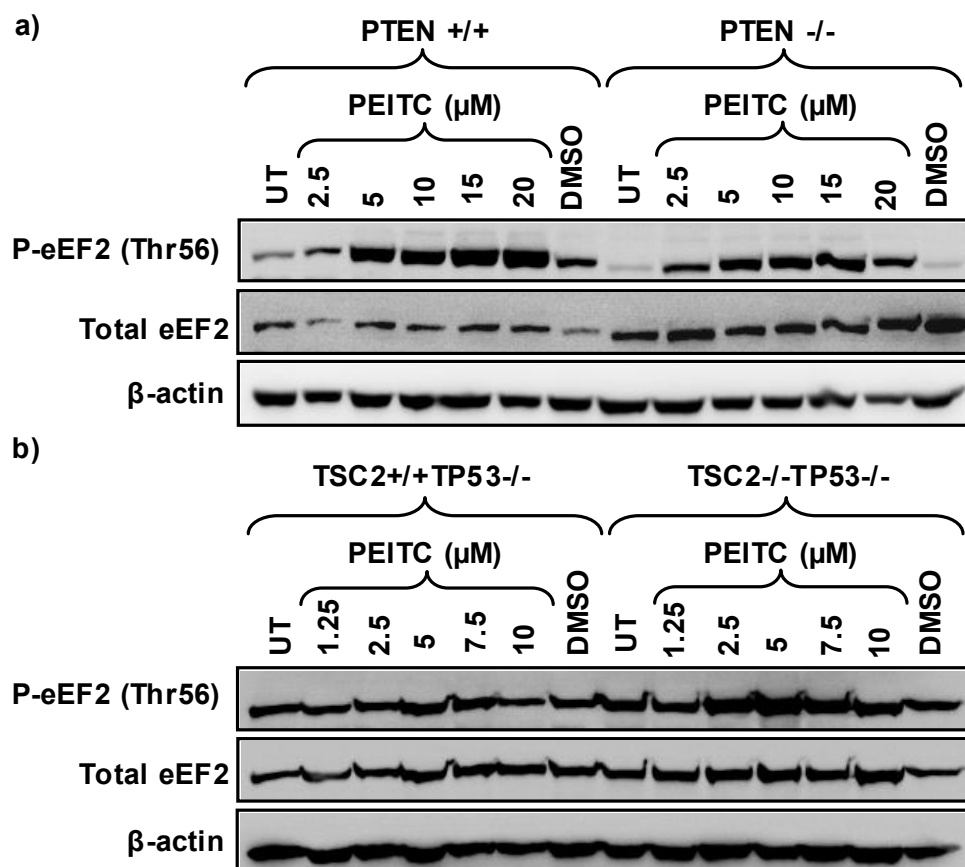
Figure 5.8: Effect of PEITC on phosphorylation of eEF2 in different MEFs.
(a) PTEN wild type (+/+) and deficient (-/-), or (b) TSC2 wild type (+/+TP53-/-) and deficient (-/-TP53-/-) MEFs cells were treated with indicated concentrations of PEITC or DMSO (equivalent to 20 µM PEITC) for 3 h. 25 µg of whole cell lysate was subjected to SDS-page and analysed by western blot for phospho-eEF2 (Thr56; 95 kDa) then stripped and re-probed for total eEF2. Equal loading was determined by probing for β-actin (42 kDa). Lysates used for blot (a) are the same as those shown in Figure 4.6.

phosphorylation occurred independently of changes in mTORC1 activity as loss of 4E-BP1 and p70 S6K phosphorylation is seen in both cell lines. However, in contrast to what was observed in MCF7 cells, PEITC caused a dose-dependent increase in eEF2 phosphorylation in both the eIF2α MEF genotypes. This difference will be discussed later. Importantly, I have shown that eIF2α phosphorylation does seem to contribute to PEITC-induced inhibition of total mRNA translation as the unphosphorylatable eIF2 AA MEFs were less sensitive than the wild type MEFs to inhibition of global protein synthesis by PEITC. Although, at higher concentrations of PEITC global protein synthesis was statistically significantly inhibited in both eIF2α MEF genotypes suggesting that other mechanisms are involved.

eIF2 is a heterotrimeric protein, and when bound to GTP recruits Met-tRNA$_i$ and transfers it to the 40S ribosomal subunit in a process that requires hydrolysis of GTP and release of eIF2-GDP. For continued initiation of translation and protein synthesis, eIF2 needs to exchange GDP for GTP and this is facilitated by the GEF eIF2B. However, phosphorylation of Ser51 on the α subunit of eIF2 stabilises the interaction between eIF2B and GDP bound eIF2, preventing nucleotide exchange (Webb and Proud, 1997). In mammals, eIF2α is known to be phosphorylated by four distinct kinases in response to a variety of environmental and cellular stresses; heme-regulated inhibitor (HRI), protein kinase RNA-dependent (PKR), PKR-like ER (endoplasmic reticulum) kinase (PERK), and nutrient-regulated general control non-depressible 2 (GCN2) (Wek *et al.*, 2006).

HRI kinase is abundantly expressed in erythroid tissues and was initially identified to phosphorylate eIF2α in response to heme deficiency, matching synthesis of globulin proteins with heme availability (Han *et al.*, 2001). A single heme molecule directly interacts with HRI kinase through a cysteine residue in the C-terminal kinase domain and a histidine residue in the N-terminal domain, inhibiting HRI kinase activity when heme is abundant (Miksanova *et al.*, 2006). However, oxidative stress induced by arsenite and heat stress has also been demonstrated to activate HRI kinase in erythroid cells, and although the exact mechanism remains unclear it seems to be independent of heme (Lu *et al.*, 2001). In addition, HRI kinase is expressed in non-erythroid cells (Berlanga *et al.*, 1998), and using HRI knockout MEFs was shown to be the principal kinase responsible for eIF2α phosphorylation induced by 26S proteasome inhibition in these cells (Yerlikaya *et al.*, 2008).

PKR plays a role in the antiviral defence mechanism, phosphorylating eIF2α to prevent translation of viral mRNA. Expression of PKR is upregulated by interferon and enzymatic activation occurs by binding of double stranded RNA (dsRNA) that accumulates during viral infection. PKR contains two dsRNA motifs that when are bound to dsRNA promotes homodimerisation and subsequent autophosphorylation to activate PKR (Cole, 2007). However, PKR can also be activated in the absence of viral infection, independent of dsRNA, by heterodimerisation with PKR-associated activator (PACT). Phosphorylation of PACT can be triggered by a variety of cellular stresses including oxidative stress, ER stress and serum deprivation, and leads to increased affinity for PKR (Patel *et al.*, 2000; Singh *et al.*, 2009a).

PERK is an ER transmembrane kinase that phosphorylates eIF2α in response to a buildup of misfolded proteins in the ER (Harding *et al.*, 2000). This forms part of the unfolded protein response (UPR), halting protein synthesis to prevent further burden on the chaperones required for proper folding of newly synthesised membrane and secretory proteins. PERK is maintained in an inactive form through binding with ER chaperone Grp78/BiP. However, accumulation of unfolded proteins sequesters Grp78/BiP away from PERK, activating the eIF2α kinase by allowing homodimerisation and autophosphorylation. Changes to the luminal ER environment by altering redox state or calcium levels, or disruption to posttranslational modification such as N-linked glycosylation can all impair proper protein folding and lead to a buildup of misfolded proteins (Lai *et al.*, 2007).

GCN2 phosphorylates eIF2α in response to amino acid deprivation, coordinating protein synthesis with amino acid availability (Zhang *et al.*, 2002). GCN2 possess a domain homologous to histidyl-tRNA synthetases (HisRS) enzyme and is believed to be activated by binding of uncharged tRNA molecules that increase in concentration during amino acid starvation. However, GCN2 has also been shown to be involved in eIF2α phosphorylation in response to UV irradiation and proteasome inhibition (Deng *et al.*, 2002; Jiang and Wek, 2005).

Considering the redundancy of these kinases to many of the cellular stresses and the pleiotrophic effects of PEITC, it is likely that PEITC activates several if not all the eIF2α kinases. Perhaps the most obvious means of PEITC to cause eIF2α phosphorylation is through oxidative stress, as it is known depletion of ROS-scavenger, glutathione (GSH), by PEITC leads to increased intracellular concentrations of ROS (Trachootham

et al., 2006). The exact mechanism by which oxidative stress causes activation of the eIF2α kinases HRI and PKR is unknown, however, as with modulation of other redox sensitive proteins it is likely to involve oxidation of key cysteine residues that alter protein activity. Indeed, a cysteine residue is required to coordinate heme to HRI kinase and thiol oxidation may disrupt this interaction, removing the inhibitory influence of heme (Miksanova *et al.*, 2006). The presence of key regulatory cysteines means it is also possible that PEITC activates HRI through direct protein modification. Furthermore, conjugation of PEITC to protein cysteinyl thiols in the ER may prevent proper formation of disulphide bridges, resulting in an accumulation of misfolded proteins and activation of PERK.

Both HRI and GCN2 have also been implicated in eIF2α phosphorylation in response to proteasome inhibition in MEFs, and 26S proteasome inhibitors MG132 and bortezomib have further been shown to induce phosphorylation of eIF2α in murine B16F10 melonama and 4T1 breast cancer cell lines (Yerlikaya and Dokudur, 2010). It has recently been shown that PEITC can inhibit both 20S and 26S proteasome activity in multiple myeloma cells (Mi *et al.*, 2011a). This inhibition occurs independently of ROS generation or ITC-induced protein aggregation therefore, the authors propose that PEITC inhibits the proteasome through direct binding. Consequently it is possible PEITC thiocarbamoylation indirectly activates eIF2α kinases via the 26S proteasome.

In addition to inhibiting general protein synthesis, eIF2α phosphorylation selectively induces translation of a subset of mRNAs that both promote cell survival, through adaptation to stress, and induce apoptosis (Harding *et al.*, 2003). Coincident signalling pathways activated alongside eIF2α phosphorylation in response to cellular stresses can also contribute to cell survival and apoptosis. Seemingly, the ultimate fate of the cell depends on the balance between the cytoprotective and pro-apoptotic pathways, and these can be influenced by the intensity, duration and type of stress (Muaddi *et al.*, 2010; Rutkowski *et al.*, 2006; Scheuner *et al.*, 2006). I found that MEFs homozygous for an unphosphorylatable form of eIF2α were more sensitive to PEITC-induced growth inhibition than their wild type counterparts, suggesting that in this context eIF2α confers some protection from PEITC-induced stress.

eEF2 is required for the translocation of the peptidyl chain along the ribosome from A to P site during elongation and is negatively regulated through eEF2 kinase directed phosphorylation, which prevents eEF2 from binding to the ribosome (Carlberg *et al.*,

1990). eEF2K is a calcium/calmodulin (CAM)-dependent kinase also regulated by multiple phosphorylation sites which have both activatory and inhibitory influences. Several mTOR-dependent eEF2K phosphorylation sites have been identified that in response to insulin inhibit eEF2K thereby decreasing eEF2 phosphorylation and promoting mRNA translation. mTORC1 substrate p70 S6K directly phosphorylates eEF2K at Ser366, impairing its activity particularly at low calcium concentrations. This site can also be phosphorylated by downstream ERK target, p90RSK (Wang *et al.*, 2001). Cdc2/cyclin B has been shown to phosphorylate eEF2K at Ser 359 in a manner that is consistent with being regulated by mTORC1, although exactly how mTORC1 controls Cdc2/cyclin B is unknown (Smith and Proud, 2008). Phosphorylation at Ser78 inhibits the binding of calmodulin to eEF2K and is sensitive to rapamycin, although the kinase responsible has yet to be identified (Browne and Proud, 2004). On the other hand, phosphorylation of Ser398 by AMPK promotes eEF2K activity, inhibiting the energy consuming process of elongation when ATP levels are low (Browne *et al.*, 2004), and phosphorylation of Ser500 (Ser499 in rabbit reticulocytes) by cAMP-dependent protein kinase (PKA) allows calcium-independent activation of eEF2 (Diggle *et al.*, 2001).

Despite PEITC inhibiting p70 S6K phosphorylation, in human MCF7 breast cancer cells, there does not appear to be any effect of PEITC on eEF2 phosphorylation. In Chapter 4 I demonstrated that PEITC enhanced phosphorylation of upstream p90RSK activator, ERK, and therefore it is possible that this compensates for the loss of p70 S6K activity. While several other mTOR-dependent eEF2K phosphorylation sites have also been identified and PEITC does affect mTORC1 activity, as shown by loss of 4E-BP1 and p70 S6K phosphorylation, it is possible that not all functions are inhibited. Surprisingly, when eEF2 phosphorylation was analysed in the eIF2α SS and eIF2α AA MEFs, PEITC resulted in a dose-dependent increase in phospho-eEF2 in both MEF genotypes. The effect of ERK phosphorylation was not examined in these cells, but as with MCF7 cells, PEITC similarly inhibited p70 S6K phosphorylation in both the wild type and unphosphorylatable eIF2α MEFs. Interestingly, the MEFs seemed to display lower basal levels of eEF2 phosphorylation than the MCF7 cells, which may be a consequence of different intracellular levels of calcium. As mentioned, phosphorylation by p70 S6K affects eEF2K activity particularly at low calcium concentrations and may explain why loss of p70 S6K phosphorylation relieves eEF2K inhibition, increasing eEF2 phosphorylation in the MEFs but not the MCF7 cells. I also examined the effect of PEITC on eEF2 phosphorylation in the other MEFs used in this study. Similar to the

eIF2α MEFs, the PTEN MEFs of both genotypes had low basal levels of eEF2 phosphorylation which increased with PEITC treatment. However, basal eEF2 phosphorylation appears to be higher in both genotypes of the TSC2 MEFs and PEITC does not have any effect. Notably, unlike the other MEFs, the TSC2 MEFs are also knockout for p53.

Chapter 6

In vitro analysis of anticancer effects of watercress extracts: proof-of-principle studies

6.1   Introduction

An important aim of this project was to determine whether it was possible to enhance the *in vitro* anticancer activity of watercress. Based on results described in Chapter 3, growth inhibition and activation of Nrf2-dependent transcription were selected as candidate assays for *in vitro* analysis of crude watercress extracts and in this chapter, I describe a series of proof-of-principle experiments using these assays. The goals of these experiments were to (i) optimise methods for preparation of watercress extracts, (ii) determine the suitability of the assays for the analysis of crude watercress extracts, and (iii) to gain any insight into whether different watercress crops might possess distinct *in vitro* anticancer properties, as a precursor to direct agronomic experiments.

Two varieties of watercress were selected for these proof-of-principle experiments. First, the commercially available 'regular' green watercress from the Vitacress seed and second, an American 'red' variety of watercress (Figure 6.1). Apart from the obvious difference in colour and the fact that red watercress is sterile little is known about the differences between these two varieties. Although, red watercress may have similar origins to brown watercress which up until the Second World War was commercially cultivated alongside the 'regular' green watercress.

6.2   Results

6.2.1 Preparation of watercress extracts

Before I could assess the *in vitro* anticancer activity of crude watercress extract it was necessary to optimise preparation of the extract. Previous studies investigating the *in vitro* anticancer potential of watercress prepared extracts by homogenising samples in a commercial juice processor (Boyd *et al.*, 2006; Kassie *et al.*, 2003). Cell debris was subsequently removed by centrifugation. This technique is not quantitative and therefore unsuitable for comparing between different watercress samples. The method obtained from colleagues in Biological Sciences, University of Southampton, UK involved grinding snap frozen samples to a fine powder in a pestle and mortar. Ground watercress was then decanted into a QIAshredder homogeniser (Qiagen) consisting of a mini-spin column and a collection tube. The sample was centrifuged to collect the extract. While this technique is quantifiable the grinding of samples is time-consuming. To determine whether grinding is necessary I compared crude watercress extracts

Figure 6.1: Images of the two different watercress varieties tested.
(a) Green watercress, (b) red watercress.

prepared using the QIAshredder method from either ground or whole snap frozen material in the MTS growth inhibition assay. I found no difference in IC_{50} values between the two samples (Figure 6.2). Snap freezing the sample seems sufficient to break open the plant cell walls and allow myrosinase to catalyse the conversion of glucosinolates to isothiocyanates. Therefore, in future experiments I used whole watercress. Another limitation to using the QIAshredder method was that due to its size only 1 g of sample could be prepared, producing approximately 500 µl crude watercress extract. Sterile filtering of the extract further reduced sample volume. Essentially the QIAshredder homogeniser consists of a column with a filter, sat within a collection tube. To create a larger version that could process more watercress, I placed a 20 ml syringe (BD Biosciences) that had had the plunger removed and a circular 25 mm glass microfibre filter (Whatman) inserted in the bottom, inside a 50 ml centrifuge tube. Like with the QIAshredder homogenizer, centrifugation allowed for collection of crude watercress extract. Crude watercress extracts prepared using either the QIAshredder or syringe method were compared in the MTS growth inhibition assay. There was no discernable difference in IC_{50} values between the two samples (Figure 6.3). As a result, the syringe method was used for the preparation of subsequent crude watercress extracts.

