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FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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Differential Responses of Human Breast Cancer Cells to Phenethyl Isothiocyanate (PEITC)

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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DIFFERENTIAL RESPONSES OF HUMAN BREAST CANCER CELL LINES TOWARDS PHENETHYL ISOTHIOCYANATE

By Sharifah Sakinah Syed Alwi

Phenethyl isothiocyanate (PEITC), a natural dietary isothiocyanate, has anti-cancer activity in *in vitro* and *in vivo* models. PEITC is highly electrophilic and has been shown to deplete the key cellular antioxidant glutathione, leading to an increase in reactive oxygen species (ROS). The aim of this study was to characterize the molecular effects of PEITC in human breast cancer cells. PEITC inhibited the growth of all cell lines and was more effective against the highly invasive MDA-MB-231 cell line compared to oestrogen receptor positive MCF7 cells (IC_{50} 7.18 ± 1.64 v 13.80 ± 0.89 μM respectively). Pretreatment with the antioxidant N-acetyl-L-cysteine decreased the growth inhibitory effects of PEITC, especially in MDA-MB-231 cells, suggesting that ROS are important for the growth inhibition. Consistent with this, PEITC-induced ROS accumulation was detected in MDA-MB-231 cells but not in MCF7 cells. Thus, MDA-MB-231 cells may be less capable of buffering PEITC-induced ROS, leading to enhanced sensitivity to this compound. This was confirmed by the elevated basal expression of Nrf2 in MDA-MB-231 cells while Nrf2 was induced in response to PEITC in MCF7 cells.

Molecular analysis demonstrated that MDA-MB-231 cells are more sensitive towards PEITC treatment with higher percentages of apoptotic cells induced via intrinsic pathway compared to MCF7 cells. Differential responses of PEITC effects also demonstrated in G2/M arrest in MDA-MB-231 cells and G1 arrest in MCF7 cells. However, NAC pretreatment lowered the percentages of apoptotic cells induced by PEITC, and the extent of G2/M arrest in MDA-MB-231 cells. By contrast, NAC had no significant effects in MCF7 cells. NAC also prevented a decrease in intracellular GSH levels and ROS accumulation upon PEITC treatment in MDA-MB-231 cells. By contrast, NAC had a modest effect on Nrf2 expression as well as in the levels of intracellular GSH in MCF7 cells suggesting a low level of ROS was perhaps involved in PEITC treatment. Downstream analysis demonstrated increased AKT activation does not appear to contribute to differential responses, since it occurred similarly in both cell lines, whereas 4E-BP1 phosphorylation may contribute specifically in MCF7 cells. 4EBP1 phosphorylation analysis was selected as a potential biomarker to measure *in vivo* exposure to PEITC. A small feeding study was performed and analysis of plasma and peripheral blood cells demonstrated accumulation or PEITC and decreased phosphorylation of 4EBP1 following ingestion of watercress in healthy volunteers.

These studies demonstrate that PEITC has *in vitro* anti-cancer effects in human breast cancer cell lines. Differential responses of individual cell lines appears to be linked to differential sensitivity to apoptosis, perhaps linked to the basal levels of ROS and Nrf2 in these cells. The feeding study suggests that normal dietary intake of PEITC via ingestion of watercress may be sufficient to modulate such pathways *in vivo*.

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DECLARATION OF AUTHORSHIP

I, Sharifah Sakinah Syed Alwi declare that the thesis entitled Differential Responses Of Human Breast Cancer Cells to Phenethyl Isothiocyanate (PEITC) 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:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as: *In vivo* modulation of 4EBP1 phosphorylation by watercress: a pilot study, 104[9], 1288-1296

Signed:

Date: 24th February 2012

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ABBREVIATIONS

AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor-1
AITC	Allyl isothiocyanate
APC	Adenomatosis polyposis coli
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BITC	Benzyl isothiocyanate
BRCA1	Breast cancer type 1 susceptibility
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
CARD	Caspase recruitment domain
CO_2	Carbon dioxide
CNS	Central nervous system
CV	Cruciferous vegetable
DCIS	Ductal carcinoma *in situ*
DED	Death effector domain
DFF45	DNA factor fragmentation 45
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

GSH	Glutathione
GSSG	Oxidized glutathione
GSTM1	Glutathione S-transferase mu 1
GSTT1	Glutathione S-transferase theta 1
γ-GCL	Gamma glutamylcysteine synthetase
HER2	Human epidermal growth factor receptor
HRP	Horseradish peroxidase
ITC	Isothiocyanate
JNK	C-Jun N-terminal kinase
kDa	Kilo Dalton
LCIS	Lobular carcinoma *in situ*
mRNA	Messenger ribonucleic acid
NAC	N-acetyl cysteine
NAD^+	Nicotinamide adenine dinucleotide
NF-κB	NF-kappa B
NNK	Nicotine-derived nitrosamine ketone
OD	Optical density
PARP	Poly(ADP-ribose) polymerase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEITC	Phenylethyl isothiocyanate
PR	Progesterone receptor
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylenediamine
TNF	Tumor necrosis factor
TRADD	TNFRSF1A-associated via death domain
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
VEGF	Vascular endothelial growth factor

Chapter 1

Introductions

1.1 Cancer

1.1.1 Introduction

Cancer is a leading cause of death worldwide with approximately 10.9 million cases diagnosed and 6.7 million deaths each year. There are more than 200 types of cancers with different causes, symptoms and treatments. All these types of cancer are named after the organ or type of cells in which they developed (National Cancer Institute). The most common types of cancer are lung (1.35 million), breast (1.15 million) and colorectal (1 million).

Cancers are defined as a set of diseases characterized by unregulated cell growth, invasion to surrounding tissues and metastasis to other parts of the body. The 2 main types of genes that are recognized to play role in cancer forming are oncogenes and tumor suppressor genes.

Oncogenes are mutated form of genes that contribute to the cancer development (Osborne et al. 2004). Mutation of proto-oncogene into an oncogene is due to point mutation, amplification or rearrangement which leads to the changes in the expression of corresponding protein product (Todd & Wong 1999; Weinberg 1983). Proto-oncogenes usually control normal cell growth. These genes code for proteins that facilitate the cell growth regulation and differentiation which involved in signal transduction and execution of mitogenic signals such as Ras, Wnt, Myc, Erk and Trk (Todd & Wong 1999).

Tumor suppressor genes are genes that act as negative regulators and code for antiproliferative proteins to suppress mitosis and cell growth, repair DNA errors and kill the cells when necessary (Osborne et al. 2004). Upon cellular stress or DNA damage, tumor suppressor genes will be activated to arrest the progression of cell cycle and prevent continuous replication of cells with damaged DNA. The malfunction of tumor suppressor genes causes the cells to grow uncontrollably and form cancer. Thus, significance different between oncogenes and tumor suppressor genes are oncogene is a consequence from the activation of proto-oncogene while tumor suppressor genes that cause formation of cancer resulted from the inactivation of the genes.

Though all tumors have the similarities of proliferating beyond the constraint limit of normal growth, they are diverse and heterogeneous. Tumors can be either benign or malignant. Benign tumor does not invade other parts of the body and do not metastasize while malignant tumor has the ability to invade and metastasize beyond the primary sites

through the bloodstream or lymphatic system. These abnormal behaviors are the result from a series of genetic mutations and epigenetic changes.