I also compared the effect of crude watercress extracts prepared from either leaves or stems on growth inhibition in MCF7 cells. Interestingly, extracts from the leaves of watercress exhibited approximately 2.5-fold more *in vitro* anticancer activity than that from the stems (Figure 6.4). It is important the watercress extracts come from both the leaf and stem to be representative of what an individual may consume however, in order to avoid differences in leaf/stem ratio skewing results crude watercress extracts for future experiments were prepared from equal parts leaf and stem.

6.2.2 Effect of crude watercress extract from different varieties of watercress on the growth inhibition of MCF7 cells

I have already demonstrated that the MTS growth inhibition assay is suitable for the analysis of a crude watercress extract; however, I also wanted to investigate whether different varieties of watercress possess distinct *in vitro* anticancer properties. To compare the growth inhibitory activity of different varieties of watercress, MCF7 breast cancer cells were treated with crude watercress extract from either the green or red variety. Extracts from both varieties inhibited the growth of MCF7 cells, but crude watercress extract from red watercress was nearly 10-fold more potent than that from
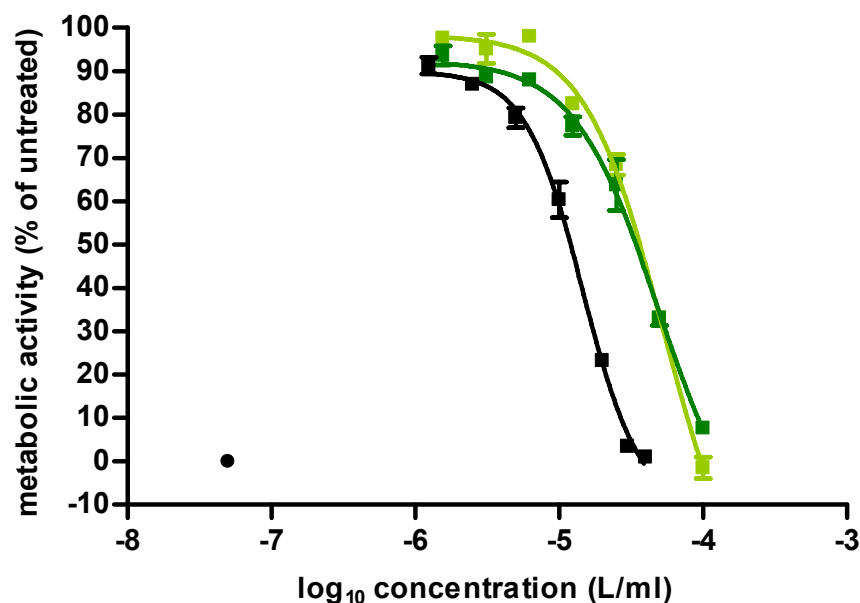
IC$_{50}$ Value (mean ± SD)		
Ground	**Whole**	**PEITC**
36.7 µl/ml ± 18.3	43.8 µl/ml ± 13.7	12.3 µM ± 2.1

Figure 6.2: Comparison of the ability of crude watercress extract prepared from either ground or whole sample to inhibit the growth of MCF7 cells.
Representative growth inhibition experiments. Cells were treated with various concentrations of crude watercress extract prepared from ground (▪) or whole (▪) frozen samples of green watercress, PEITC for use as a standard (▪) or 0.5 µM STS as a positive control (●). Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). Table showing IC$_{50}$ values represent mean values ± SD from three experiments.
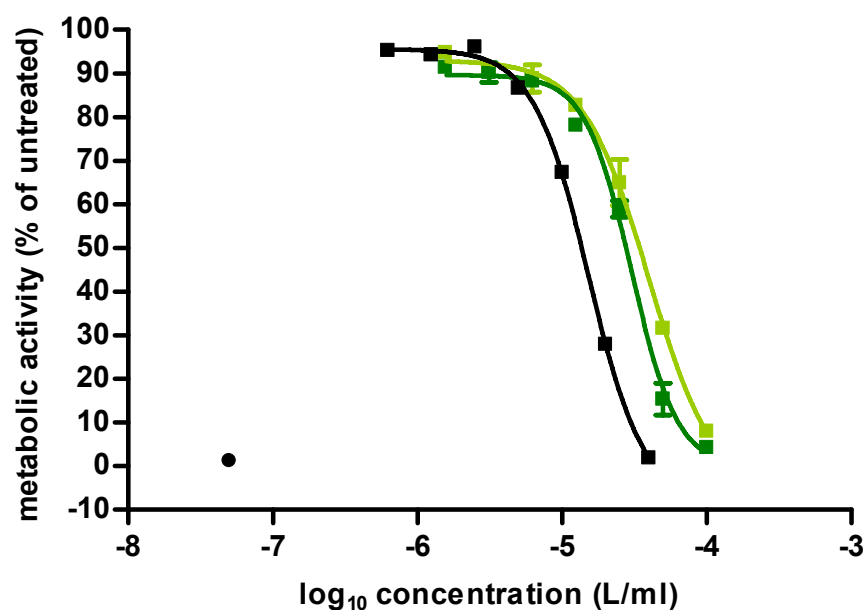
IC$_{50}$ Value (mean ± SD)		
QIAshredder	**Syringe**	**PEITC**
36.6 µl/ml ± 20.1	37.2 µl/ml ± 20.6	8.6 µM ± 5.8

Figure 6.3: Comparison of the ability of crude watercress extract prepared by the QIAshredder or syringe technique to inhibit the growth of MCF7 cells.
Representative growth inhibition experiments. Cells were treated with various concentrations of crude watercress extract from green watercress prepared by the QIAshreder (■) or syringe (■) technique, PEITC for use as a standard (■) or 0.5 µM STS as a positive control (●). Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96$^{®}$ AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). Table showing IC$_{50}$ values represent mean values ± SD from three experiments.
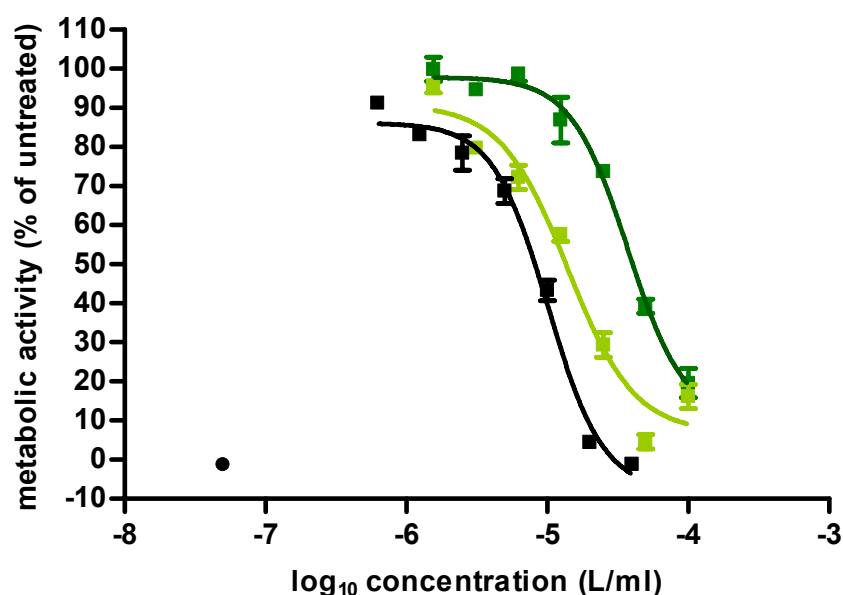
IC$_{50}$ Value (mean ± SD)		
Leaf	**Stem**	**PEITC**
45.4 µl/ml ± 11.0	16.5 µl/ml ± 5.2*	14.4 µM ± 3.8

Figure 6.4: Comparison of the ability of crude watercress extract from either the leaf or stem of green watercress to inhibit the growth of MCF7 cells.
Representative growth inhibition experiments. Cells were treated with various concentrations of crude watercress extract from the leaf (■) or stem (■) of green watercress, PEITC for use as a standard (■) or 0.5 µM STS as a positive control (●). Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). Table showing IC$_{50}$ values represent mean values ± SD from three experiments. A statistically significant difference between IC$_{50}$ values for crude watercress extracts derived from the leaf and stem is indicated (* $p<0.05$).

green watercress (Figure 6.5).

6.2.3 Effect of crude watercress extract on Nrf2 activity

To investigate effects of watercress extracts on Nrf2 activity, AREc32 cells were treated with crude watercress extract from green or red varieties for 24 h before luciferase was measured (Figure 6.6). Extracts from the green and red watercress both caused a dose-dependent increase in Nrf2-dependent transcription. Similar to observations from growth inhibition assays, extract from the red cultivar was 10-fold more potent than the green, with a 2-fold increase in Nrf2 activity seen at 0.3125 µM and 3.125 µM respectively.

6.2.4 Effect of crude watercress extract on HIF activity

In addition to growth inhibition and Nrf2 activation, I also investigated the effects of watercress extracts on other important cancer pathways. In particular, I analysed effects on HIF activity and total mRNA translation, shown to be modulated by PEITC in previous work (Chapters 4 and 5).

To explore effects on HIF transcriptional activity, MCF7 cells were transiently transfected with a luciferase construct containing an upstream hypoxia response element (HRE) (Wang *et al.*, 2009). To control for non-specific effects, the activity of the SV40-promoter based reporter plasmid pGL3-promoter was also analysed. MCF7 cells were transfected with the HIF or pGL3-promoter construct and treated with increasing amounts of crude watercress extract from the green (Figure 6.7) or red (Figure 6.8) varieties, and water as a control. Cells were then exposed to the hypoxia mimetic DFO, an iron chelator that inhibits prolyl hydroxylase activity required to target HIF for degradation in normoxia.

In both experiments, pretreatment with DFO resulted in strong induction in HIF activity with little change observed in the expression of luciferase controlled by the pGL3-promoter construct. Crude watercress extract from the green variety caused a dose-dependent decrease in the activity of the HIF reporter construct that is statistically significant at 50 µl/ml treatment. There does not seem to be any effect of the green watercress extract on the activity of the pGL3-promoter construct. Treatment with crude watercress extract from the red variety also resulted in a dose-dependent
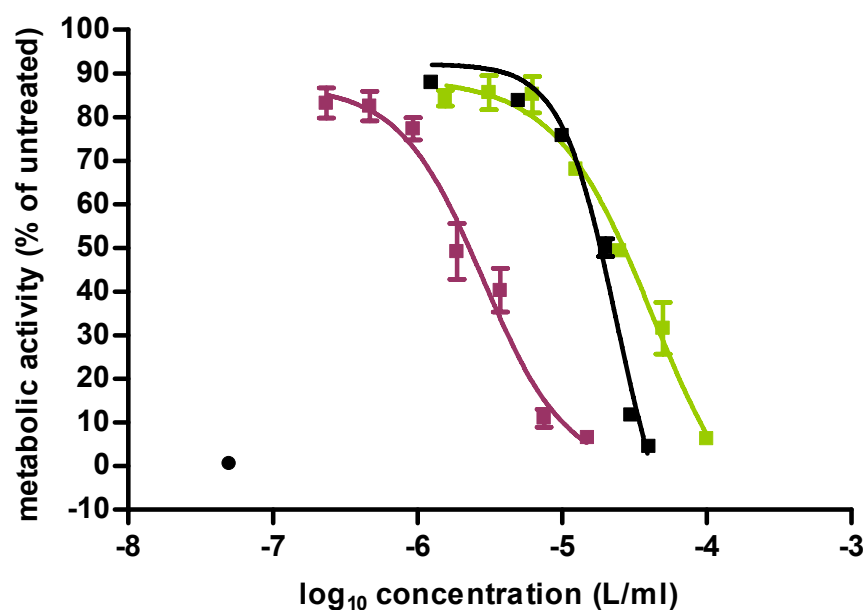
IC$_{50}$ Value (mean ± SD)		
Green Wx	**Red Wx**	**PEITC**
34.58 µl/ml ± 9.11	3.25 µl/ml ± 1.77**	17.12 µM ± 4.87

Figure 6.5: Effect of crude watercress extract on the growth of MCF7 cells.
Representative growth inhibition experiments. Cells were treated with various concentrations of crude watercress extract either from the green cultivar (■) or the red cultivar (■), PEITC for use as a standard (■) or 0.5 µM STS as a positive control (●). Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). Table showing IC$_{50}$ values represent mean values ± standard deviation from three experiments. A statistically significant difference between IC$_{50}$ values for green and red crude watercress extracts is indicated (** $p < 0.01$).
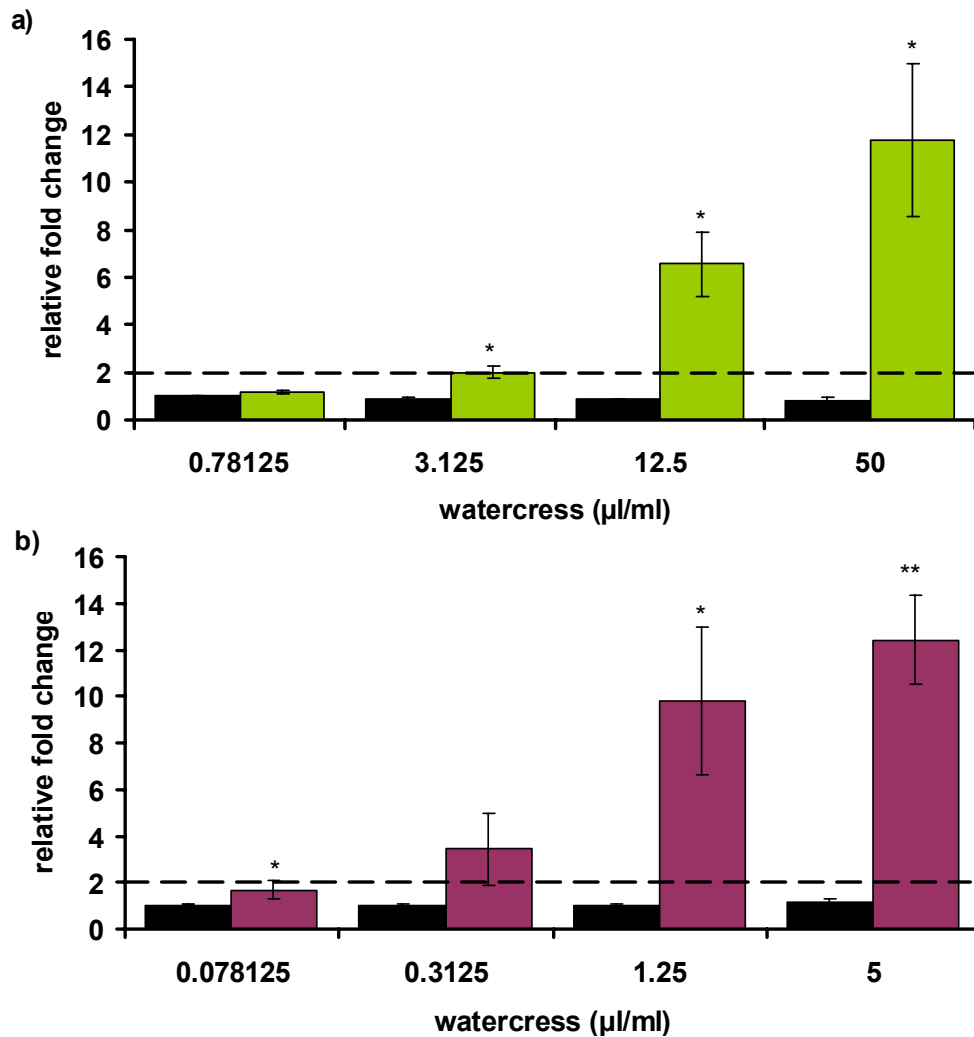
Figure 6.6: Effect of crude watercress extract on ARE-driven expression.
MCF7 cells stably transfected with ARE were treated with the indicated concentrations of crude watercress extract either from (a) green cultivar (■) or (b) red cultivar (■) and equivalent amounts of H_2O as a control (■) for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments. Statistically significant differences between H_2O and watercress treated cells are indicated (* $p < 0.05$; ** $p < 0.01$). All other comparisons were not statistically significant.
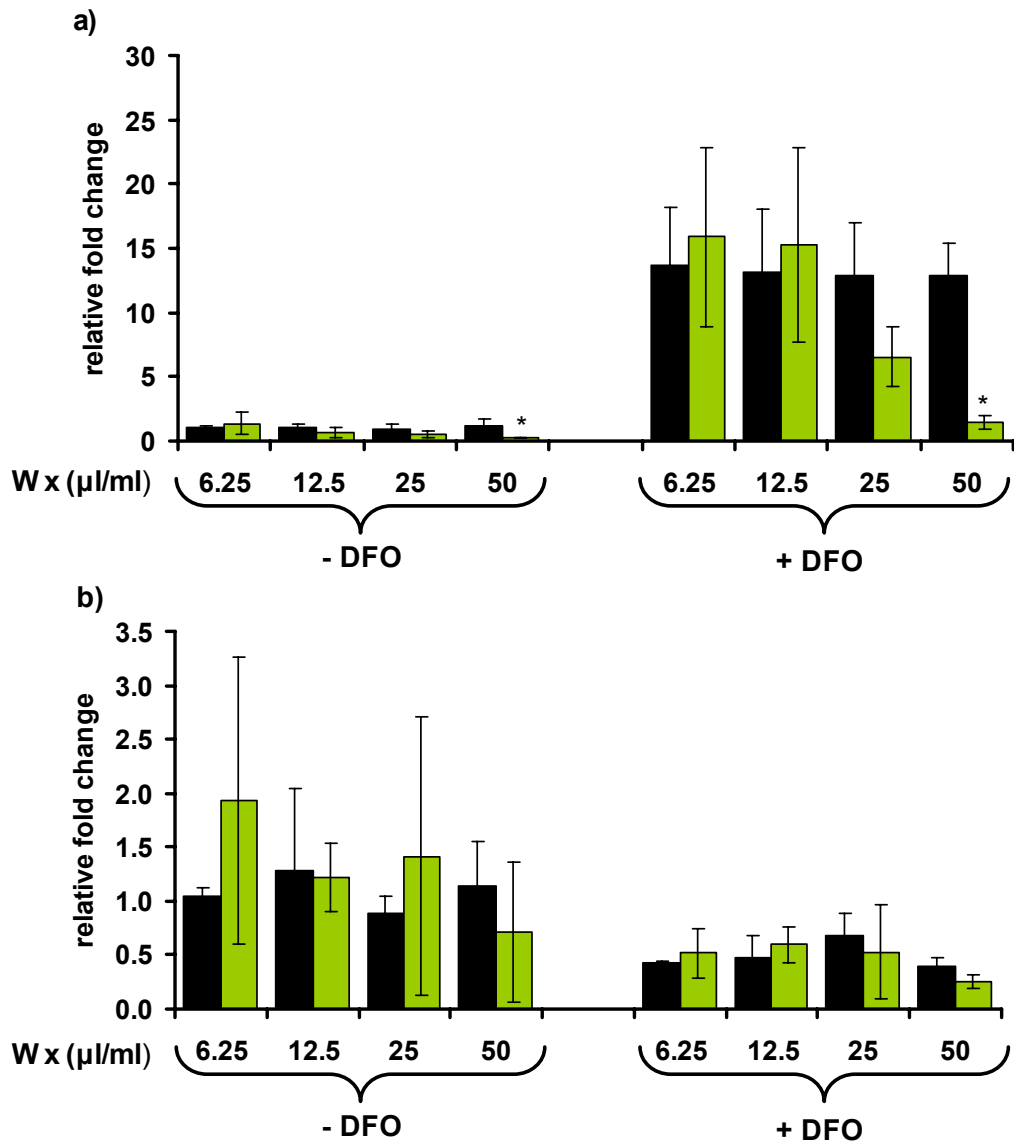
Figure 6.7: Effect of a crude extract from green watercress on HIF activity.
MCF7 cells were transfected with (a) pGL2-TK-HRE or (b) control pGL3-promoter reporter constructs and treated with the indicated concentrations of crude watercress extract from the green cultivar (■) or equivalent amounts of H_2O as a control (■) for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments. Statistically significant differences between H_2O and watercress treated cells are indicated (* $p < 0.05$). All other comparisons were not statistically significant.
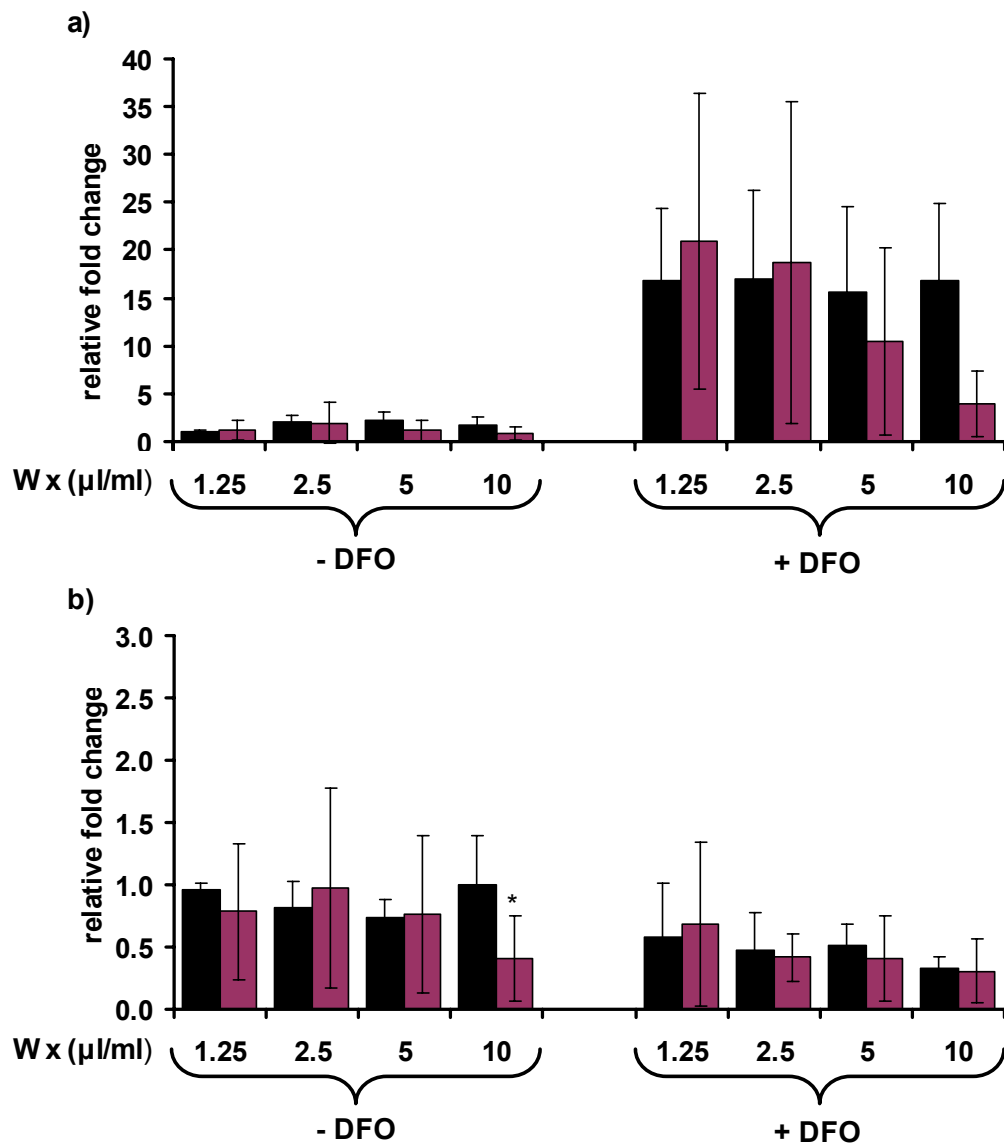
Figure 6.8: Effect of a crude extract from red watercress on HIF activity.
MCF7 cells were transfected with (a) pGL2-TK-HRE or (b) control pGL3-promoter reporter constructs and treated with the indicated concentrations of crude watercress extract from the red cultivar (■) or equivalent amounts of H_2O as a control (■) for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments. Statistically significant differences between H_2O and watercress treated cells are indicated (* $p < 0.05$). All other comparisons were not statistically significant.

decrease in the activity of the HIF reporter construct but at much lower concentrations. This is in agreement with the growth inhibition and Nrf2 activity data collected from the green and red watercress varieties. However, results were more variable with the red watercress extract and the reduction in HIF activity was not statistically significant. In addition, extract from the red watercress seemed to reduce activity of the control pGL3-promoter construct suggesting there may be non-specific effects.

6.2.5 Effect of crude watercress extract on total mRNA translation

To investigate effects of watercress extracts on total mRNA translation, MCF7 cells were treated with crude watercress extract from the green and red varieties. Cells were then metabolically labelled with $[^{35}S]$Met/Cys and protein synthesis determined following TCA precipitation (Figure 6.9). Extracts from both green and red watercress caused a dose-dependent decrease in total mRNA translation. However, crude watercress extract from the red variety appears to be only 4-fold more potent than that from the green variety, as opposed to the 10-fold difference observed in the growth inhibition and Nrf2 activity assays.

6.3 Discussion

The goals of this chapter were to optimize preparation of a crude watercress extract, determine whether assays developed in previous chapters could be used to analyse the *in vitro* anticancer activity of a crude watercress extract, and investigate whether different varieties of watercress might possess distinct *in vitro* anticancer properties. I found that a crude watercress extract can inhibit the growth of MCF7 breast cancer cells using the MTS assay and enhance Nrf2 activity as assessed by ARE-driven gene expression in a luciferase reporter system. Moreover, I demonstrated that the extract from red watercress has more potent *in vitro* anticancer activity than extracts from the green watercress. Therefore, the MTS growth inhibition assay and Nrf2 activity assay appear to be suitable to investigate the *in vitro* anticancer activity of watercress grown under different agronomical conditions. I also looked at HIF activity, analysed by a HIF-dependent construct in a luciferase reporter assay, as a potential means to assess the *in vitro* anticancer activity of crude watercress extract. Crude watercress extract was shown to inhibit HIF activity in this assay, and similar to the MTS and Nrf2 activity assays showed a difference in the potency of the two varieties of watercress tested. However, this assay produced large error bars which meant inhibition of HIF activity
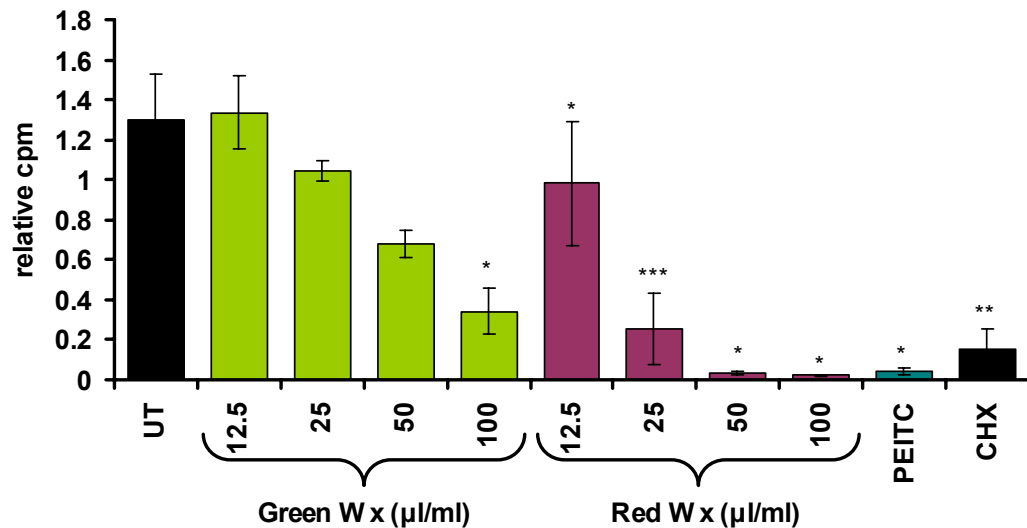
Figure 6.9: Effect of crude watercress extract on total mRNA translation.
MCF7 cells were incubated in the absence (UT) or presence of indicated concentrations of green (■) or red (■)watercress extract, 20 µM PEITC (■), or 10 µg/ml cycloheximide (CHX) as a positive control for 1 h. 100 µCi/ml of [^{35}S]Met/Cys protein labelling mix was added for 2 h. Cells were harvested and protein extracted using TCA precipitation. Radioactivity was measured on a scintillation counter. Experiments were performed in duplicate and normalised to UT, graph shows means from three independent experiments ± SD. Statistically significant differences between UT and the treatments are indicated (* $p<0.05$; ** $p<0.01$; *** $p<0.005$). All other comparisons were not statistically significant.

was not always statistically significant, and there is also some question as to whether crude watercress extract causes non-specific inhibition of the control pGL3-promoter. As a result, I decided not to use this assay when determining the *in vitro* anticancer activity of my field trial samples.

Very few studies have actually examined the effect of crude watercress extract on cell proliferation. In peripheral blood mononuclear cells (PBMCs) *in vitro*, 10 µl/ml crude watercress extract caused approximately 50% inhibition of metabolic activity while having little effect on membrane integrity, suggesting the cells are undergoing apoptosis (Hofmann *et al.*, 2009). On the other hand, analysis of cell cycle progression following treatment of colorectal carcinoma HepG2 cells with crude watercress extract seemed to result in an accumulation in S phase (Boyd *et al.*, 2006). Much of the work on crude watercress extract has focused on its effect on drug metabolising enzymes. Crude watercress extract has been shown to increase the activity of multiple phase II drug metabolising enzymes *in vitro*, including NQO1 and GST which are under the control of AREs (Kassie *et al.*, 2003; Rose *et al.*, 2000). This laboratory has previously published data demonstrating the inhibitory effect of crude watercress extract on HIF activity in an ARE luciferase system, and showed that consumption of 80 g watercress by healthy individuals may be sufficient to cause a transient decrease in 4E-BP1 phosphorylation *in vivo* (Syed Alwi *et al.*, 2010). To my knowledge no other groups have looked at the effect of watercress or any other cruciferous vegetable on HIF or angiogenesis.

Notably, in the MTS and Nrf2 activity assays the crude watercress extract from the red variety was found to display considerably more *in vitro* anticancer activity than that from the green variety, being approximately 10-fold more potent. One obvious difference between the two varieties is colour. Despite being known as red watercress the leaves I tested actually looked more purple in colour, while the green variety of watercress was the typical light green colour generally associated with watercress. Indeed, the crude watercress extract collected were purple and green respectively. The reddy purple colours observed in the red watercress would suggest an increased presence of anthocyanins (Mol *et al.*, 1998). It is well known that anthocyanins are powerful antioxidants and their presence may be a marker of stress induced changes.

In addition to being antioxidants, there is evidence emerging that anthocyanins and their aglycones, anthocyanidins, may also play a more specific anticancer role (Wang and Stoner, 2008). Both pure anthocyanins and extracts from anthocyanin rich fruits

and vegetables have been shown to inhibit the growth of a variety of human cancer cell lines (Chen *et al.*, 2005; Reddivari *et al.*, 2007; Seeram *et al.*, 2006). This is thought to be due to cell cycle arrest in G2/M phase, which is associated with down-regulation of a range of cell cycle regulatory proteins, as well as apoptosis initiated via both the intrinsic and extrinsic pathways. It has also been demonstrated that anthocyanins can induce the activity of several phase II metabolising enzymes including GST and NQO1 in rat liver clone 9 cells by promoting ARE-driven gene expression (Shih *et al.*, 2007). Anthocyanins have also been reported to possess anti-angiogenic properties. Both the anthocyanidin delphinidin and an anthocyanin-rich berry extract were shown to inhibit angiogenesis in *in vivo* models and this was associated with a decrease in VEGF expression which is a target for HIF1α (Bagchi *et al.*, 2004; Favot *et al.*, 2003). However, it should be noted that these experiments were carried out using much higher concentrations of anthocyanins than are likely to be found in red watercress.

If the red watercress does in fact have higher concentrations of anthocyanins than the green watercress this may cause the enhanced *in vitro* anticancer effect observed. However, it would be interesting to determine any differences in ITC content between the red and green watercress. Anthocyanin presence is an indicator stress and therefore other stress-induced changes may have occurred, and these are likely to also contribute to the differences in *in vitro* anticancer activity.

As far as I am aware there has not been any previous data published linking extracts from watercress or any other cruciferous vegetable to a decrease in mRNA translation. Interestingly, while red crude watercress extract is still more potent than green at inhibiting total protein synthesis, the difference in activity is only 4-fold, opposed to the 10-fold difference observed in the assays looking at growth inhibition and Nrf2 activity. It could also be said that crude watercress extract from red watercress is only 5-fold more potent than that from the green at inhibiting HIF activity. However, as already mentioned there is some difficulty in interpreting the results from this assay.

When assessing the effect of crude watercress extracts on total mRNA translation, growth inhibition experiments were carried out alongside using the same extracts. This was to confirm that the difference in potency was specific to the effect of watercress on total mRNA translation and not an overall decrease in potency due to instability of phytochemicals in storage. While storage slightly decreased the potency of the watercress samples there was still a relative 10-fold difference between the green and red crude watercress extracts in the MTS growth inhibition assay (Appendix 1). These

results suggest that the compound in red watercress responsible for the increased growth inhibitory and Nrf2 activity could be different from that responsible for the increased inhibition of total mRNA translation. Alternatively, the increased potency in the growth inhibition and Nrf2 activity assays could be due to a synergistic effect with other phytochemicals, while inhibition of protein translation is caused by a single compound acting alone.