Epigenetic refers to heritable changes in the phenotype or gene expression caused by a mechanism that are not coded in DNA sequence itself (Baylin 2005; Feinberg et al. 2006; Rountree et al 2001). Meanwhile genetic mutation is a failure to correct DNA damage and any changes to the DNA and the chromosomes. Mutations can be induced by mutagen, a chemical agent that changes the genetic information though probably not triggering cancer formation. However, mutations can also occur spontaneously with several different forms of mutations such as point mutation which involved alteration in single nucleotide, deletion where part of gene is deleted, gene amplification which more than one copies of gene exist in a chromosome and chromosomal translocation with parts of the chromosome break and join abnormally. Mutations that occur in germ cells may be transmitted to subsequent generations, whereas mutations in somatic cells are generally of consequence only to the affected individual. As many mutagens cause cancer, mutagens are typically also carcinogens and approximately 90% of chemical carcinogens have been shown to be mutagenic such as tobacco smoke and ultraviolet.

Carcinogen refers to any substance that involved in enhancing the growth of cancer. It will interfere with the biological processes via altering cellular metabolism or damaging DNA which lead to cancer. The most common cause of genetic changes are contributed by induced mutations which involved exposure to three categories of external agents such as physical carcinogens (ultraviolet and ionizing radiation), chemical carcinogens (asbestos, component from tobacco smoke, aflatoxin which related to food contamination and arsenic) and biological contamination (infection from certain viruses, bacteria or parasites). About 10% to 20% of cancer can be caused by viruses and there are two mechanisms by which viruses causing cancer; direct and indirect (Eckhart 1998). Direct mechanism involved infection of virus in a cell and induced transformation in the infected cell by expressing its own gene that later enhance the survival and resistant of the cell. Meanwhile indirect mechanism involved virus act as a cofactor for the tumor but not necessary present in the tumor cell. Examples of the virus which promote tumor formation are Epstein-Barr virus (EBV) associated with lymphoma and human papilloma virus (HPV) that cause cervical cancer (Dalton-Griffin & Kellam 2009).

Research studies have also established the role of inherited genes as one of the significant risk factor in developing cancer. Hereditary cancer is a consequence of a gene mutation that passed down from a parent to a child. However, the carrier might not

necessarily develop the cancer but this will increase the risk factor of developing cancer. It is also well documented that most type of cancer increase progressively with age in both animals and humans (Anisimov 2007). It is suggested that aging may increase the susceptibility to the induction of new neoplasms which the growth has already exist but stayed dormant. This may due to the hormonal imbalance at later age and increased in the number of loci chronic proliferation (Ukraintseva & Yashin 2003) which later triggers the cancer formation. Moreover, cancer-prone phenotype in older age may correspond to the pathogenic effects of mutational load, dysfunctional telomeres and altered stromal milieu which explained the increasing in the vulnerability to cancer with age (Rubin 2001; Krtolica & Campisi 2002).

It has been suggested that there are six essential hallmarks of cancers that differentiate the normal and tumor cells (Table 1.1). The six essential alterations to normal cell physiology are self-sufficiency for growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless ability to replicate, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg 2011).

A tumor also has the ability to change the microenvironment which affects its growth. Two of the six cancer hallmarks that depend on the surrounding microenvironment to maintain the cell proliferation are angiogenesis and invasion and metastasis. Angiogenesis which had been postulated by Dr Judah Folkman in 1971, is defined as the formation of new blood vessels which is necessary for tumor to grow larger and metastasis. However, the factors that contribute to the creation of angiogenesis depends on the counterbalance of pro and anti-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, platelet-derived endothelial cell growth factor (PD-GF), angiopoietins and ephrins (Yadav & Aggarwal 2011). Angiogenesis will later increase the capabilities of cancer cells to invade and metastasis to distant sites and escaped the primary tumor mass where more nutrients and spaces can be found.

Table 1.1. Differences between normal cells and cancer cells from the view of six essential cancers hallmark (Hanahan & Weinberg 2011)

6 essential hallmarks	Normal cells	Cancer cells
Self sufficiency in growth signals	-growth of normal cells required mitogenic growth signals which is important for cells to move out of quiescent state to active proliferation state and this involved signaling molecules such as diffusible growth factors, extracellular matrix components and interaction within cells. -normal proliferation depends upon growth factors that usually made by one cell type to stimulate proliferation and largely instructed to grow by paracrine signals (neighbours) or endocrine signals (systemic).	-able to generate many of their own growth signals thus avoid the dependence on growth factors from other cells within a tissue.
Insensitive to antigrowth signals	-normal cells proliferation is maintained by multiple antigrowth signals that block and inhibit cell proliferation and differentiation	-avoiding all the antigrowth signals in order to survive and keep on replicating.
Evading apoptosis	-normal cell proliferation is tightly regulated by process known as programmed cell death or apoptosis.	-resistance to apoptosis and maintain cells proliferation.
Endless potential of replication	-normal cells population have limit in the growth and replication. These normal cells will stop at certain number of doublings and this process is known as senescence.	-In contrast, tumor cells have unlimited proliferation.

Sustained angiogenesis	-The survival of normal cells depend on the accessibility of oxygen and nutrients which supplied by the vasculature.	-to develop into larger, potentially metastatic of cancer cells, new blood vessels are formed termed as angiogenesis.
Tissue invasion and metastasis		-the ability of cancer cells to invade and metastasis from primary sites to distant organ enables the cells to obtain unlimited nutrients and space.

Although there were plenty of differences between normal and tumor cells, they still share the most common and essential ability to self-renew (Li & Neaves 2006). Many pathways that are classically associated with cancer may also regulate normal stem cell development. Cancer stem cells may arise from normal stem cells. Normal stem cells that were damaged due to mutation may transform into cancer cells and this lead to the uncontrollable reproduction thus forming tumors (Clarke et al. 2006). Leukemia disease was first to be identified as cancer stem cells in 1997.

1.2 Breast Cancer

1.2.1 Normal breast development

The breast is composed of many different tissue types including adipose tissue, connective tissue and glandular tissue. Each breast has 15 to 20 sections called lobes and each lobe consists of 20 to 40 lobules (Kawamura 1997; Harris et al. 2000). The lobes, lobules (milk producing glands) and bulbs are linked by ducts that lead to the nipple in the centre of a dark area of skin known as areola. Each breast also contains blood vessels that lead to the small bean-shaped organs known as lymph nodes and vessels that carry lymph (National Cancer Institute). Most of the lymphatic vessels' flow moves toward the axiliary and internal mammary lymph nodes (Turner-Warwick 1959) which located around the breast's edges, in the underarm, above the collarbone and in the chest. Axiliary lymph nodes located underarm are often the major route of regional spread in the metastasis of the primary breast cancer metastasis (Harris et al. 2000) (Figure 1.1).

Breast development occurs in several stages in a woman's life and undergoes cyclical changes throughout reproduction, menstruation and menopause (Kumar et al. 2005). These development involved combination of systemic hormones and local cellular interactions which are mediated by a variety of growth factors including epidermal growth factor, transforming growth factor beta, fibroblast growth factor and *Wnt* gene families (Snedeker et al. 1991; Liscia et al. 1990; Jackson et al. 1997; Daniel et al. 1996; Nusse & Varmus, 1992). Some of these growth regulators were reported to affect mammary cell growth. However, the involvement of hormonal cues together with the growth regulators gives effect to the development of normal glandular breast (Harris et al. 2000).