One limitation of this study is that I was unable to successfully determine PEITC levels in the different watercress samples. Previous studies have highlighted the difficulty in measuring breakdown products of glucosinolates due to the volatile nature of many of the compounds (Boyd *et al.*, 2006; Rose *et al.*, 2000). However, through use of the cyclocondensation assay this problem can be overcome. This method relies on the quantitative reaction of ITCs with an excess of 1,2-benzenedithiol to yield the stable cyclic-ring product, 1,3-benzodithole-2-thione, and a free amine (Zhang *et al.*, 1992).The 1,3-benzodithole-2-thione product can then be measured using high-performance liquid chromatography (HPLC) (Zhang *et al.*, 1996). It should be noted that this assay can not distinguish between different isothiocyanates, or their conjugation products, therefore the cyclocondensation assay measures total isothiocyanates and their metabolites. Nonetheless, this assay could still provide valuable insight on whether the differences in *in vitro* anticancer activity I observed were a consequence of altered isothiocyanate content.

In experiments not reported here, aimed at developing the cyclocondensation assay for analysis of watercress extracts, I was able to detect 1,3-benzodithole-2-thione in the PEITC standard. However, this peak was not seen in the crude watercress extract, even when this was spiked with the PEITC standard. The cyclocondensation assay has largely been used to determine total ITC content from urinary and plasma samples following ingestion of cruciferous vegetables, but crude extracts from broccoli sprouts have been analysed by this method (Conaway *et al.*, 2000; Shapiro *et al.*, 2001). During my analysis of crude watercress extract an extremely large peak is initially seen that was not present in the PEITC standard. This suggests that there is a large amount of non-specific binding derived from the watercress extracts that is quickly eluted. The non-specific binding may inundate the HPLC column preventing 1,3-benzodithole-2-thione binding. Differences in the length and diameter of the C_{18} HPLC column could account for why others were still able to achieve 1,3-benzodithole-2-thione binding and thus detect ITCs in a crude vegetable extract.

Chapter 7

Watercress: Field trials

7.1   Introduction

This chapter explores whether it is possible to enhance the *in vitro* anticancer activity of watercress. As described in the introduction, manipulation of both genetics and environmental factors provide possible routes to enhance the concentration of glucosinolates in crops. Colleagues in Biological Sciences, Southampton University, UK are currently establishing a germplasm collection with the view to improve the anticancer potential of watercress through selective breeding. Therefore this study will investigate the impact of manipulating environmental factors. Altering various aspects of the growth environment such as temperature, photoperiod, light quality, sulfur fertilisation and water availability have all previously been shown to influence the glucosinolate content of cruciferous vegetables. As the changes I make have to be suitable for large scale cultivation of watercress in the field, temperature, photoperiod and light quality were excluded as variables that could be manipulated. However, increasing sulfur fertilisation and withholding water are changes that can be easily implemented in the field. Furthermore, it is known that the expression of some plant genes follow a circadian rhythm that can be influenced by environmental cues such as light and temperature (Schaffer *et al.*, 2001). The fact that glucosinolate content can also be affected by these environmental cues suggests that glucosinolate biosynthesis may follow a circadian rhythm. Consequently, I investigated whether (i) time of day harvest, (ii) increased sulfur fertilisation, and (iii) drought-induced stress, effect the *in vitro* anticancer activity of watercress.

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7.2   Results

7.2.1 First field trial

On 6th August 2009 watercress seedlings were transplanted into three 50 yd^2 gravel lined concrete beds at Vitacress' Fobdown farm in Alresford. To increase sulfur fertilisation, Microthiol Special fertiliser, containing 80% w/w sulfur was applied as a foliar spray to one of the beds two weeks after planting (Figure 7.1a). In accordance to the manufacturer's guidelines the 50 yd^2 bed was sprayed with 42 g Microthiol Special dissolved in 2 L water. After a further two weeks of growth, the crop was ready to harvest (Figure 7.1b). To introduce drought stress, water supply to the second watercress bed was cut off at 18:00 h the day before harvesting (see Figure 7.2 for an overview of the trial). Typically, harvesting would begin at approximately 07:00-08:00 h
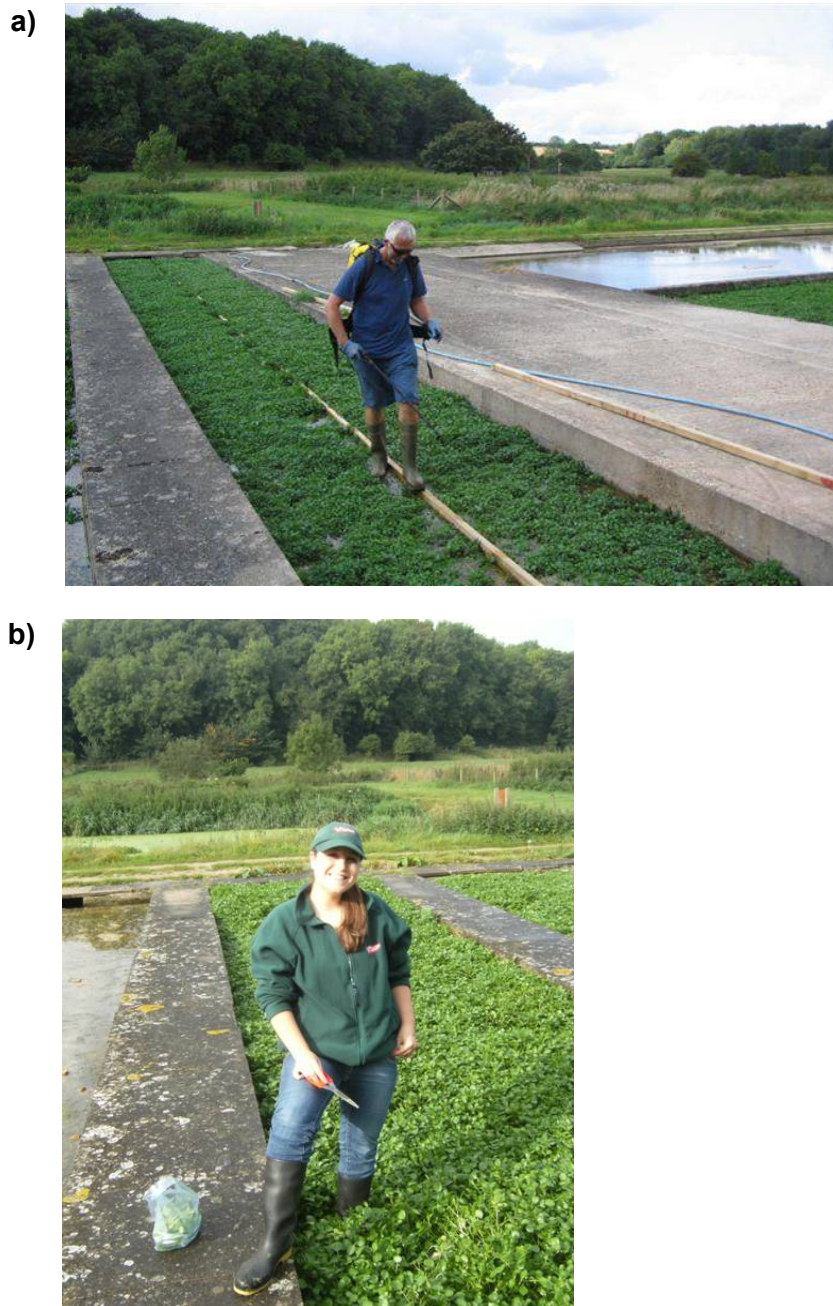
Figure 7.1: Images from first field trial.
(a) Fobdown farm manager, Ron Bainbridge applying a foliar spray of Microthiol Special to the sulfur fertilisation bed two weeks after the watercress seedlings were transplanted into the bed. (b) Myself harvesting watercress from the control bed four weeks after the watercress seedlings were transplanted into the bed.
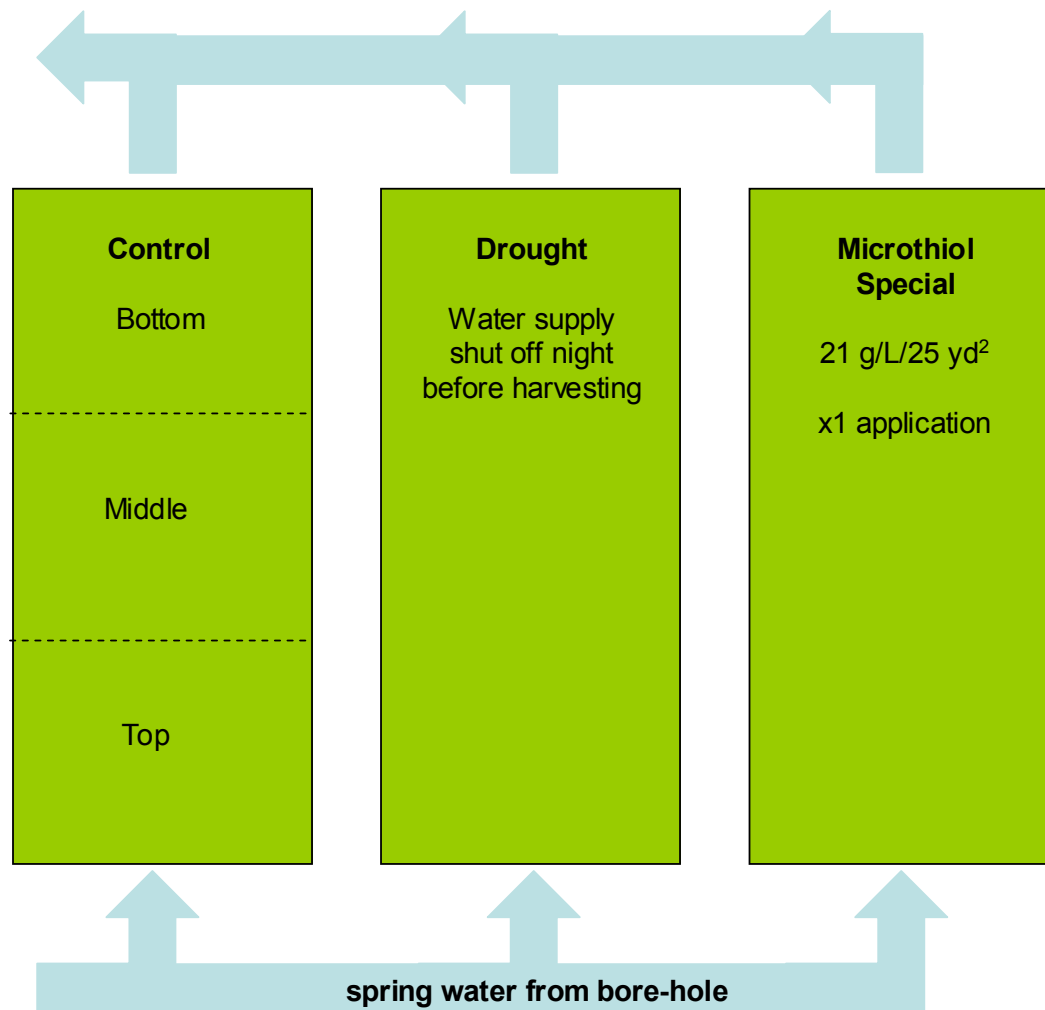
Figure 7.2: Overview of first field trial.
Three 50 yd^2 gravel lined concrete beds at Vitacress' Fobdown farm in Alresford, supplied with spring water from a bore-hole were transplanted with seedlings. Two weeks later sulfur-rich fertiliser Microthiol Special was sprayed onto the watercress in the sulfur fertilisation bed. Four weeks later the watercress was ready to harvest. The night before harvesting the water supply was cut off to the drought bed. On the day of harvest, nine samples from each of the beds were collected, three from each of the top, middle and bottom sections of the beds at 6:00 h and then every three hours until midnight. Samples were also taken at 6:00 h and 9:00 h the following morning from all three beds and at 18:00 h for two days following the first day of harvest from the control and drought beds.

with exact start and finish time depending on weather and size of order. I decided to harvest at first light, which was 6:00 h and then every three hours until midnight, repeating the 6:00 h and 9:00 h harvests the following morning to confirm any pattern was circadian.

Although there was no rain, shutting off the water supply to the drought bed did not completely dry out the bed and there was still water at the 9:00 h harvest on 9th September 2009. Consequently, I decided to continue to take samples from the drought and control beds at 18:00 h everyday until the bed had dried out and the appearance of watercress no longer met quality standards. Samples were collected at 18:00 h on the 9th and 10th September 2009 when there was still some water in the beds. However, on arriving at Fobdown at 18:00 h on 11th September 2009 I discovered that the water supply had erroneously been returned to the bed and as a result no further samples were collected.

Initially I compared the *in vitro* anticancer activity of watercress samples harvested at different times throughout the day in the MTS growth inhibition assay (Figure 7.3) and Nrf2 activity assay (Figure 7.4). While I carried out experimental repeats on the same samples, I also carried out a biological repeat on samples harvested at the same time but from a different part of the bed. There was no difference observed in the ability of any of the samples to inhibit the growth of MCF7 cells or enhance Nrf2 activity.

As time of day harvest had no effect on *in vitro* anticancer activity of watercress, when examining the effect of sulfur fertilisation only watercress samples harvested at 6:00 h and 18:00 h from the control and sulfur treated beds were compared. A single treatment of watercress with a foliar spray of Microthiol Special, at an application rate of 21 g/L/25 yd^2 two weeks into a four week growing period, had no effect on the ability of watercress to inhibit the growth of MCF7 cells (Figure 7.5) or enhance Nrf2 activity (Figure 7.6). When comparing the control and drought stressed watercress I also analysed samples at 6:00 h and 18:00 h as well as samples harvested at 18:00 h on the following two days. Shutting off the water supply to watercress for three days to induce drought stress had no effect on the ability of watercress to inhibit the growth of MCF7 cells (Figure 7.7) or enhance Nrf2 activity (Figure 7.8). Although, it is important to note that the bed was never totally depleted of water, and as water supply was erroneously returned to the bed after four days the experiment ended prematurely.

a)

b)
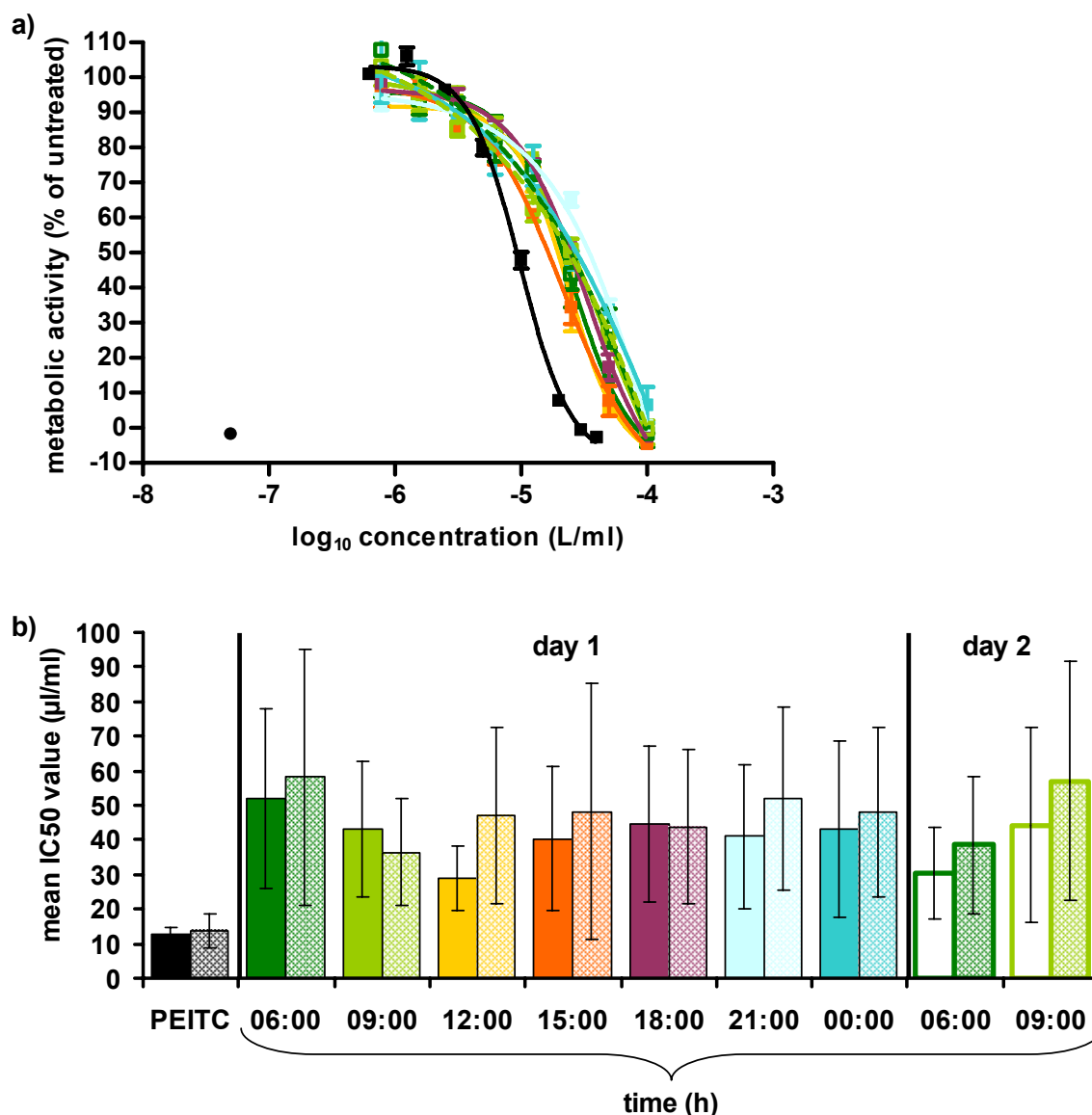
Figure 7.3: Effect of time of harvest on the ability of crude watercress extract to inhibit the growth of MCF7 cells.

(a) Representative growth inhibition experiment. MCF7 cells were treated with various concentrations of crude watercress extract from samples harvested at different times throughout the day (06:00 h (■), 09:00 h (■), 12:00 h (■), 15:00 h (■), 18:00 h (■), 21:00 h ( ), 00:00 h (■), and 06:00 h (▫) and 09:00 h (▫) the following day), PEITC (■) for use as a baseline or 0.5 μM STS (●) as a positive control. Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). (b) Mean IC$_{50}$ values ± SD. Block and hatched bars represent biological repeats, each performed in triplicate.

a)

b)
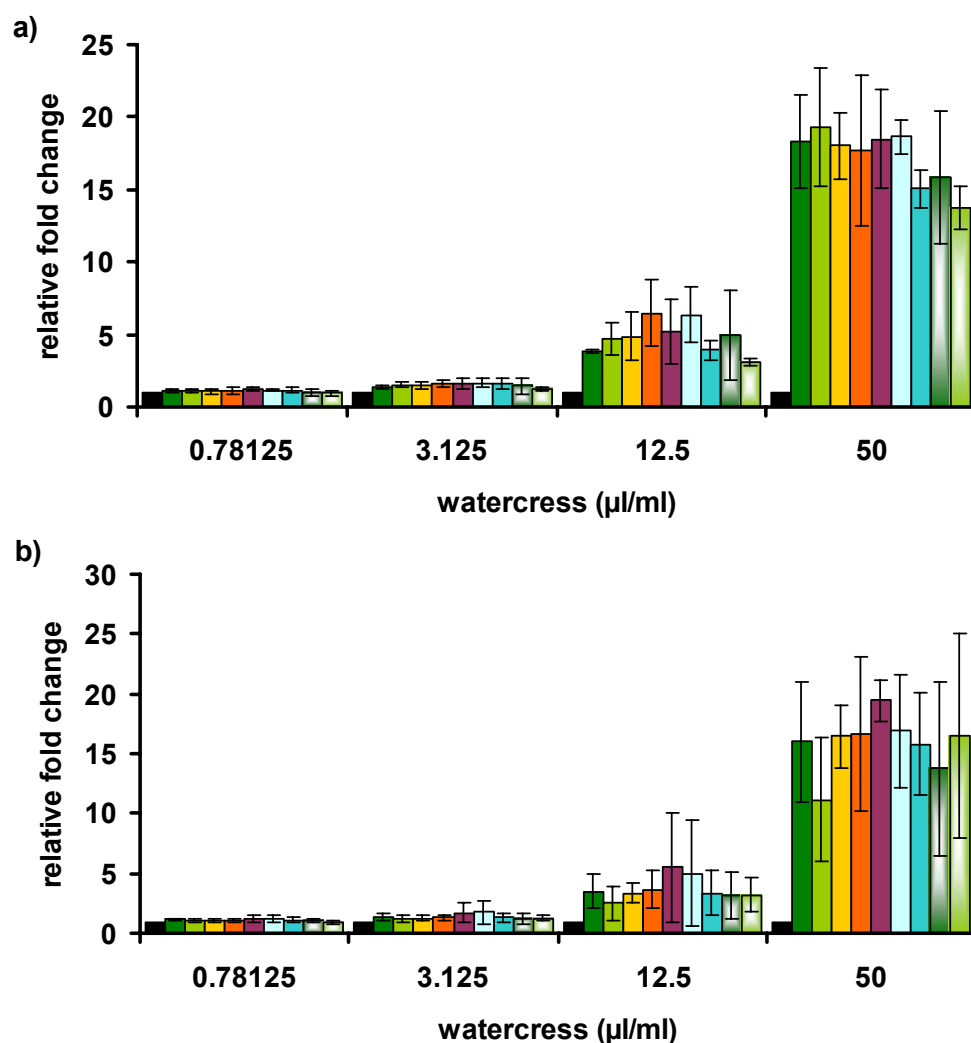
Figure 7.4 Effect of time of harvest on the ability of crude watercress extract to induce ARE-driven expression.
MCF7 cells stably transfected with ARE were treated with the indicated concentrations of crude watercress extract from samples harvested at different times throughout the day (06:00 h (■), 09:00 h (■), 12:00 h (■), 15:00 h (■), 18:00 h (■), 21:00 h ( ), 00:00 h (■), and 06:00 h (□) and 09:00 h (□) the following day), or equivalent amounts of H_2O (■) as a control for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments. (a) and (b) represent biological repeats.
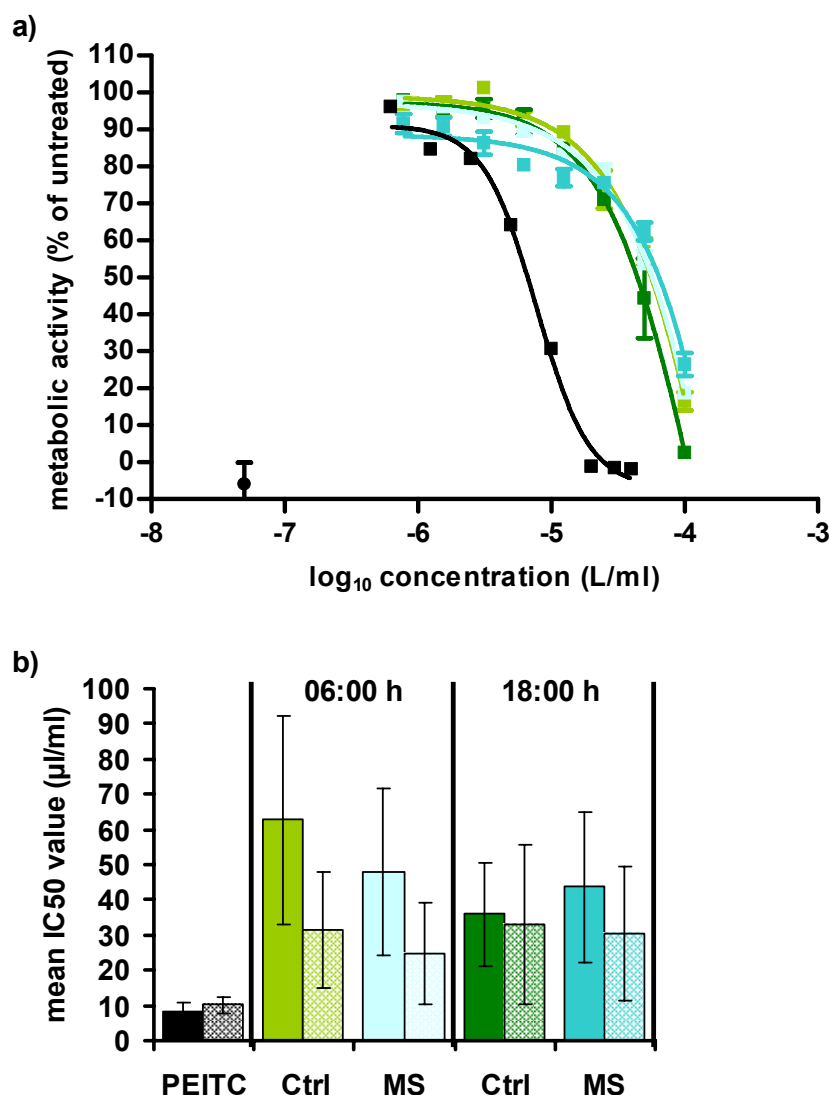
Figure 7.5: Effect of sulfur on the ability of crude watercress extract to inhibit the growth of MCF7 cells.
(a) Representative growth inhibition experiment. MCF7 cells were treated with various concentrations of crude watercress extract from control beds (Ctrl) harvested at 06:00 h (■) or 18:00 h (■), Microthiol Special (MS) treated beds at 06:00 h ( ) or 18:00 h (■), PEITC (■) for use as a baseline or 0.5 µM STS (●) as a positive control. Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). (b) Mean IC$_{50}$ values ± SD. Block and hatched bars represent biological repeats, each performed in triplicate. Data from the internal PEITC standard and control bed are the same as that used in Figure 7.7 when looking at the effect of drought-induced stress.

a)

b)
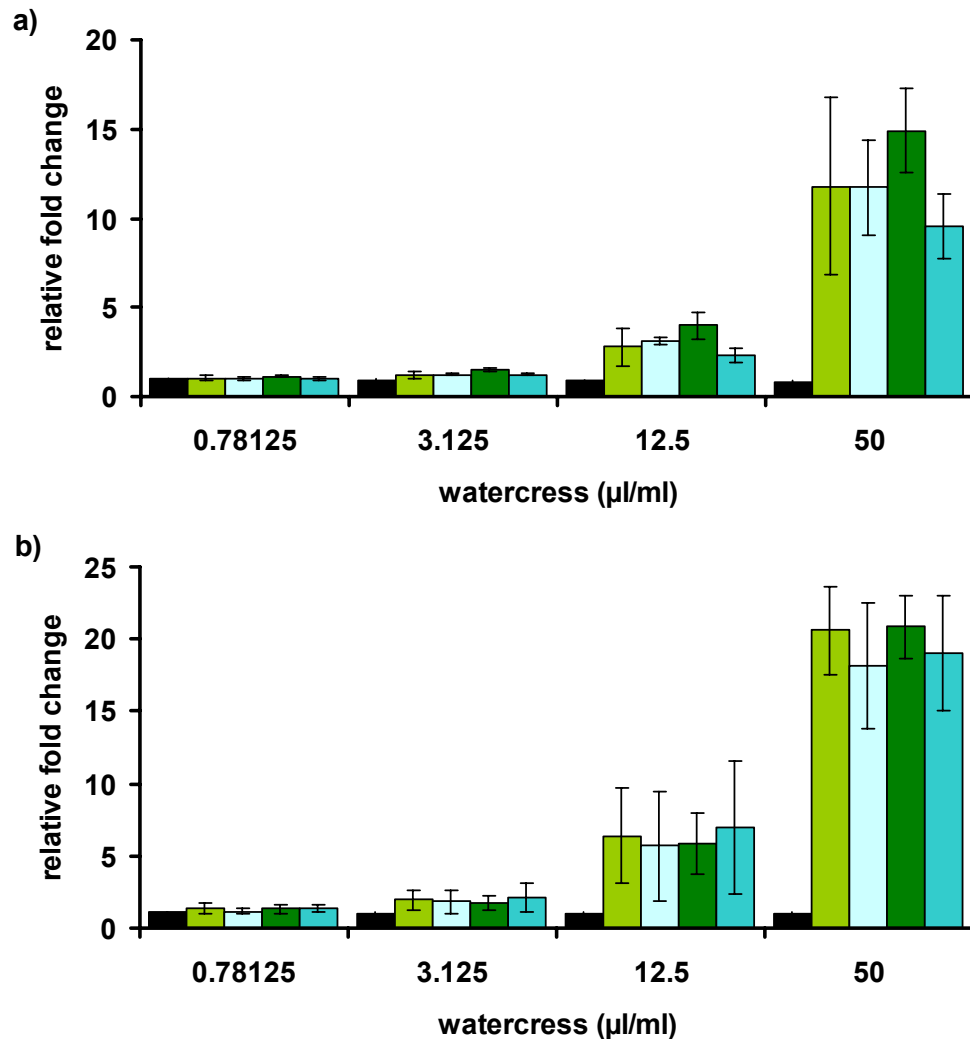
Figure 7.6: Effect of sulfur on the ability of crude watercress extract to induce ARE-driven expression.
MCF7 cells stably transfected with ARE were treated with the indicated concentrations of crude watercress extract from control beds harvested at 06:00 h (■) or 18:00 h (■), Microthiol Special (MS) treated beds at 06:00 h ( ) or 18:00 h (■), or equivalent amounts of H_2O (■) as a control for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments. (a) and (b) represent biological repeats. Data from the H_2O control and control bed are the same as that used in Figure 7.8 when looking at the effect of drought-induced stress.
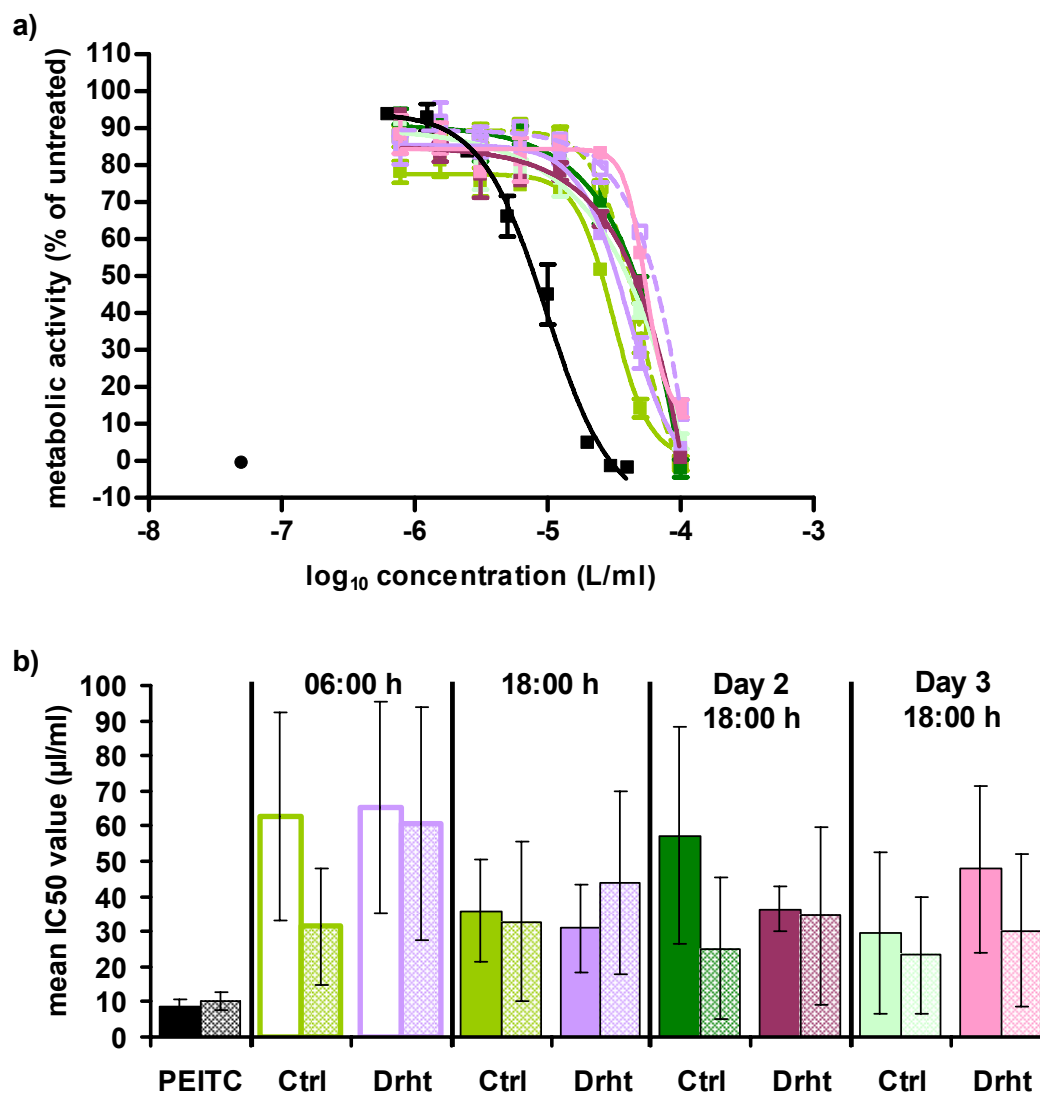
Figure 7.7: Effect of drought on the ability of crude watercress extract to inhibit the growth of MCF7 cells.