Systemic hormones had been reported play more important role at puberty with the physiological effects of estrogen in maturing the breast. The combination of both hormones estrogen and progesterone produced full ductular-lobular-alveolar development of mammary tissue (Vorherr 1974). As a consequence, the terminal ductules will form buds that precede further breast lobules (Harris et al. 2000). At the beginning of the menstruation cycle, the levels of estrogen and progesterone hormones will decline followed by apoptosis of the epithelial cells and regression in the lobules size. The breast will grow and gradually decline in size with each menstrual

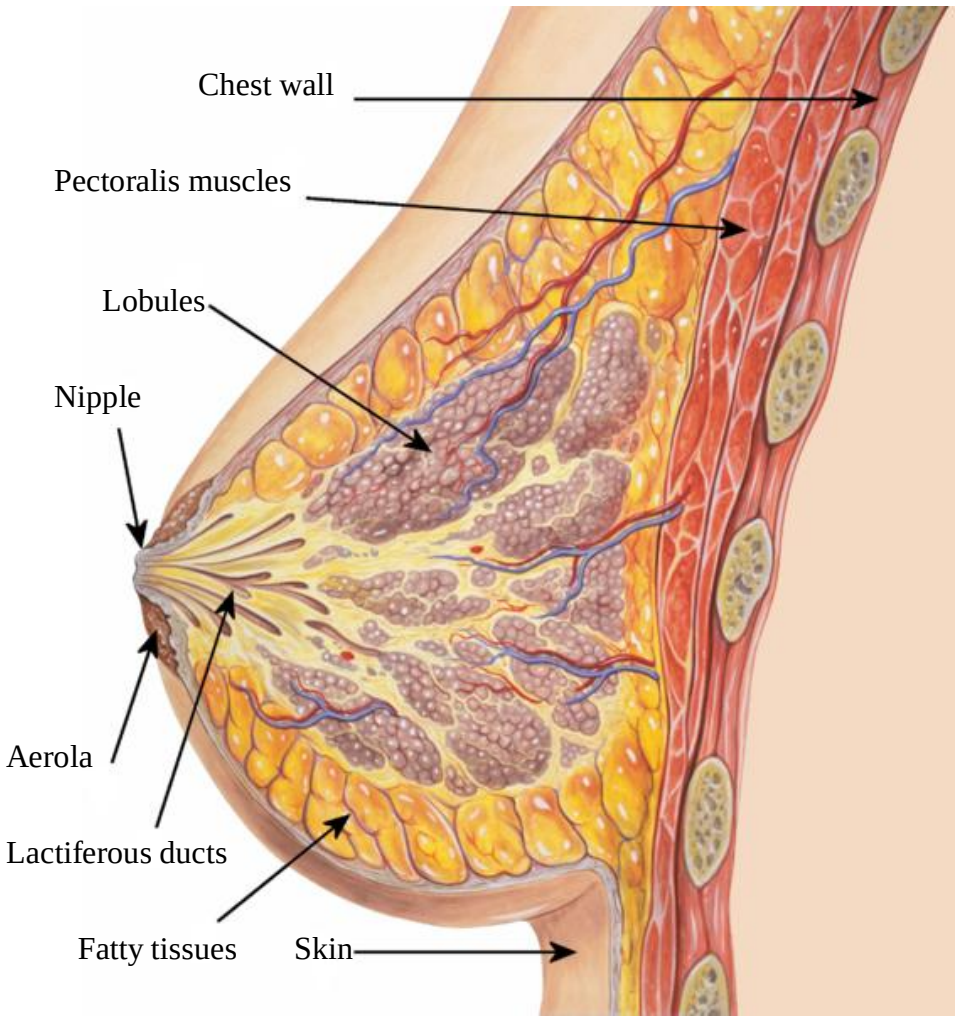


Figure 1.1. The anatomy of the normal human breast (Lynch 2007)

cycle. However, after ovulation, the cell proliferation will increase as well as vacuolization of epithelial cells and these are due to the influence of estrogen and changes in progesterone hormones (Kumar et al 2005).

The complete morphologic maturation and functional activity of the breast can be seen on the onset of pregnancy with numerous dermal glands in the aerola became more prominent and the ability of mammary epithelial cells to synthesize milk (Harris et al. 2000). These involved several hormones such as estrogen, progesterone, epidermal growth factor, growth hormone, prolactin, insulin like growth factor and parathyroid growth factor (Geddes et al. 2007). After the birth, lactation is induced due to the lower level of placental lactogen, steroid sex and progesterone (Russo et al. 2004; Kumar et al. 2005). However, after lactation process, the systemic hormones will combine with local cellular to promote involution of the mammary gland where the lobules will revert and the total breast size diminishes noticeably.

1.2.2 Cancer of the breast

Breast cancer is the most common malignancy throughout the world which afflicts millions of people especially women. This disease is very rare among men and about 140 times more frequent in women as in men. Breast cancer arises from a single neoplastic cell whose growth is not regulated either by internal or external inhibitory signals. Therefore, this gives a survival advantage to invade the surrounding cells (King 1996). There are several different forms of breast cancer including ductal carcinoma *in situ* (DCIS), lobular carcinoma *in situ* (LCIS) and invasive breast cancer.

1.2.3 Different forms of breast cancer

1.2.3.1 Histopathological view of breast cancer

The histological type, grade, tumor size, involvement of lymph nodes, estrogen receptor and HER2 expression had all been contributed to the prognostic information. Histological grading is based on the evaluation of morphologic and cytologic features of tumor cells via microscope. Moreover, it is a combination of nuclear grade, mitotic count and tubule formation with each of them scored 1 to 3 (Bloom & Richardson 1957). Therefore, the total combination of these scores is then divided into 3 groups that are graded (G) into G1

(well-differentiated and slow growing), G2 (moderately differentiated) and grade G3 (poorly differentiated and highly proliferative) (Ivshina et al. 2006). Though, the histological grading has not been accepted mainly due to the reproducibility and consistency (Elston and Ellis 1991), the classification into subtypes of tumors had been done based on the histopathological characteristic or cytological appearance or both of the tumors. It has been reported that tumor histology is significant in some cases (Stenkvist et al. 1983). There are two main histological classifications that are non-invasive and invasive

1.2.3.2 Noninvasive

Noninvasive breast cancer is also known as *in situ*. The term *in situ* is defined as the abnormal cells remain 'in place' inside the ducts where they first develop with no invasion or metastasis (Veronesi et al. 1999). Noninvasive breast cancer can be divided into two major subgroups that are lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS)

1.2.3.3 Invasive breast cancer

Invasive breast cancer is a heterogenous group and showed a wide variation in clinical presentation (Weigelt et al. 2008). The prognosis for invasive breast cancer is worse than carcinoma *in situ*. Invasive breast cancer occurs when the abnormal cells from inside the ducts or lobules invade the surrounding tissue. Although the cancer originates in the breast, it has high potential to metastasize to other parts of the body via the lymphatic or circulatory system. Histologically distribution of invasive breast cancer into several types is based on the growth pattern and cytologic features of the invasive tumor cells. About 25% of invasive breast cancers had been identified as 'special type' with special histologic features such as invasive ductal carcinoma, invasive lobular carcinoma, medullary carcinoma, mucinous carcinoma and tubular carcinoma (Tavassoli & Devilee 2003; Schnitt & Guidi 2004; Page 2003).

1.2.4 Gene expression profiling

Recently, gene expression profiling has been one of the paradigm shifts for identifying profiles of tumor subtypes in breast cancer biology (Sotiriou et al. 2006; Pusztai et al. 2009). This new emerging technology examines the composition of cellular messenger ribonucleic acid (mRNA) populations. The breast carcinoma is subdivided based on the difference in overall gene-expression profile (Kreike et al. 2007). Systematic investigation has been done on the gene expression patterns in human breast tumors which provided the basis of the molecular taxonomy of breast cancer (Perou et al. 2000).

The largest difference in overall gene-expression profile is the estrogen receptor status which represents distinct phenotype, treatment and prognosis (Sunami et al. 2008). Estrogen is an important regulator for growth and differentiation in normal mammary gland as well as in the development and progression of breast carcinoma (Gruvberger et al. 2001). The differences between ER statuses were assessed via methylation of several breast tumor related genes such as RASSF1A, CCND2, GSTP1, TWIST and APC genes that were found in higher proportion in ER positive compared ER negative tumor group.