(a) Representative growth inhibition experiment. MCF7 cells were treated with various concentrations of crude watercress extract from control (Ctrl) beds harvested at 06:00 h (□), 18:00 h (■), 18:00 h 24 h later (■) or 48 h later (○), or beds where water supply was stopped at 18:00 h the day before (Drht) and harvested at 06:00 h (□), 18:00 h (■), 18:00 h 24 h later (■) or 48 h later (■), PEITC (■) for use as a baseline or 0.5 µM STS (●) as a positive control. Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96[®] AQ~ueous~ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). (b) Mean IC_{50} values ± SD. Block and hatched bars represent biological repeats, each performed in triplicate. Data from the internal PEITC standard and early control bed time points are the same as that used in Figure 7.5 when looking at the effect of sulfur fertilisation.

a)

b)
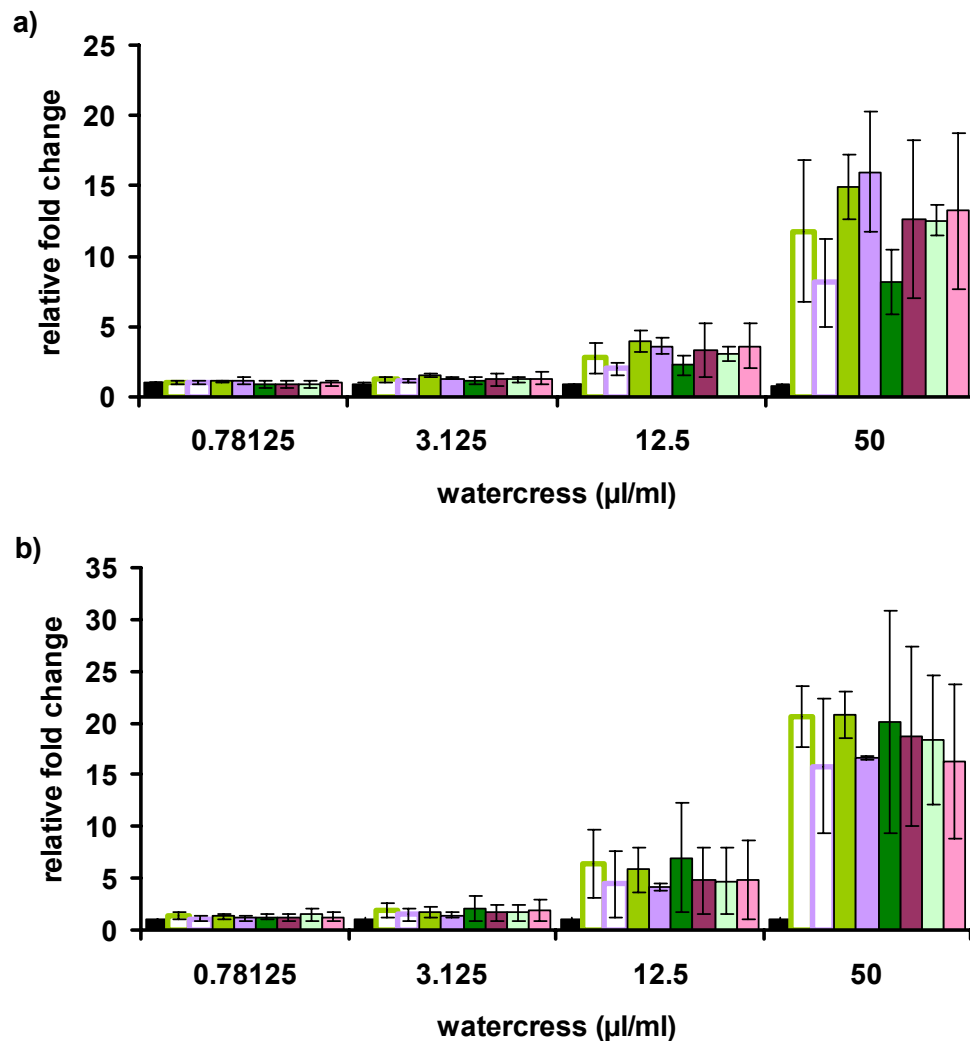
Figure 7.8: Effect of drought on the ability of crude watercress extract to induce ARE-driven expression.
MCF7 cells stably transfected with ARE were treated with the indicated concentrations of crude watercress extract from control beds harvested at 06:00 h (▫), 18:00 h (■), 18:00 h 24 h later (■) or 48 h later ( ), or beds where water supply was stopped at 18:00 h the day before and harvested at 06:00 h (▫), 18:00 h (■), 18:00 h 24 h later (■) or 48 h later (■), or equivalent amounts of H_2O (■) as a control for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments. (a) and (b) represent biological repeats. Data from the H_2O control and early control bed time points are the same as that used in Figure 7.6 when looking at the effect of sulfur fertilisation.

7.2.2 Second field trial

Due to the difficulties encountered in drying out the watercress bed in the first trial and existence of potential problems of keeping the crop dry in wet weather, using drought-induced stress to increase *in vitro* anticancer activity of watercress was not pursued further. Instead, the second field trial focused on developing the method to increase sulfur fertilisation. As the trial beds at Fobdown were unavailable, the second field trial was carried out at Doddings Farm in Bere Regis on 7th September 2010. The beds at Doddings Farm were larger and only two were available for my use, therefore in order to maximise the number of sulfur treatments, one of the beds was transplanted with 50 yd^2 strips of watercress seedlings, separated by a 25 yd^2 strip. In the separate bed, 400 g/25yd^2 of Palm Brand sulfur powder, which is 99.8% sulfur, was added as a base application prior to transplanting the watercress seedlings (Figure 7.9a and Figure 7.9b). It was decided to also try the Microthiol Special fertiliser again, but using repeated applications as well as higher application rates. Due to the cold weather the watercress was growing slowly so 4 weeks after planting one of the beds was sprayed with 42 g Microthiol Special dissolved in 2 L water, the same as the first trial, and another with 84 g dissolved in 2 L water (Figure 7.9c). Two weeks later this was repeated, see Figure 7.10 for an overview of the trial. After a further two weeks of growth, the crop was ready to harvest.

Crude watercress extracts from samples exposed to different sulfur fertilisation treatments were compared in the MTS growth inhibition assay (Figure 7.11) and Nrf2 activity assay (Figure 7.12). There was no difference observed in the ability of any of the samples to inhibit the growth of MCF7 cells or enhance Nrf2 activity. In order to determine whether the applications of sulfur fertilisation were indeed increasing sulfur in the watercress, fresh samples from each of the beds were sent to Natural Resource Management Ltd. (NRM; Berkshire, UK) for chemical analysis of total sulfur content. Unfortunately there was no difference in total sulfur content between the different treatments (Figure 7.13). As a result, it was not thought necessary to analyse biological repeats.

7.2.3 Third field trial

The third field trial was again carried out at Doddings Farm in Bere Regis on 12th April 2011. Over two beds, six 25 yd^2 strips of watercress seedlings were transplanted, separated by a 25 yd^2 strip (Figure 7.14a). Analysis of the sulfur content of watercress

a)

b)

c)

Figure 7.9: Images from second field trial. (a) Mark Newton applying a base application of sulfur powder to one of the beds. (b) Neal Thorn and Marc Miller transplanting the watercress seedlings. (c) Mark Newton applying a foliar spray of Microthiol Special.

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Figure 7.10: Overview of second field trial.
A base application of Palm Brand sulfur powder was applied to one bed prior to transplantation of four 50 yd^2 strips of watercress seedlings in gravel lined concrete beds supplied with spring water from a bore-hole at Vitacress' Doddings farm in Bere Regis. Four weeks later a low and high application of sulfur-rich fertiliser Microthiol Special was sprayed onto the watercress. This was repeated Two week later, and the watercress was harvested at eight weeks. Samples were taken from each of the top, middle and bottom sections of the beds.
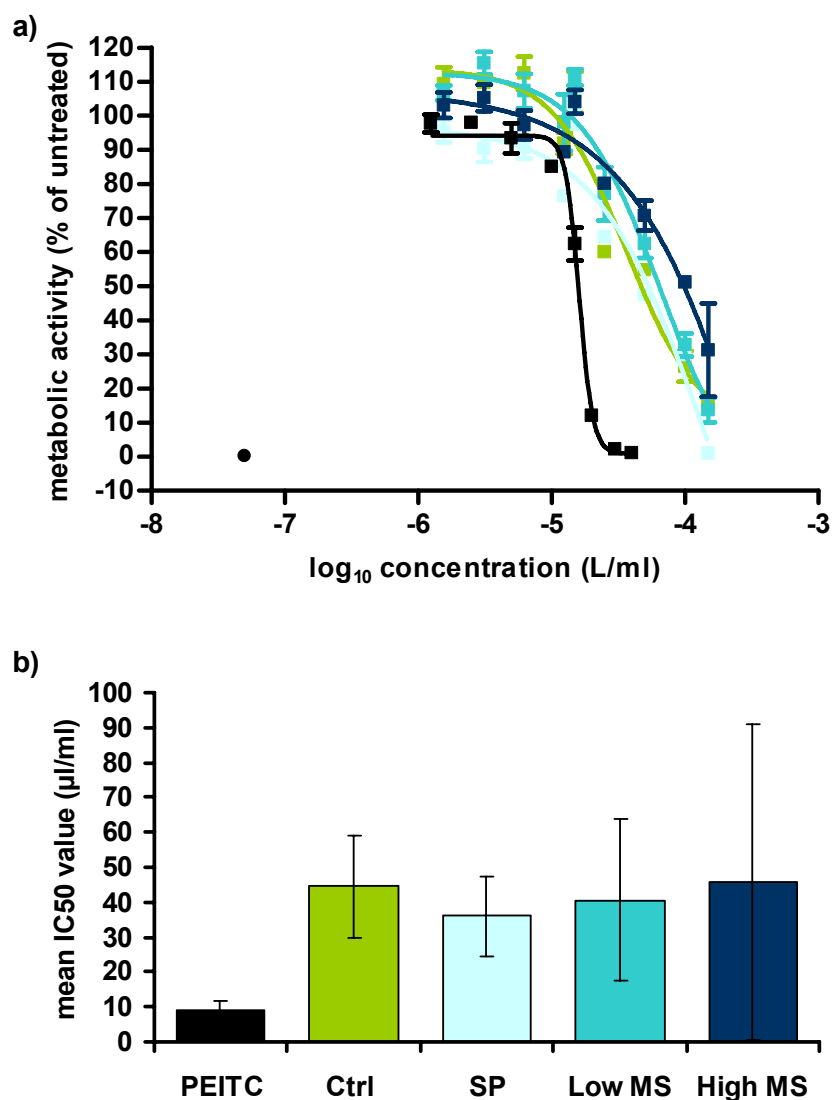
Figure 7.11: Effect of different sources of sulfur on the ability of crude watercress extract to inhibit the growth of MCF7 cells.
(a) Representative growth inhibition experiment. MCF7 cells were treated with various concentrations of crude watercress extract from control (Ctrl; ■), Palm Brand sulfur powder (SP; ■), 21 g/L/25 yd^2 Microthiol Special (Low MS; ■) or 42 g/L/25 yd^2 Microthiol Special (High MS; ■) treated beds, PEITC (■) for use as a baseline or 0.5 µM STS (●) as a positive control. Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD).
(b) Mean IC$_{50}$ values ± SD from three independent experiments.
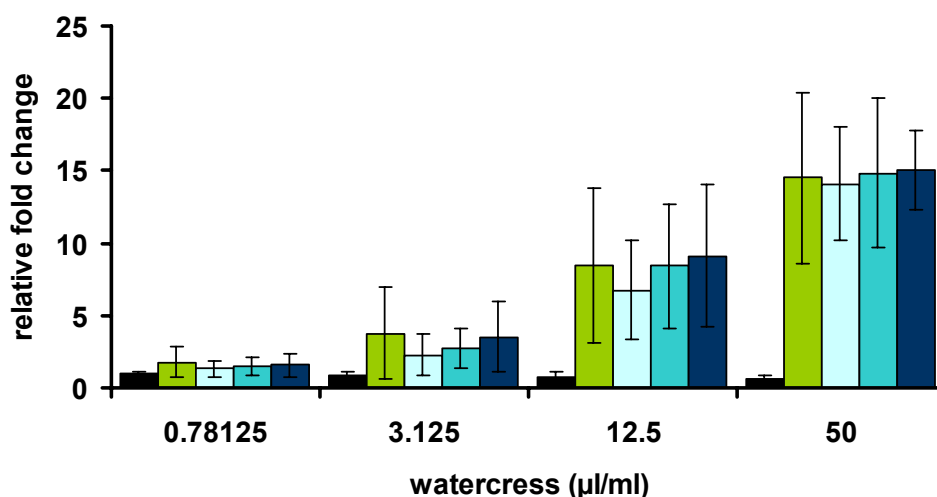
Figure 7.12: Effect of different sources of sulfur on the ability of crude watercress extract to induce ARE-driven expression.
MCF7 cells stably transfected with ARE were treated with the indicated concentrations of crude watercress extract from control (■), Palm Brand sulfur powder (□), 21 g/L/25 yd^2 Microthiol Special (■) or 42 g/L/25 yd^2 Microthiol Special (■) treated beds, or equivalent amounts of H_2O (■) as a control for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments.
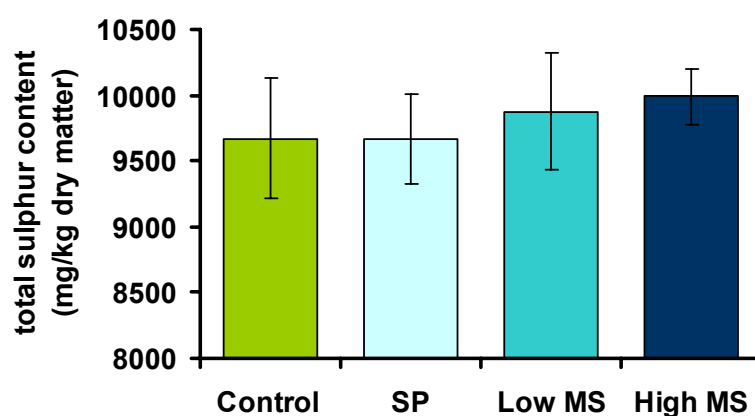
Figure 7.13: Total sulfur content of sulfur treated beds in the second field trial.
Fresh samples from control, Palm Brand sulfur powder (SP), 21 g/L/25 yd^2 Microthiol Special (Low MS) or 42 g/L/25 yd^2 Microthiol Special (High MS) treated beds were sent to Natural Resource Management Ltd. (NRM) for chemical analysis of total sulfur content. Data shown is mean (± SD) of two samples, one taken from the middle section of the bed and the other from the bottom.

samples from the previous trial revealed that sulfur treatments failed to increase sulfur concentration. As a result I decided to try sulfur from a different source, added with and without citric acid anhydrous (CA) which has been shown to increase nutrient uptake (Baldotto *et al.*, 2011). 200 g or 400 g of the sulfur rich fertiliser, SoluPotasse, containing 55.8% SO_4, 50.9% K_2O and 0.6% Cl, was applied as a top dressing to the beds in the absence or presence of 25% w/w CA one week after planting the beds (Figure 7.14b). As a control, one of the beds was treated with only the highest volume of CA. This was repeated every week for four weeks; see Figure 7.15 for an overview of the trial. After another weeks growth the crop was ready to harvest (Figure 7.14c).

The *in vitro* anticancer activity of watercress samples treated with increasing amounts of SoluPotasse, in the absence or presence of CA, were compared in the MTS growth inhibition assay (Figure 7.16) and Nrf2 activity assay (Figure 7.17). All the watercress samples inhibited the growth of MCF7 cells and enhanced Nrf2 activity, but there were no difference in their ability to do so. Again, fresh samples from each of the beds were sent to Natural Resource Management Ltd. (NRM; Berkshire, UK) for chemical analysis of total sulfur content. There was no change in total sulfur content between samples taken from the different beds (Figure 7.18).

7.3   Discussion

To investigate whether the *in vitro* anticancer activity of watercress can be increased by altering environmental factors, I introduced drought stress prior to harvesting and increased sulfur fertilisation throughout the growing period. In addition I examined whether time of day harvest influenced *in vitro* anticancer activity of watercress.

Circadian rhythms cycle through a 24 h period and are maintained endogenously by what is known as the circadian clock. However, these rhythms are synchronised to the light/dark cycle by environmental inputs such as light and temperature. In plants, many biochemical and physiological processes are regulated by circadian rhythms and confer an adaptive advantage (McClung, 2006). For example, the emission of attractant volatiles are controlled by circadian rhythms so that they occur when pollinators are at their most active (Helsper *et al.*, 1998). The influence of light and temperature on glucosinolate concentration in cruciferous vegetables suggests that glucosinolate biosynthesis may be regulated by circadian rhythms. However, I found

a)

b)

c)

Figure 7.14: Images from third field trial. (a) Neal Thorn and Mark Newton transplanting the watercress seedlings. (b) Neal Thorn applying a top dressing of SoluPotase in the absence or prescence of citric acid anhydrous. (c) After five weeks growth, the watercress beds are ready to harvest.

Figure 7.15: Overview of third field trial.
Six 25 yd^2 strips of watercress seedlings were transplanted in gravel lined concrete beds supplied with spring water from a bore-hole at Vitacress' Doddings farm in Bere Regis. One week later a low and high application of sulfur-rich fertiliser SoluPotasse in absence or presence of 25% w/w citric acid anhydrous (CA) was applied as a top dressing to the watercress. This was repeated every week for four weeks, before the watercress was harvested at five weeks. Samples were taken from each of the top, middle and bottom sections of the beds.
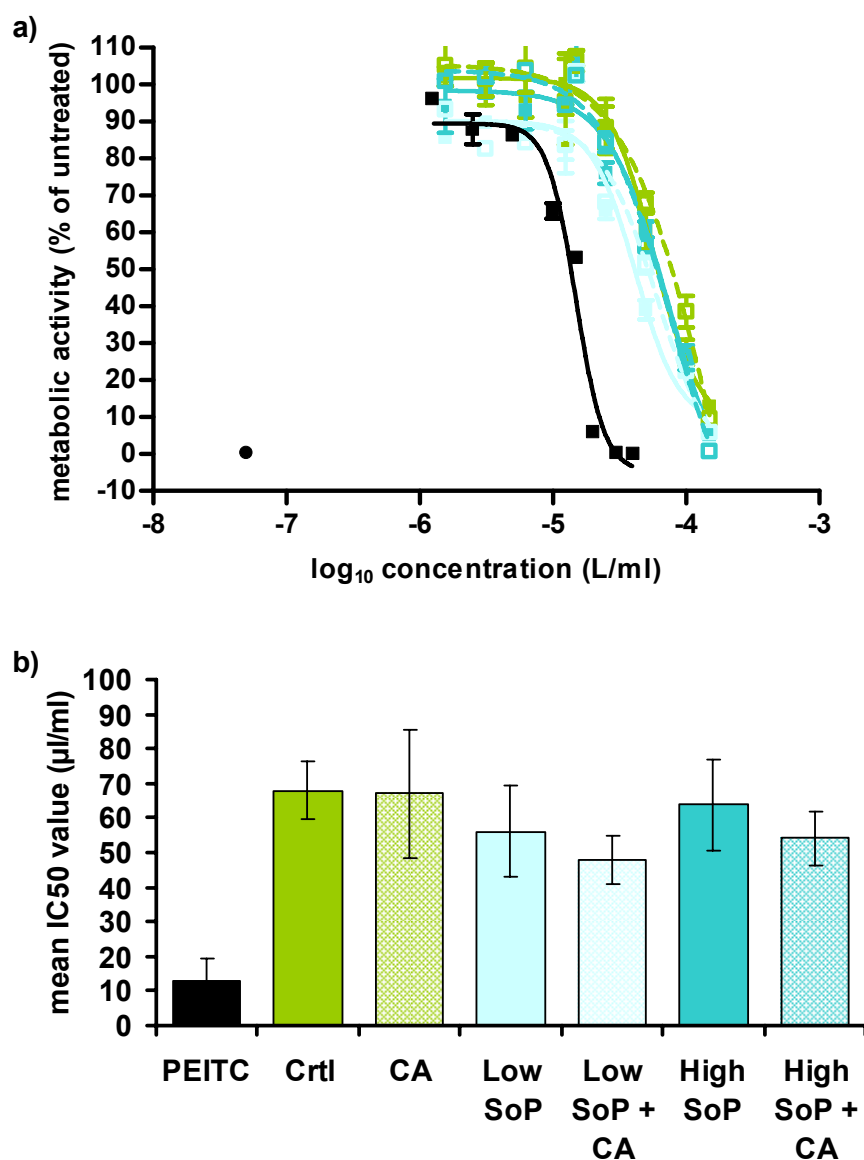
Figure 7.16: Effect of different application rates of sulfur on the ability of crude watercress extract to inhibit the growth of MCF7 cells.

(a) Representative growth inhibition experiment. MCF7 cells were treated with various concentrations of crude watercress extract from control (Ctrl; ■), 200 g/25 yd^2 SoluPotasse (Low SoP; □) or 400 g/25 yd^2 SoluPotasse (High SoP; ■) treated beds, with (solid lines) or without (dashed lines) the addition of citric anhydrous (CA), PEITC (■) for use as a baseline or 0.5 µM STS (●) as a positive control. Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96® AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). (b) Mean IC$_{50}$ values ± SD from three independent experiments.
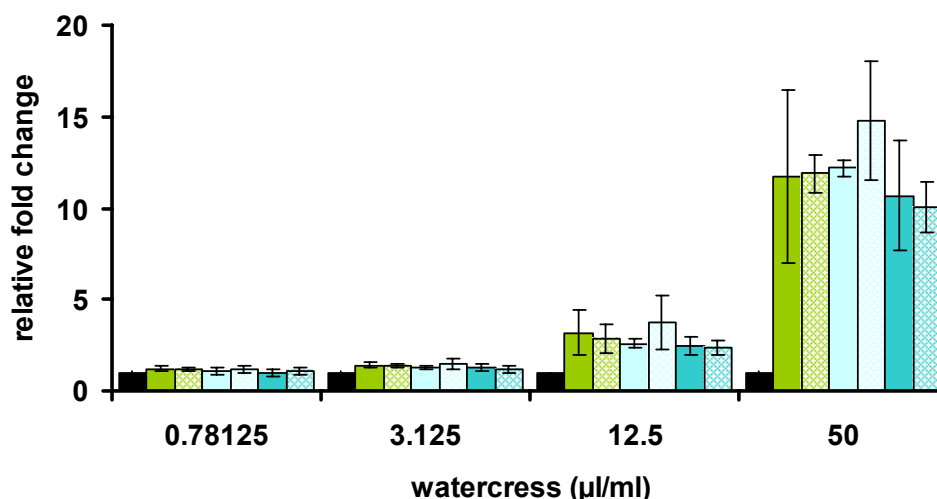
Figure 7.17: Effect of different application rates sulfur on the ability of crude watercress extract to induce ARE-driven expression.
MCF7 cells stably transfected with ARE were treated with the indicated concentrations of crude watercress extract from control (■), 200 g/25 yd^2 SoluPotasse ( ) or 400 g/25 yd^2 SoluPotasse (■) treated beds, with (solid bars) or without (hatched bars) the addition of citric anhydrous (CA), or equivalent amounts of H_2O (■) as a control for 24 h and then luciferase activity determined. Data shown is mean (± SD) of three independent experiments.
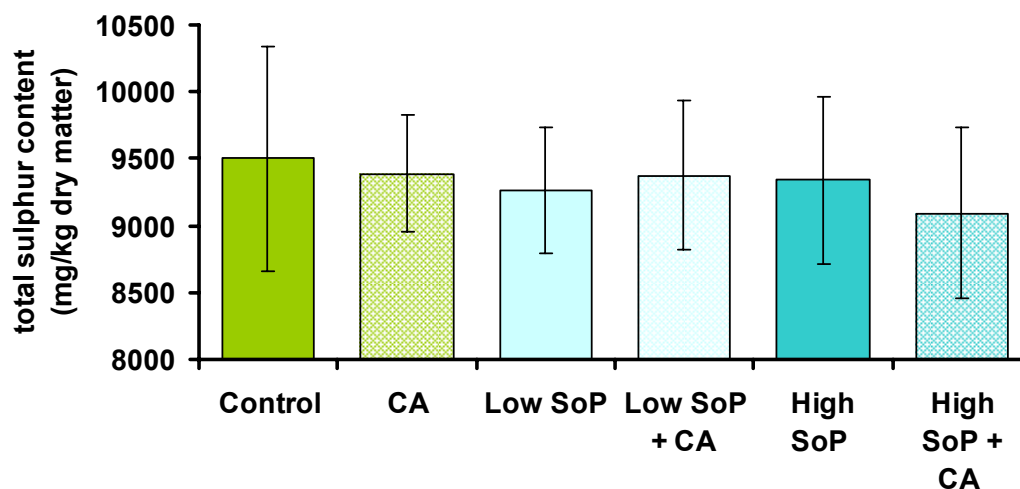
Figure 7.18: Total sulfur content of sulfur treated beds in the third field trial.
Fresh samples from control, 200 g/25 yd^2 SoluPotasse (Low SoP) or 400 g/25 yd^2 SoluPotasse (High SoP) treated beds, with or without the addition of citric anhydrous (CA) were sent to Natural Resource Management Ltd. (NRM) for chemical analysis of total sulfur content. Data shown is mean (± SD) of two samples, one taken from the middle section of the bed and the other from the bottom.

no difference in the effect of watercress samples harvested at different times throughout the day and night to inhibit the growth of MCF7 cells or enhance Nrf2 activity. Interestingly, it has been reported that transcription of genes encoding enzymes involved in glucosinolate biosynthesis are in fact circadian regulated, peaking between 21:00 - 01:00 h (Pan *et al.*, 2009). However, even if glucosinolate biosynthesis does follow a circadian rhythm in watercress this does not seem to translate to increased *in vitro* anticancer activity. There might not be a sufficient increase in glucosinolate concentration to make a detectable difference in the assays used, or despite an increase in glucosinolate concentration there might not be optimal conversion to isothiocyanates.

The production of plant secondary metabolites is known to increase in response to water stress, specifically the drought-stress induced rise in anthocyanin content in grapevine has been shown to be the result of enhanced biosynthesis (Castellarin *et al.*, 2007). It has previously been shown that water deprivation can also increase glucosinolate concentration in cultivated rapeseed (Bouchereau *et al.*, 1996; Jensen *et al.*, 1996). Glucosinolate levels may be sensitive to abiotic stress as the production of glucosinolates is believed to be adapted as a defence mechanism to biotic stresses, making the plant bitter and unpalatable to predators (Rask *et al.*, 2000). However, I found shutting off the water supply to watercress for three days to induce drought-stress had no effect on the ability of the crop to inhibit the growth of MCF7 cells or enhance Nrf2 activity. Although, it is important to note that the bed was never totally depleted of water, therefore it is unlikely that the watercress was exposed to any drought-induced stress. In future, water deficit could be confirmed by analysis of water content within the plant.

In initial attempts to increase sulfur fertilisation I used a foliar spray of Microthiol Special. This had no effect on the ability of the crude watercress extract to inhibit the growth of MCF7 cells or enhance Nrf2 activity. To determine whether this was due to an insufficient application of fertiliser in the subsequent trial I increased both application rate and concentration of Microthiol Special. I also tried a different source of sulfur in the form of Palm brand sulfur that is applied as a base fetiliser prior to transplanting seedlings. However, again none of the sulfur treatments had any effect on the ability of the crude watercress extract to inhibit the growth of MCF7 cells or enhance Nrf2 activity. Chemical analysis of samples by an outside company, NRM, revealed that the sulfur treatments failed to increase sulfur concentrations in the watercress. As a result, in the final trial I used another source of sulfur in the form of

sulfur-rich fertiliser SoluPotasse at various application rates. This was also added with and without citric acid anhydrous, which has been shown to increase nutrient uptake (Baldotto *et al.*, 2011). Nonetheless, total sulfur content within the watercress did not change following any of the treatments, and there was no change in the ability of the crude watercress extract to inhibit the growth of MCF7 cells or enhance Nrf2 activity.

It has been widely reported that increasing sulfur fertilisation can increase glucosinolate concentrations in cruciferous vegetables (Falk *et al.*, 2007; Kopsell *et al.*, 2007; Rosen *et al.*, 2005). This is not wholly unsurprising as glucosinolates are sulfur containing compounds. Most of these studies were carried out in controlled growth rooms, and of those carried out in the field, the crops were grown in soil. My failure to increase sulfur concentrations in watercress is possibly a consequence of the open hydroponic system of watercress cultivation, with any nutrients introduced being quickly leached away.

However, it should be noted that various attempts by groups to increase glucosinolate concentrations in broccoli by sulfur fertilisation have also been largely unsuccessful (Aires *et al.*, 2006; Vallejo *et al.*, 2003). This suggests that not all cruciferous vegetables may benefit from a fertilisation approach to increase glucosinolate content. Although, in the case of the study by Aires *et al.* (2006), the limited effect of sulfur (and nitrogen) fertilisation may be due to a concomitant increase in potassium chloride in the fertiliser. The authors suggest that salt-stress is responsible for the low glucosinolate levels. However, potassium has also been shown to specifically influence glucosinolate concentration. In *Arabidopsis Thaliana*, a small flowering plant often used as a model organism in plant biology, potassium deprivation was shown to increase glucosinolate content (Troufflard *et al.*, 2010). As plants deficient in potassium exhibit traits that make them more attractive to herbivores, such as elevated sugar levels, it is proposed that increased glucosinolate biosynthesis has evolved as a means to enhance the plants resistance to attack under such conditions. In addition, while nitrogen fertilisation has also been shown to increase some glucosinolates in watercress, there is an optimum nitrogen concentration before levels start to decline again (Kopsell *et al.*, 2007). This is believed to be due to the additional effect of nitrogen to increase tissue biomass. Therefore, as the rate of plant growth overtakes the rate of glucosinolate biosynthesis, the glucosinolates become diluted within the plant. This is likely to also account for why a study investigating the combined effect of nitrogen and sulfur fertilisation in cabbage, reported an increase in nitrogen to sulfur ratio decreased glucosinolate content (Rosen *et al.*, 2005).

This data taken together indicates a complex interplay between different minerals to influence overall concentrations of glucosinolates within a crop. Therefore, when applying fertilisers the impact of each component needs to be considered. Furthermore, attempts to increase glucosinolate content via nutrient fertilisation would perhaps achieve greater results by identifying an optimum ratio between multiple minerals, rather than altering a single mineral. However ultimately, with regards to watercress, a method to successfully increase sulfur in the crop has to be established before it can be determined whether this translates to increased *in vitro* anticancer activity.

Chapter 8

Final discussion

8.1   Summary

To investigate my hypothesis that the *in vitro* anticancer activity of watercress can be enhanced by altering environmental factors, I outlined four aims. The first aim was to select assays to assess the *in vitro* anticancer effects of watercress-derived compounds. This was addressed in Chapter 3. Growth inhibition and activation of Nrf2-dependent transcription were selected as candidate assays for *in vitro* analysis of crude watercress extracts.

The exact mechanism by which PEITC exhibits its anticancer activity is poorly understood, and therefore my second aim was to explore the anticancer effects of PEITC. Previously in this laboratory Wang *et al.* (2009) demonstrated that PEITC can inhibit HIF activity, therefore in Chapter 4 I investigated the effect of PEITC on HIF. The key findings are summarised below:

- PEITC inhibits the expression of a variety of HIF target genes, as well as HIF1α accumulation in hypoxia treated MCF7 cells.
- PEITC inhibits mTORC1 activity as assessed by loss of substrate phosphorylation (p70 S6K and 4E-BP1). It has previously been shown that impaired activity of mTORC1 can effect HIF1α mRNA translation (Bernardi *et al.*, 2006).
- PEITC-induced inhibition of mTORC1 is dependent on TSC1/2 activity, a negative regulator of mTORC1, but PEITC does not seem to work via any of the known pathways that control TSC1/2; PI3K/Akt, ERK, AMPK or REDD1.
- PEITC-induced inhibition of mTORC1 contributes to its growth inhibitory activity, albeit modestly.
- PEITC increases the activity of a second mTOR containing complex, mTORC2, seemingly independent of TSC1/2 and its effect on mTORC1.
- PEITC inhibits protein synthesis of HIF1α as demonstrated by ^{35}S-metabolic cell labelling.

Following on from the discovery in Chapter 4 that PEITC decreases HIF1α mRNA translation, in Chapter 5 I investigated the effect of PEITC on total mRNA translation. The key findings are summarised below:

- PEITC inhibits general protein synthesis.

- PEITC-induced inhibition of total mRNA translation is not a consequence of its inhibition of mTORC1 activity.
- PEITC increases phosphorylation of key translational modulator, eIF2α, in MCF7 cells.
- PEITC also increases eIF2α phosphorylation in MEFs, and this occurs independently of PEITC-induced inhibition of mTORC1 activity.
- eIF2α phosphorylation contributes to PEITC-induced inhibition of total mRNA translation.
- eIF2α phosphorylation confers modest protection from PEITC-induced growth inhibition.
- PEITC increases phosphorylation of another regulator of translation, eEF2, in a cell specific manner.

My third aim was to discover whether the assays selected in Chapter 3 were suitable for the analysis of a crude watercress extract. I also explored whether different watercress crops might possess distinct *in vitro* anticancer properties, as a precursor to direct agronomic experiments. This was addressed in Chapter 6. I demonstrated that a crude watercress extract can inhibit the growth of MCF7 breast cancer cells using the MTS assay, and enhance Nrf2-dependent transcription in a luciferase reporter assay. Moreover, through the analysis of two different varieties of watercress, green and red, I established that different watercress crops posses more *in vitro* anticancer activity than others. I also investigated the effect of a crude watercress extract on other important cancer pathways, shown to be modulated by PEITC in previous work. Similar to PEITC, I found a crude watercress extract inhibited both HIF activity and total protein translation. However, due to issues with practicality or assay sensitivity, neither assay was used as a means to assess the *in vitro* anticancer activity of crude watercress extracts collected from the field trials.