In gene-expression profiling it has been observed that ER positive was mostly luminal-like. Luminal breast cancer is define as an ER positive breast cancer cell which tends to grow slowly and due to relatively high expression of many genes expressed by breast luminal cells (Gruvberger et al. 2001). Moreover, antibodies against the luminal cell keratin 8/18 were applied to distinguish the tumors and subdivided them into luminal A and luminal B. On the other hand, the ER negative tumors were mostly basal-like. Basal-like breast cancer is define as an ER negative breast cancer cell which is characterized by an expression of the basal or myoepithelial cells of the breast (Rakha et al. 2008). Basal-like tumors have been further subdivided into several subgroups known as molecular subtypes with gene characteristics of HER2-positive tumors, normal breast-like tissue and basal epithelial/myoepithelial cells (Perou et al. 2000; Kreike et al. 2007) (Table 1.2).

HER2 positive tumors were characterized by the high expression of genes located in the HER2 amplicon on 17q21 (Rakha et al. 2008). Normal breast-like groups were identified with the characteristic of high expression of many genes of adipose cells, basal epithelial cells and other non-epithelial cell types as well as low level expression of luminal epithelial cell genes (Perou et al. 2000). Meanwhile, immunohistochemically, basal-like tumors were characterized with ER negative, HER2 negative, expression of genes with basal epithelial cells characteristic (Rakha et al. 2008) and keratin 5/6 (KRT5/6)

and/or EGFR positive with worst prognosis (Sorlie et al. 2003). However, whether basal-like tumors and triple-negative tumors (ER-/PR-/HER2-) were synonymous or not remains unclear.

Table 1.2. The four subgroups of the gene expression profiles of the breast cancer.

Expression profiles	**Explanations**
Luminal A and B	-These luminal subtypes are the most common subtypes in breast cancer. Genetic activity of these cancers is similar to the normal lumen cells that line the breast ducts and glands. -Luminal cancers are estrogen receptor positive and usually grow slowly -The luminal cluster can be divided into two other subtypes that are luminal A and luminal B. -**Luminal A –** the phenotype which classified into the low-grade invasive cancers and has higher expression of estrogen receptor. However, luminal A is less proliferative compared to luminal B. -**Luminal B –** Luminal B has poor prognosis and more aggressive compared to luminal A.
Basal-like	-This group of carcinoma was identified by the lack of ER expression and low expression of HER2 -However, this group expressed basal-like keratin 5/6 and 17 as well as proliferation related genes and poorly identified subtypes.

HER2+/estrogen receptor (ER)-	-This group of carcinomas was identified by the overexpression of HER2/neu protein. -This group also express E-cadherin and ERBB2 and tend to grow quickly and poorly identified. -However, this group responds very well to the herceptin treatment.
Normal breast-like	-Contain normal amount of HER2/neu, lack of estrogen and progesterone receptors and expressing basal-like keratins.

In order to establish more details, DNA microarray profiling studies have been done on the breast tumors to identify subtypes of breast carcinoma cell lines. Thus, description into the insight of the molecular and biological features of 51 breast cancer cell lines had been done by Neve and colleagues. These cell lines reflect the genomic and transcriptional abnormalities that present in the primary breast tumors. In the classification by Neve et al. basal-like cell lines has been subdivided into two different clusters that are basal A and basal B. The existence of these two distinct clusters maybe due the uncontaminated cell line expression profiles with normal epithelial or stromal cells. It is also might be due to the absence of stromal or physiological interactions and/or signaling cell culture (Neve et al. 2006).

Table 1.3. Clinical and pathological features of the tumors used to derive breast cancer cell lines used in this study (Neve et al. 2006)

Cell line	Gene cluster	ER	PR	HER2	Source	Tumor type
MDAMB231	Basal B	-	-	Normal	Pleural effusion	Adenocarcinoma
BT549	Basal B	-	-	Normal	Primary breast	Invasive ductal carcinoma
SKBR3	Luminal	-	-	Amplified	Pleural effusion	Adenocarcinoma
MCF-7	Luminal	+	+	Normal	Pleural effusion	Invasive ductal carcinoma
T47D	Luminal	+	+	Normal	Pleural effusion	Invasive ductal carcinoma
ZR75.1	Luminal	+	-	Normal	Ascites fluid	Invasive ductal carcinoma

1.3 Apoptosis

1.3.1 Hallmark of apoptosis

In the steady state, cell division must be counterbalanced by cell death which is known as apoptosis or programmed cell death. Apoptosis is part of the cell development, maturation in multicellular organisms (Clavien et al. 2000) and as a homeostasis process. Apoptosis has been recognized as a tightly controlled mechanism involving cell death factors and death receptor in the control of cell proliferation (Sakinah et al. 2007). It is a major form of cell death which is characterized by molecular, biochemical and morphological alterations involving caspases activation, generation of reactive oxygen species, calcium flux and self-destruction (Fulda et al. 2010; Vander Heiden et al. 1997). The 'term' apoptosis was first introduced by Kerr and colleagues (1972) and is Greek in origin, meaning 'falling off or dropping off', in analogy to leaves falling from trees or petals from flowers (Kerr et al. 1972).

Morphologically, in the early event of apoptosis, shrinkage of cells subsequently causing decreasing in cell volume, condensation of cytoplasm and chromatin as well as losing the normal intercellular network. Following the shrinkage, chromatin moves towards the nucleus membrane and undergoes fragmentation at the linker regions between nucleosomes. At the later stage of apoptosis, extensive plasma membrane blebbing and cytoplasmic vacuolization will occur followed by separation of cell fragments via a process known as 'budding' (Elmore 2007; Fulda et al. 2010).

The fragmented cellular contents are then packaged into compact membrane-enclosed structures known as 'apoptotic bodies'. These apoptotic bodies will be phagocytosed by neighbouring cells or macrophages, preventing exposure of cytosolic contents that could initiate inflammatory response. Apoptotic cells also undergo biochemically modifications which involved alterations of proteins such as cleavage and cross-linking and DNA breakdown (Hengartner 2000) which resulted in DNA fragments into 180 until 200 base pair (Didier et al. 1996). These fragmented DNA can be visualized in the form of DNA laddering by agarose gel electrophoresis. However detection of DNA fragments via gel electrophoresis has served as a general hallmark (Burzstajin et al. 2000). In contrast to apoptosis, necrosis is an uncontrolled cell death process occur when cell exposed to the chemical or physical insults such