The final aim, which is ultimately the crux of my hypothesis, was to investigate whether altering environmental factors can increase the *in vitro* anticancer activity of watercress. This was addressed in Chapter 7. I found the *in vitro* anticancer activity of crude watercress extract was unaffected by time of day harvest, increased sulfur fertilisation, or drought-induced stress. However, experiments were likely unsuccessful due to methodology, sulfur treatments failed to increase sulfur concentrations in the watercress, and true drought conditions were not achieved.

Overall, this study had two main goals, to investigate the mechanisms underlying the anticancer activity of PEITC, and to increase the *in vitro* anticancer activity of watercress by manipulating environmental factors.

In exploring the anticancer effects of PEITC I have uncovered a novel role for PEITC in inhibiting HIF1α and total protein synthesis, and identified several key regulators of these processes, mTORC1 and eIF2α, that are modulated by PEITC. Inhibition of mTORC1 activity was found to contribute, albeit modestly, to PEITC-induced growth inhibition, while phosphorylation of eIF2α contributes in part to PEITC-induced inhibition of total mRNA translation. I also demonstrated that in response to PEITC treatment there is an increase in eEF2 phosphorylation, although this effect seems to be cell type specific therefore its biological relevance is unknown.

mTORC1 via its substrate 4E-BP1 regulates the availability of cap binding protein eIF4E for association with the eIF4F complex at the 5'cap of mRNA to initiate cap-dependent translation. It has been shown that mRNAs with complex secondary structures within their 5'UTR are more sensitive to changes in eIF4E (Koromilas *et al.*, 1992). As a result, it seems that 4E-BP1 only has translational control over a small subset of mRNAs, with many of these being involved in growth, proliferation and survival (Mamane *et al.*, 2007). This selective inhibition of translation makes the mTORC1 pathway an attractive target for cancer therapy, as cancer cells are more dependent on growth and survival signals. Indeed, treatment with the mTORC1 inhibitor rapamycin only reduces total protein synthesis by approximately 10-15% (Jefferies *et al.*, 1994). Although, while rapamycin does inhibit phosphorylation at Thr70 and Ser65 sites on 4E-BP1, it does not have any effect on phosphorylation of Thr37 and 46, which are believed to act as priming steps for further phosphorylation at Thr70 and Ser65. Despite this, Torin1, which inhibits the catalytic kinase activity of mTOR and suppresses phosphorylation of 4E-BP1 even at the rapamycin-insensitive sites, only reduces total protein synthesis by a maximum of 50% (Thoreen *et al.*, 2009). mTORC1 can also increase the protein synthesis capacity of cells by upregulating specific mRNAs (often 5'TOP mRNAs) such as those encoding ribosomal proteins. However, although this function is sensitive to rapamycin, the exact mechanism by which mTORC1 increases translation of these mRNAs remains unknown (Pende *et al.*, 2004).

I have also demonstrated that PEITC acts as a global inhibitor of protein synthesis, at least in part by inducing the phosphorylation of eIF2α. Normal and transformed cells

are likely to be equally sensitive to inhibition of total mRNA translation, and therefore this action by PEITC may prove to be toxic. As a result, it is important to appreciate the balance between selective and general inhibition of protein synthesis by PEITC. It is possible that while PEITC does inhibit global protein synthesis, a small subset of mRNAs with highly structured 5'-UTRs are particularly sensitive to decreased efficiency of translation.

Furthermore, I also found that PEITC increases the phosphorylation of Akt and ERK. These pathways are activated in response to mitogenic signalling and therefore their upregulation is often associated with promoting carcinogenesis. Nonetheless, PEITC displays many anticarcinogenic activities *in vitro* and *in vivo*, therefore the functional consequences of enhanced Akt and ERK signalling are unclear. However, it should be noted that I only looked at early time points (2-3 h), and the increase in Akt and ERK phosphorylation may only be transient, possibly the result of PEITC-induced inhibition of mTORC1 and loss of negative feedback loops. An initial increase in Akt phosphorylation followed by a more sustained decrease in Akt phosphorylation has previously been observed in response to PEITC (Jakubikova *et al.*, 2011).

However, the key question is whether normal dietary intake of cruciferous vegetables is sufficient to deliver effective concentrations of ITCs to induce these molecular changes *in vivo*. In fact, a pilot study carried out in this laboratory found that consuming a single 80 g portion of watercress was sufficient to cause a transient decrease in 4E-BP1 phosphorylation in PMBCs (Syed Alwi *et al.*, 2010). Other groups have also demonstrated that consumption of cruciferous vegetables can modulate pathways associated with anticancer effects of ITCs *in vivo*. HDAC activity was found to be inhibited in PBMCs of healthy human subjects after 3 h of consuming 68 g of SFN-enriched broccoli sprouts (Myzak *et al.*, 2007). Whereas consumption of 85 g of raw watercress once a day for 8 weeks decreased several measures of DNA damage in peripheral blood lymphocytes (Gill *et al.*, 2007).

The second goal of this study was to try to generate a watercress cultivar with enhanced anticancer activity. Even though I was unsuccessful, the above data is encouraging and suggests that efforts to increase glucosinolate concentrations in crops could indeed be beneficial. Within this study I found the open hydroponic system used in watercress cultivation makes it difficult to increase nutrient availability or introduce drought-stress. As a result, altering environmental factors may not be the best approach to increase the *in vitro* anticancer activity of watercress grown on a

large scale. Manipulation of genetics through selective breeding may be a better strategy, as used for the development of an ITC-enriched variety of broccoli (Mithen *et al.*, 2003). However, enhancing the level of glucosinolates in watercress may reduce palatability as these compounds are responsible for the hot bitter taste of cruciferous vegetables (Drewnowski and Gomez-Carneros, 2000). Furthermore, while the intention is to generate a watercress variety with enhanced anticancer activity by increasing glucosinolate concentration, there is the possibility that this could in fact cause adverse effects, especially when considering PEITC can inhibit total protein synthesis.

In addition, like 4E-BP1 phosphorylation, the molecular changes identified in this study could also be used as biomarkers to assess PEITC bioavailability. In that respect the work described in this study is also relevant in the context of clinical trials. There are currently two cancer clinical trials evaluating the potential chemopreventive/ chemotherapeutic effect of PEITC (http://www.cancer.gov/clinicaltrials). PEITC has been found to induce apoptosis in primary chronic lymphocytic leukaemia cells *in vitro* (Trachootham *et al.*, 2008) and consequently a phase I trial (NCT00968461) is planned to determine the maximum tolerated dose of oral PEITC in patients with previously treated lymphoproliferative disorders. There is also a randomised and placebo-controlled phase II trial (NCT00691132) in progress to assess the effects of oral PEITC in lung cancer in smokers. The primary objective of this trial is to determine the effect of PEITC on metabolism of the cigarette smoke procarcinogen 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK). Secondary objectives include histopathological evaluation of lung lesions and analysis of cell cycle and apoptosis biomarkers in bronchial tissue. Phosphorylation of p70 S6K and eIF2α may be useful biomarkers to monitor PEITC exposure.

8.2 Suggestions for future work

8.2.1 Identifying targets of PEITC

Phosphorylation of eIF2α is mediated by four known kinases that are each stimulated by distinct environmental and cellular stresses, HRI, PKR, PERK, and GCN2 (Wek *et al.*, 2006). The next step would be to determine if PEITC acts to increase eIF2α phosphorylation via any of these four kinases. MEF cell lines each deficient in one of the kinases already exist (Yerlikaya *et al.*, 2008) and could be used to establish whether loss of a particular kinase prevents eIF2α phosphorylation in response to

PEITC. However, considering the diverse targets of PEITC it likely activates several of the eIF2α kinases. As a result, it may be necessary to use small interfering RNAs (siRNA) or inhibitors such as hemin (HRI inhibitor) in conjunction with the MEFs, or indeed MEFs that are deficient for multiple eIF2α kinases, in order to identify which of the kinases are involved.

With regards to identifying how PEITC suppresses mTORC1 activity, this study discovered that PEITC-induced inhibition of mTORC1 occurs in a TSC1/2-dependant manner. However, PEITC does not seem to work via any of the known pathways that control TSC1/2, namely PI3K/Akt, ERK, AMPK or REDD1. It is therefore likely that at this time we have gone as far as we can go to dissect this pathway. Furthermore, following elucidation of the kinase(s) responsible for PEITC-induced phosphorylation of eIF2α, the exact mechanism of their activation by PEITC will remain unknown. The ability for PEITC to directly modify proteins is thought to be important in PEITC-mediated apoptosis and cell cycle arrest, however only a few proteins have been shown to be direct targets for ITCs. Perhaps future studies exploring the anticancer activity of PEITC, and indeed other ITCs, should employ new strategies to identify these molecular targets.

Brown *et al.* (2009) used immobilised PEITC and affinity purification to identify proinflammatory cytokine macrophage migration inhibitory factor (MIF) as a binding targets. Lysates were resolved by SDS-PAGE and bands either analysed by immunoblotting, or excised and analysed by mass spectrometry. On the other hand, tubulin was identified as a direct binding partner for PEITC by treating cells with radiolabelled PEITC ([C^{14}]PEITC). Total protein profiles obtained by coomassie blue staining 2D-electrophoresis gels were overlaid with radioactivity patterns of the gels, allowing proteins that directly conjugate with PEITC to be identified. Proteins from spots of interest can then be identified by mass spectrometry (Mi *et al.*, 2008). Using this method Mi *et al.* (2011b) identified approximately 50 potential binding targets for PEITC in human lung cancer A549 cells. However, a limitation of these approaches is that they are unable to detect transient dithiocarbamate adducts. In order to examine the cysteines modified in Keap-1 by SFN, a stable adduct was achieved by replacing the isothiocyanate group of SFN with a sulfoxythiocarbamate moiety (Ahn *et al.*, 2010). These sulfoxythiocarbamate analogs are less reactive electrophiles so the adducts formed are more stable. However, as they are structurally different from SFN caution should be taken when interpreting results. McMahon *et al.* (2010) employed a biotin switch technique to convert unstable cysteine adducts to stable biotin-cysteine

adducts, which could then be pulled out with streptavidin beads.

Breast cancer is a highly heterogeneous disease, with distinct molecular subtypes giving rise to differential responses to target therapies. The majority of experiments in this study were carried out using only MCF7 cells, particularly those examining the novel role of PEITC in inhibiting HIF1α and total protein synthesis, which represent only one molecular subclass of breast cancer. Therefore, further work should be conducted to determine whether these findings are consistent with other molecular subtypes of breast cancer. Any variations in sensitivity between the different subtypes may also provide additional insight into the molecular mechanisms behind PEITCs mode of action. Furthermore, as mutations in the regulatory pathways of both HIF1α and total protein synthesis are not specific to breast cancer, this work could also be expanded into cancer cell lines derived from other tissues.

8.2.2   Increasing the anticancer activity of watercress

As already proposed, a selective breeding strategy may be a better than an environmental approach to increase the *in vitro* anticancer activity of watercress grown on a large scale. An ITC-enriched variety of broccoli was developed by cross breeding commercial broccoli with a wild variety of broccoli containing higher glucosinolate concentrations (Mithen *et al.*, 2003).

Similar to the wild broccoli, I found crude watercress extract from the red variety of watercress was more potent at inhibiting the growth of MCF7 cells and enhancing Nrf2 activity than that from the commercial green watercress. It would be interesting to determine if this difference in potency was a consequence of a higher glucosinolate concentration. Little is known about the genetics of the red variety of watercress, but it may have similar origins to brown watercress, a hybrid between *Nasturtium officinale* and a wild watercress, *Nasturtium microphyllum*. Both are sterile and are characterised by purple/brown/red leaves (Stevens, 1983). The sterile nature of these varieties of watercress mean they can only be propagated vegetatively and are more susceptible to diseases. Indeed, the susceptibility of brown watercress to crook root disease is the reason it is no longer cultivated today. Due to disease susceptibility and coarser texture of red watercress, which makes it less appealing to the consumer, it might be beneficial to identify the genetic traits behind the enhanced *in vitro* anticancer activity of red watercress and introduce these into the genetic background of the commercial green watercress. Although as red watercress is sterile conventional cross-breeding

techniques can not be used. The relevant gene or genes would have to be inserted artificially via genetic engineering. However, it should be considered that GM crops carry negative connotations with the consumer, which may outweigh the perceived benefit of enhanced anticancer activity. Subsequently it might be more favorable to identify other wild watercress varieties which might display increased *in vitro* anticancer activity but are not sterile so can be bred using conventional techniques.

Experiments carried out during optimisation of crude watercress extract preparation, found watercress leaves are slightly more potent than stems at inhibiting the growth of MCF7 cells. Increasing the ratio of leaf to stem may therefore may be a beneficial trait for selective breeding. This could be achieved by reducing internodal length, the length of the stem between each branch of leaves, which incidentally is already part of Vitacress' breeding program as a shorter internodal length makes the crop more aesthetically pleasing. Alternatively, plants with a higher number of leaves could be selected for.

Furthermore, a limitation to examining crude watercress extracts is that the phytochemical concentrations of the 'juice' are undefined. As described in Section 6.3, total isothiocyanate content of crude watercress extracts could be quantified using a cyclocondensation assay. Alternatively, crude watercress extracts could be analysed by high-performance liquid chromatography electro-spray mass spectrometry to determine the types and concentrations of phytochemicals present.

8.3   Final comment

In addressing the hypothesis of this study, that the *in vitro* anticancer activity of watercress can be enhanced by altering environmental factors, I have demonstrated the *in vitro* anticancer activity of PEITC and crude watercress extract. In particular, I have uncovered a novel role for PEITC in inhibiting HIF1α and total protein synthesis, and identified several key regulators of these processes that are modulated by PEITC. This work contributes to the understanding of the mechanisms underlying the anticancer activity of PEITC. However, in conclusion, manipulating environmental factors does not seem to be the best approach to increase the *in vitro* anticancer activity of watercress grown in the field. As I established that a different variety of watercress displayed more potent anticancer activity than the commercial variety, in the future selective breeding techniques would be better pursued instead.

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Appendix 1

Supplementary data
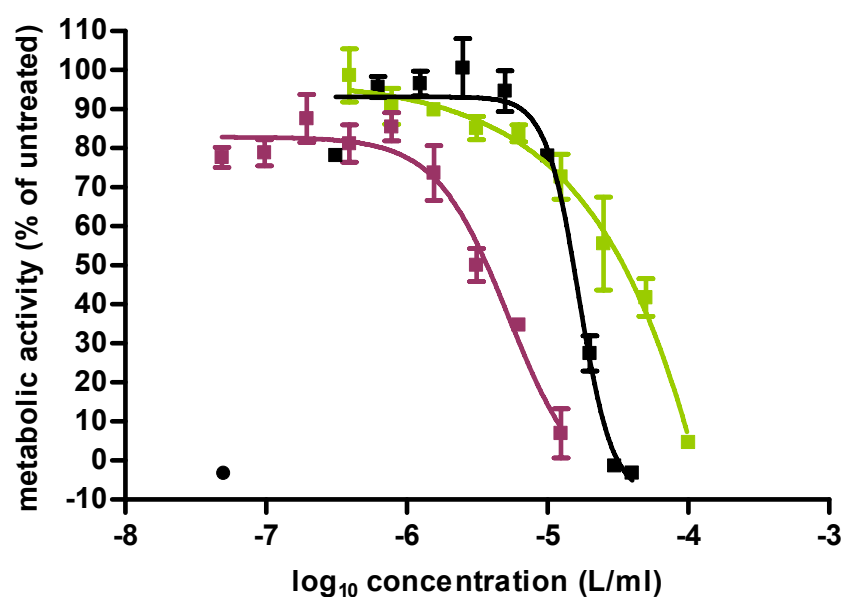
IC$_{50}$ Value (mean ± SD)		
Green Wx	**Red Wx**	**PEITC**
46.77 µl/ml ± 6.81	5.99 µl/ml ± 4.92*	15.04 µM ± 1.497

Comparison of the ability of green and red crude watercress extract, used in the experiments to analyse protein synthesis, to inhibit the growth of MCF7 cells. Representative growth inhibition experiments. Cells were treated with various concentrations of crude watercress extract either from the green cultivar (■) or the red cultivar (■) alongside the experiments analysing protein synthesis (Figure 6.6), PEITC for use as a standard (■) or 0.5 µM STS as a positive control (●).Note on the graph the units for PEITC and STS are M. After six days metabolic activity was determined by MTS assay using the CellTiter 96$^{®}$ AQ$_{ueous}$ One Solution reagent. Data shown are derived from means of duplicate wells (± SD). Table showing IC$_{50}$ values represent mean values ± SD from three experiments. A statistically significant difference between IC$_{50}$ values for green and red crude watercress extracts is indicated (* $p<0.05$).