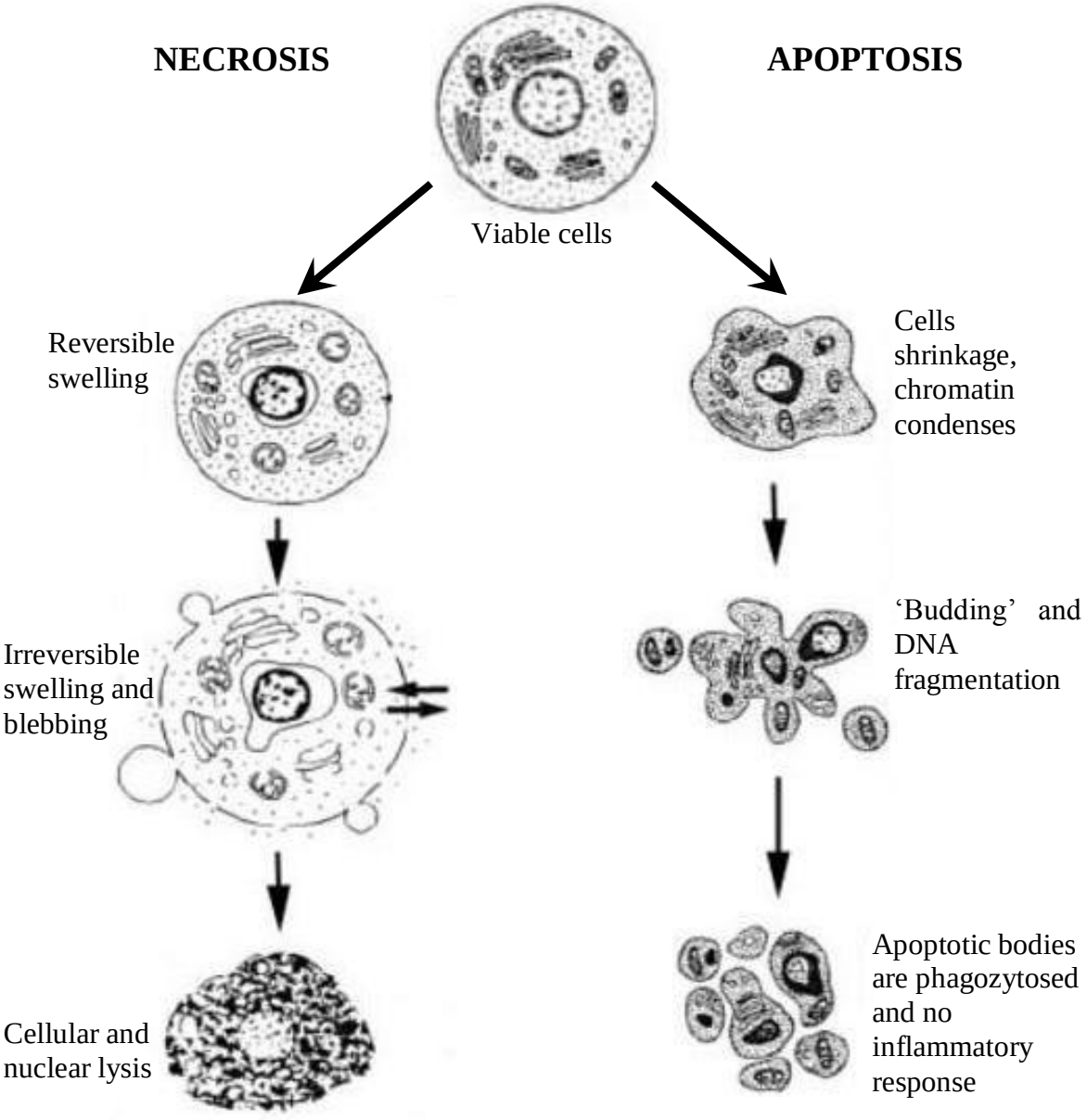


Figure 1.2. Apoptosis versus necrosis (Van Cruchten & Van de Broeck 2002).

as mechanical force, heat and cold (Zong & Thompson 2009). Necrosis is also an intracellular ATP-independent and does not involve caspases pathway (Denecker et al. 2001; Elmore 2007; Zong & Thompson 2009). There are several external factors such as exposure to hyperthermia, hypoxia, ischemia or metabolic toxins that will cause severe injury towards the cells thus lead to cell death via necrosis (Wyllie et al. 1980). The morphology of the necrotic cells can be seen clearly from the changes in the nucleus and cytoplasm structures. The cells loss the membrane integrity and swelling occurs in the intracellular organelles (Fulda et al. 2010). As a consequence, the cells lyse, releasing their cytoplasmic and nuclear contents into the intercellular environment and caused inflammatory response as well as damage to the surrounding cells (Fulda et al. 2010; Elmore 2007; Leist & Jaattela 2001).

1.3.2 Importance of apoptosis in breast cancer

The susceptibility of the mammary gland to tumorigenesis is influenced by its normal development especially during puberty and pregnancy which is characterized by the changes in the cell proliferation and differentiation. For example, after lactation there is massive restructuring and apoptosis leading to involution and a return to the primary structure (Kumar et al. 2000). The upregulation of cell proliferation or downregulation of apoptosis allow the accumulation of mutations that result in breast cancer. This will lead to the changes in the phenotype of the cell from normal to cancers through a series of malignant lesions and finally invasive disease. Apoptosis in this involution process is characterized by cellular condensation and nuclear fragmentation which occur most in the lobular unit of the terminal duct (Anderson 1999).

Involution is an essential component of the mammary gland remodeling programmed that characterized by gene products which are conditionally switched on or off during this phase of the mammary gland development. It is also accompanied by proteolytic degradation of the supporting basement membrane and the systemic reduction in hormone levels (Strange et al. 1995; Lund et al. 1996).

Moreover, in ‘premalignant’ stages, there are major alterations in apoptosis, proliferation and regulatory biomarkers of the cell cycle. In ductal carcinoma *in situ* and invasive breast cancer, apoptosis was reported to be increased (Gandhi et al. 1998; Lipponen et al. 1994). However, how normal mechanisms and signaling pathways

controlling growth and apoptosis in the human breast act in the development of tumors, in the protection from tumor development or in tumor dissemination is still unclear.

1.3.3 Molecular mechanism of apoptosis signaling pathway

Apoptosis mechanisms are highly complex and energy-dependent. Upon receiving apoptotic stimulus, cells will enter the next step where they are committed to death. This 'execution phase' of apoptosis is characterized by a series of morphological and physiological changes that finally culminate in the death of the cell. Depending on the stimulus, different signaling pathways are activated.

Evidence which has been obtained in the last few years indicating that there are many pathways and mechanisms involved in triggering apoptosis, thus avoiding the imbalance ratio of cell proliferation and apoptotic cell death. There are 2 different mechanisms which are controlled by internal signals as well as external signals from the environment that promote a cell to commit suicide via apoptosis (Elmore 2007).

1.3.3.1 Apoptosis triggered via extrinsic pathway

The extrinsic pathway is mediated by cell surface death receptors which belong to the family of tumor necrosis factor (TNF) gene included Fas (APO-1, CD95) and TRAIL-R receptors (Jin & El-Deiry 2005). Fas and TNF receptors are integral membrane proteins of which the receptor domain consists of two to six cysteine-rich extracellular domains on the cell surface (Smith et al. 1994). A cell can be committed to death by binding of a death-promoting FAS ligand to the Fas death receptors. Upon binding, Fas will be activated causing the recruitment of the adaptor molecules Fas-associated death domain (FADD) via hemophilic interaction mediated by death domain (Walzack & Krammer 2000).

FADD has another domain known as death effector domain (DED) (Cho & Choi 2002). The Fas-FADD complex then binds to and activates the protease caspase-8 via death effector domain (DED) interaction which later initiates an intracellular apoptotic signaling cascade (Cho & Choi 2002). The complex of Fas-FADD and caspase-8 is known as death-inducing signaling complex (DISC) (Jin & El-Deiry 2005; Ashkenazi 2008). The binding of procaspase-8 to DISC causing autoproteolysis thus activates procaspase-8 (Denault & Salvesen 2002) which later activates effector caspases including caspase-3, 6 and 7 which function as downstream effectors of the cell death programmed (Budiharjo et

al. 1999; Fulda & Debatin 2006). The apoptotic cells will form 'apoptotic bodies' and engulfed by neighbouring cells (Figure 1.3).

Certain receptors such as Fas/Apo-1/CD95 will transmit the signals directly to the specific apoptotic caspases. Alternatively, other receptor such as TNFR-1-TRADD complex will transmit the signals via intracellular pathway which involved NF-κB and JNK/AP-1 and the immune response system, differentiation and proliferation (Cho & Choi 2002).

1.3.3.2 Apoptotic triggered via intrinsic pathway

The intrinsic pathway is an important cell death regulator mediated by diverse apoptotic stimuli converge at the mitochondria (Lowe & Lin 2000; Jin & El-Deiry 2005). This pathway mainly involved the Bcl-2 family (Cory & Adam 2002). Thus, the ratio between pro and anti-apoptosis family protein expression levels is the control point in the regulation of apoptosis to determine the cell sensitivity towards apoptosis. The proapoptotic members of Bcl-2 family play important role in caspase activation via permeabilization of the outer mitochondrial membrane. Disruption of the outer mitochondrial membrane allowed the opening of the transition pore and releases a set of proteins that could be found in between the inner and outer mitochondrial membranes (Fulda & Debatin 2006). The first identified protein released from the mitochondrial inter-membrane space into the cytosol is cytochrome *c* which is one of the caspase activator proteins (Yang et al. 1997). However, recent studies reported there were other proteins released upon apoptosis known as Smac/DIABLO and serine protease HtrA2.Omi (Garrido et al. 2006; Cho & Choi 2002).

Subsequent released of cytochrome *c* will bind to the apoptotic protease activating factor-1 (Apaf-1) forming a complex known as apoptosome in the presence of ATP/dATP. This apoptosome complex recruit procaspase-9 in the presence of ATP or dATP thus activates it via oligomerization and initiates the executioner caspases such as caspase-3, 6 and 7 (Cho & Choi 2002) (Figure 1.3).

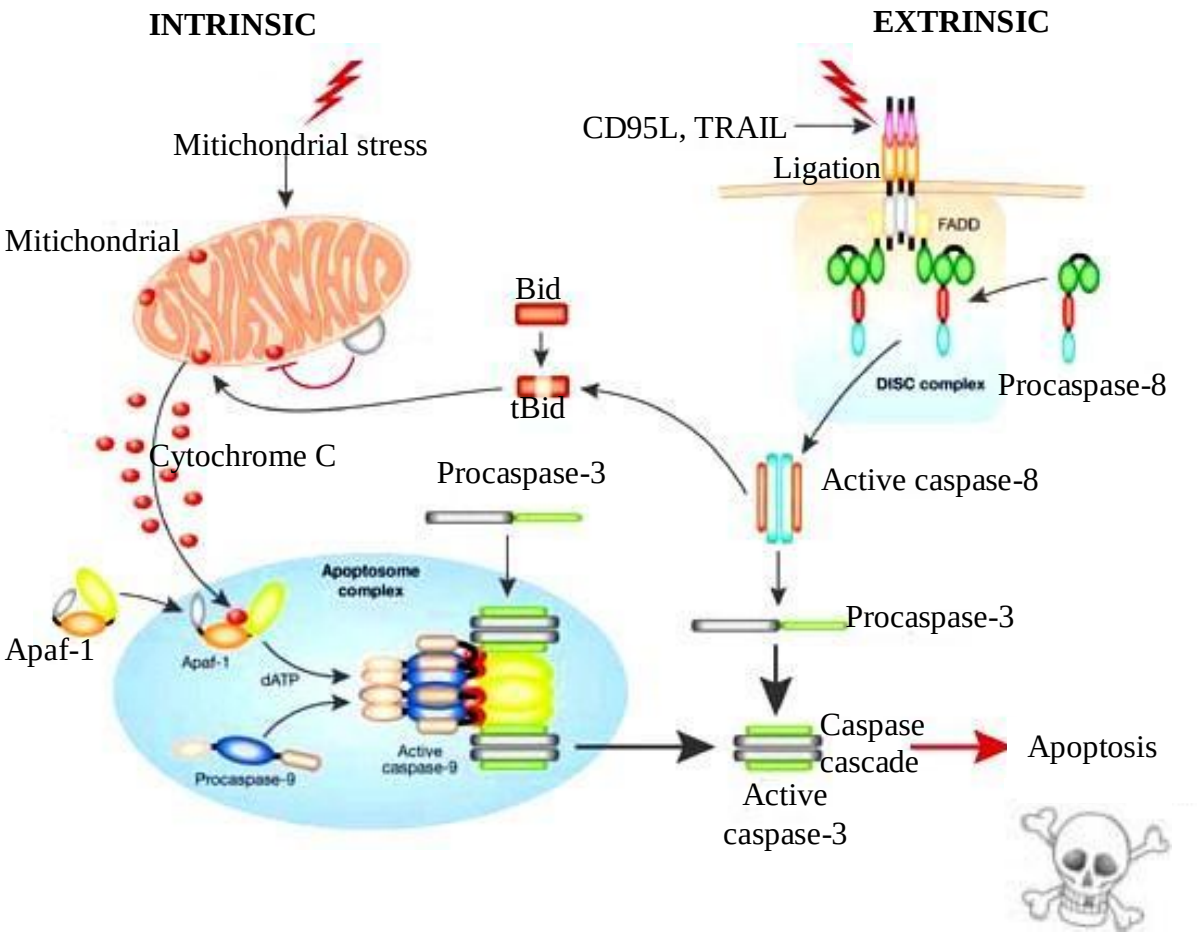


Figure 1.3. Extrinsic and intrinsic pathways involve the apoptotic regulators that later activate the caspase cascade (Adams 2003; Danial & Korsmeyer 2004).

1.3.4 The Apoptotic machinery

1.3.4.1 Bcl-2 family proteins

Bcl-2 protein is an integral membrane protein with molecular mass of ~26 kDa (Ohta et al. 1995). It was first isolated from a B-cell lymphoma that caused oncogenesis by suppressing apoptosis (Grunenfelder et al. 2002). This protein resides on the cytoplasmic surface of various organelles such as mitochondria, endoplasmic reticulum and nucleus and anchored by a hydrophobic stretch of amino acids located near its carboxyl terminus (Green & Reed 1998; Krajewski et al. 1993).

To date, about 25 genes have been identified in Bcl-2 family and these include apoptosis-promoting and apoptosis-inhibiting members which differ from the structures and functions (Elmore 2007). Apoptosis-promoting proteins including Bax and other members such as Bcl-10, Bax, Bak, Bik, Bad, Bid and Hrk are death promoters that elicit cell death by mediating cytochrome *c* release (Elmore 2007). Conversely, anti-apoptotic members including Bcl-2, Bcl-x, Bcl-XL, Bcl-XS and Bcl-w, as well as MCL-1 and Bfl-1 are potent death suppressors that promote cell survival by preventing mitochondrial disruption and cytochrome *c* release (Elmore 2007).

Bcl-2 family proteins have been reported able to form a variety of proteins interactions. The interaction between Bcl-2 itself will form homodimers and heterodimers with Bax (Yin et al. 1994). The ratio of these pro-apoptotic proteins to anti-apoptotic proteins determine the ability of these proteins to form pores in the mitochondrial membranes that allow the release of cytochrome *c* and activate caspases (Liu et al. 1996; Goldstein 1997; Kluck et al. 1997; Yang et al. 1997). The pore-forming helices are conserved in two domains that are BH1 and BH2 domains (Figure 1.4). Uniquely, each of the Bcl-2 family members shares the significant sequence homology in Bcl-2 homology (BH) domains (Annis et al. 2004) and function to promote and prevent apoptosis.

Anti-apoptotic proteins show homology in four BH domains (BH1, BH2, BH3 and BH4) while the proapoptotic proteins can be divided into ‘multidomain’ and ‘BH-3 only’ subfamilies. Multidomain proapoptotic proteins show homology in BH 1-3 domains for example Bax and Bak whereas BH-3 only proteins have a similar structure to multidomain family but the sequence similarity is limited to only BH3 domain; for example Bid and Bim (Zhang et al. 2004). BH3 domain plays important role as an

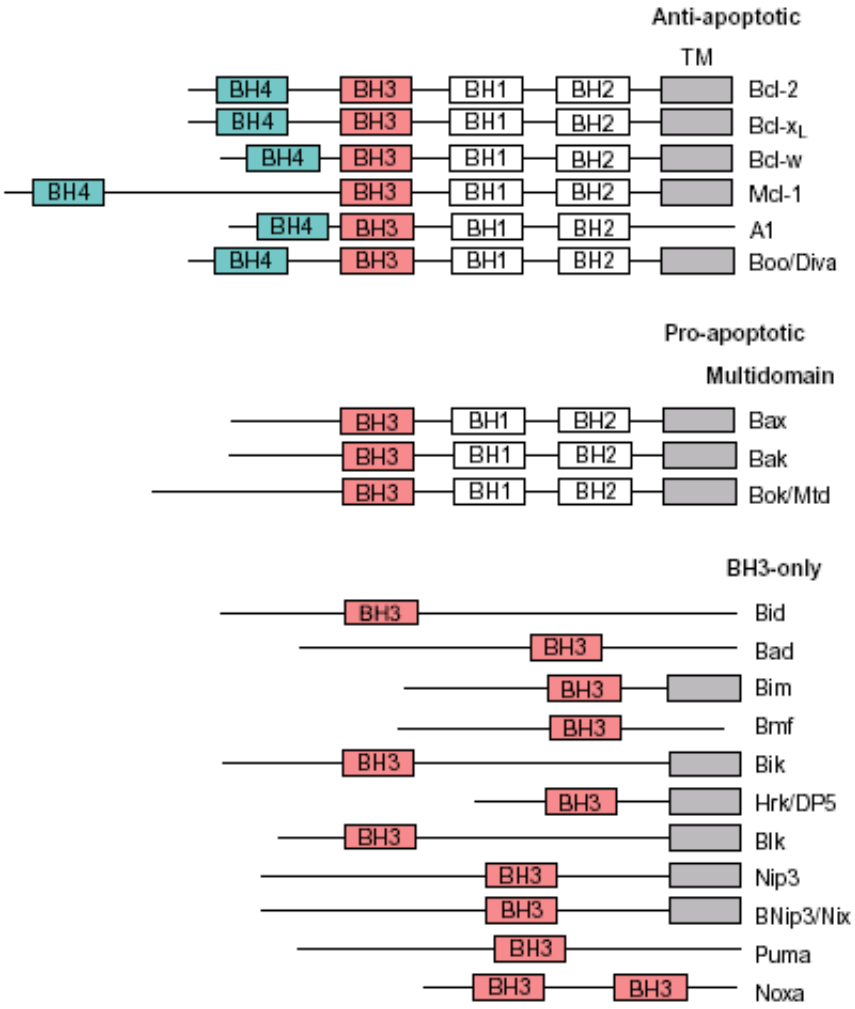


Figure 1.4. Domain structures in the Bcl-2family members. BH1, BH2 and BH3 domains are important for the physical interactions between Bcl-2 family proteins that allow the forming of homo- or heterodimer (Chittenden et al. 1995).

executioner domain and all domains are important for physical interaction especially between Bax and Bcl-2 via dimerization to allow apoptosis. High levels of BH3 domain peptides permeabilize the mitochondrial outer membrane and induce the release of cytochrome *c* (Figure 1.4) (Polster et al. 2001; 2003).

1.3.4.2 Bax, the Bcl-2 subfamily protein

Bcl-2-associated-X or Bax is a pro-apoptotic protein that belongs to the Bcl-2 family proteins (Gavathiotis et al. 2008) with molecular mass of 21 kDa. Bax normally resides in the cytosol but translocates to mitochondria upon activation to induce mitochondrial outer membrane permeabilization (Epand et al. 2002; Gavathiotis et al. 2008). Activation of Bax involves multi-step process which is highly regulated such as conformational change, mitochondrial translocation and oligomerization that leads to apoptosis (Annis et al. 2005; Gavathiotis et al. 2008). The conformational change of Bax is dependent on the exposition of C-terminal and N-terminal of Bax which are temporarily insert into the outer layer of mitochondrial membrane (Lalier et al. 2007).

Bax then oligomerizes to permeabilize membranes and induce the opening of the mitochondrial voltage-dependent anion channel (VDAC) that release cytchrome *c* and SMAC/Diabalo (Antonsson et al. 2001; Sharpe et al. 2004; Annis et al. 2005). The oligomerization of Bax is promoted by caspase-8-cut-Bid (tBid) (Eskes et al. 2000). In addition, Bax also independently promotes the movement of calcium from the endoplasmic reticulum during apoptosis that leads to the release of cytochrome *c* (Epand et al. 2002).

1.3.5 Caspases

1.3.5.1 Role of caspase in apoptosis

Proteolytic system is the core component of cell death via apoptosis programme which involved a family of proteases known as caspases. Caspases participate in a cascade that is triggered in response to pro-apoptotic signals derived from either the extracellular or intracellular milieu resulting in disassembly of the cell and ultimately, cell death (Cohen 1997; Nicholson & Thornberry 1997). These enzymes are

synthesizes as inactive proenzymes that cleave target at specific aspartate residues (Alnemri et al. 1996; Nicholson et al. 1995). This site is important for cleavage due to the existing of three amino acid residues which determine the specificity of recognition by individual caspases (Thornberry et al. 1997).

Caspases play essential role in apoptosis. It has been reported that caspase activation correlates with the onset of apoptosis while caspases inhibitors effectively attenuated apoptosis induced by diverse apoptotic stimuli. In the absence of caspase activation, tumor cells can achieve long term survival and growth (Chang & Yang 2000). Animals that lacking certain caspases exhibit profound defects in apoptosis (Cohen 1997; Nicholson & Thornberry 1997; Green 1998; Varfolomeev et al. 1998).

There are several types of caspases with different functions. Caspases 1, 4, 5, 11, 12, 13 and 14 involved in cytokine activation while caspases 3, 6 and 7 involved in apoptosis induction. Other caspases such as caspase 2, 8, 9 and 10 involved in early apoptosis process.

1.3.5.2 Caspase structure

The term 'caspase' is a short form for 'cysteine aspartate-specific proteases'. 'Cysteine proteases' because its catalytic activity depends on a critical cysteine residue within its highly conserved active site and 'aspartate-specific' due to its nature of specifically cleaving substrates after aspartatic acid residues (Lavrik et al. 2005).

Caspases are synthesized as inactive proenzymes (procaspases) and share a common structure, amino acid sequence and substrate specificity (Wolf and Green 1999). Each zymogen contains 3 domains: NH2-terminal prodomain; the large subunit of approximately 20 kDa containing the active site cysteine with a conserved QACXG motif; and the C-terminal small subunit of approximately 10 kDa. Caspases can be divided into 3 major groups that are; caspases with large prodomains that referred as inflammatory caspases, initiator caspases and caspases with a short prodomain of 20-30 amino acids known as effector caspases (Lavrik et al. 2005).

Caspase prodomains are highly variable in length and sequence and contain structural motifs that belong to the death domain superfamily (Weber et al. 2001; Martinon et al. 2001). An aspartate cleavage site separates the prodomain from the large subunit, and an interdomain linker containing one or two aspartate cleavage sites separates the large and

small subunits (Walker et al. 1994; Wilson et al. 1994; Rotonda et al. 1996; Mittl et al. 1997).

Caspases can be divided into initiator and effector caspases. Caspases with long prodomains are observed to be the upstream initiator caspases which promotes the interactions with activator molecules (Wang & Lenardo 2000; Hoffman et al. 1997). Examples of caspases with long prodomains are procaspase 8 and 10 which consist of two tandems repeat of the 'death effector domain' (DEDs) (Sprick et al. 2002) meanwhile other caspases such as caspase 1, 2, 4, 5, 9, 11 and 12 consist of 'caspase recruitment domain' (CARD) (Fuentes-Prior & Salvesen. 2004; Lamkanfi et al. 2005). Both DED and CARD known as distinct protein-proteins interaction motifs and allow caspases to be recruited once the cell receives signals. This will enable autocatalytic activation of the caspases (Lavrik et al. 2005).

However, caspase 3, 6 and 7 have shorter prodomains and has been observed to be the downstream effector caspases. The activation of these caspases depend on the upstream initiator caspases (Wang & Lenardo 2000). Proteolytic processing between domains usually results in two proteolytic steps (Thornberry & Lazebnik 1998). Firstly, the C-terminal of the protease domain is released followed by the removal of the prodomain (Wang & Lenardo 2000). The large and small subunits then associate to form a heterodimer. Two heterodimers form a tetramer with two catalytic sites that appear to function independently (Walker et al. 1994; Wilson et al. 1994; Thornberry & Lazebnik 1998).

The higher specificity of caspases allows them to perform a very limited activity and the cleavage will only occur to the selective site. Thus, caspase processing will not degrade the substrate proteins instead caspase cleavage will activate or deactivate the substrate proteins' functions.

1.3.5.3 Initiator caspases

Apoptosis process is governed by a conserved system and involved common morphological changes. Different apoptotic stimuli may trigger different initiator caspase activation. There are two major mechanisms that initiate the caspase cascade; the first mechanism initiated from the cell surface and involved caspase 8 while the other mechanism initiated from the mitochondria and involved caspase 9 which acts as the apical caspase.

1.3.5.3.1 Caspase-8

Caspase 8 involved in the Fas signaling mechanism. Fas molecules are pre-assembled into the homotrimer form in the extracellular regions (Chan et al. 2000; Siegel et al. 2000). Upon apoptotic induction, Fas ligand (cytosolic adaptor protein) is expressed and bind to the Fas receptor causing trimerization and FADD is recruited through an interaction between the Fas intracellular death domain and the FADD C-terminal death domain (Keller et al. 2009). FADD also contains death effector domain (DED) in its N-terminal region.

The exposed of DED domain in FADD is due to the aggregation of FADD which then interacts with DED domains in the prodomain of procaspase-8 and recruits procaspase-8 to Fas (Fan et al. 2005). The complex formed by Fas, FADD and procaspase-8 is known as the death-inducing signaling complex (DISC) (Ashkenazi 2008). When procaspase-8 is recruited to the DISC, autoproteolytic processing occurs and this is triggered by oligomerization (Ashkenazi 2008). The activation of downstream pathways of caspase-8 always varies with different cell types. Caspase-8 can also activate the mitochondrial pathway by truncating Bid into its active form known as tBid that leads to the released of cytochrome *c*, apoptosis-inducing factor (AIF) and other molecules from mitochondria and finally to the induction of apoptosis (Wang et al. 2005; Arnoult et al. 2003; Lu et al. 2003; Fu & Fan 2002).

1.3.5.3.2 Caspase-9

Another site that initiates intracellular signaling that mediates caspase activation following apoptotic stimuli is the mitochondria. Liu and colleagues (1996) demonstrated that a protein complex obtained from the S-100 cytosolic fraction of HeLa cells could activate executioner caspases *in vitro*. The protein complex was made up of three distinct polypeptides, which were named Apaf-1 (Apoptotic activating factor), Apaf-2 and Apaf-3 (Liu et al. 1996; Li et al. 1997; Zou et al. 1997).

Apaf-1 is a cytosolic protein and was found to be the mammalian homologue for ced-4 in *C. Elegans* (Ravagnan et al. 2002). Apaf-2 was identified to be cytochrome *c* (Aktas et al. 2006; Cho & Choi 2002). In response to apoptotic stimuli, cytochrome *c* is released from the intermitochondrial space into the cytoplasm. This suggested that mitochondria might play a central role in the mechanism of apoptosis. Apaf-3 was

identified to be caspase-9 thus suggesting that caspase-9 may function as an initiator caspase that activates the downstream effector caspases (Cho & Choi 2002).

Findings from these studies propose the following mechanism; following apoptotic stimuli, cytochrome *c* is released from the mitochondria into the cytoplasm. In the presence of ATP or dATP, cytochrome *c* in the cytoplasm induces conformational changes in Apaf-1 allowing dimerization of procaspase-9, thereby forming a complex called the apoptosome. Close proximity of procaspase-9 molecules induces autocleavage, thereby activating caspase-9 (Deneckar et al. 2001).

1.3.5.4 Effector caspases

Death commitment signals activate apoptosis via the initiator caspases. Thereon, the initiating caspases converge on the central effector caspases, caspase-3 and -7. While the initiator procaspases are activated by oligomerization, effector procaspases are often activated by other proteases, most commonly by initiator caspases (Chang & Yang 2000). In most of the models studied, caspase-3 and -7 seem to be the effector proteases responsible for the morphological manifestation of apoptosis.

1.3.5.4.1 Caspase-3

Caspase-3, a 32 kDa cysteine protease is also called CPP32 (Fernandes Alnemri et al. 1994; Nicholson et al. 1995), Yama (the Hindu god of death) and apopain (Tewari et al. 1995; Nicholson et al. 1995). Caspase-3 is one of the main executioners of apoptosis, cleaving many key substrates that contain a common Asp-Xaa-Xaa-Asp (DXXD) motif (Childs et al. 2007). Upon receiving apoptotic signals, procaspase-3 is cleaved to its active fragment (Keum et al. 2002). These signals include death receptor activation (Nagata et al. 1997), growth factor deprivation (Ohta et al. 1997), ionizing radiation (Yu & Little 1998) and chemotherapeutic agents (Mesner Jr. et al. 1997). Active caspase-3 is responsible for the proteolysis of proteins such as the inhibitor of the DNA fragmentation factor, (DFF45) and the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Jurkiewicz et al. 2004). Interestingly, caspase-3 knock out mice show major defects of apoptosis in the brain but seemed to have normal apoptosis responses otherwise (Kuida et al. 1996) suggesting the existence of the alternate pathways not requiring caspase-3.

1.3.5.4.2 Caspase-7

Caspase-7 was cloned independently in three different laboratories and named Mch3/caspase-7/CMH-1 (Fernandes-Alnemri et al. 1995; Duan et al. 1996; Lippke et al. 1996). A member of the CED-3 subfamily, it is a 303-amino acid protein most closely related to caspase-3 (75% similarity) (Fernandes-Alnemri et al. 1994; Nicholson et al. 1995; Tewari et al. 1995). Pro-caspase-7 appears to exist as dimers or higher-order oligomers (Gu et al. 1996).

Northern blot analysis revealed that caspase-7 is constitutively expressed in a variety of fetal and adult human tissues with lowest expression observed in the brain. Caspase-7, like caspase-3, preferentially cleaves PARP and the peptide substrate Ac-DEVD-AMC. Following cleavage at Asp-198 and Asp-23, procaspase-7 is activated to a form that cleaves PARP to its signature fragment of ~85 kDa in the induction of apoptosis (Chinnaiyan et al. 1996; Fernandes-Alnemri et al. 1996; Gu et al. 1996). The competitive peptide aldehyde inhibitor Ac-DEVD-CHO is also a potent inhibitor of caspase-7. As caspase-3 and caspase-7 are functionally similar and have similar substrate specificity, cleavage of PARP during apoptosis may be due to a combination of the action of both caspases (Fernandes-Alnemri et al. 1995; Cohen 1997).

It has been demonstrated *in vitro* that caspase activation requires limited cleavage segment (Thornberry & Mollineaux 1995). Active caspase-7 is made up of two subunits, similar to other caspases (Fernandes-Alnemri et al. 1996). The activity of caspase-7 is tightly regulated in mammalian cells and activation of caspase-7 requires removal of the prodomain (Duan et al. 1996). In the MCF-7 breast carcinoma cell line, overexpression of full length caspase-7 does not include apoptosis. However, caspase-7 is activated to its catalytically active subunit in cells undergoing apoptosis (Chandler et al. 1997; MacFarlene et al. 1997).

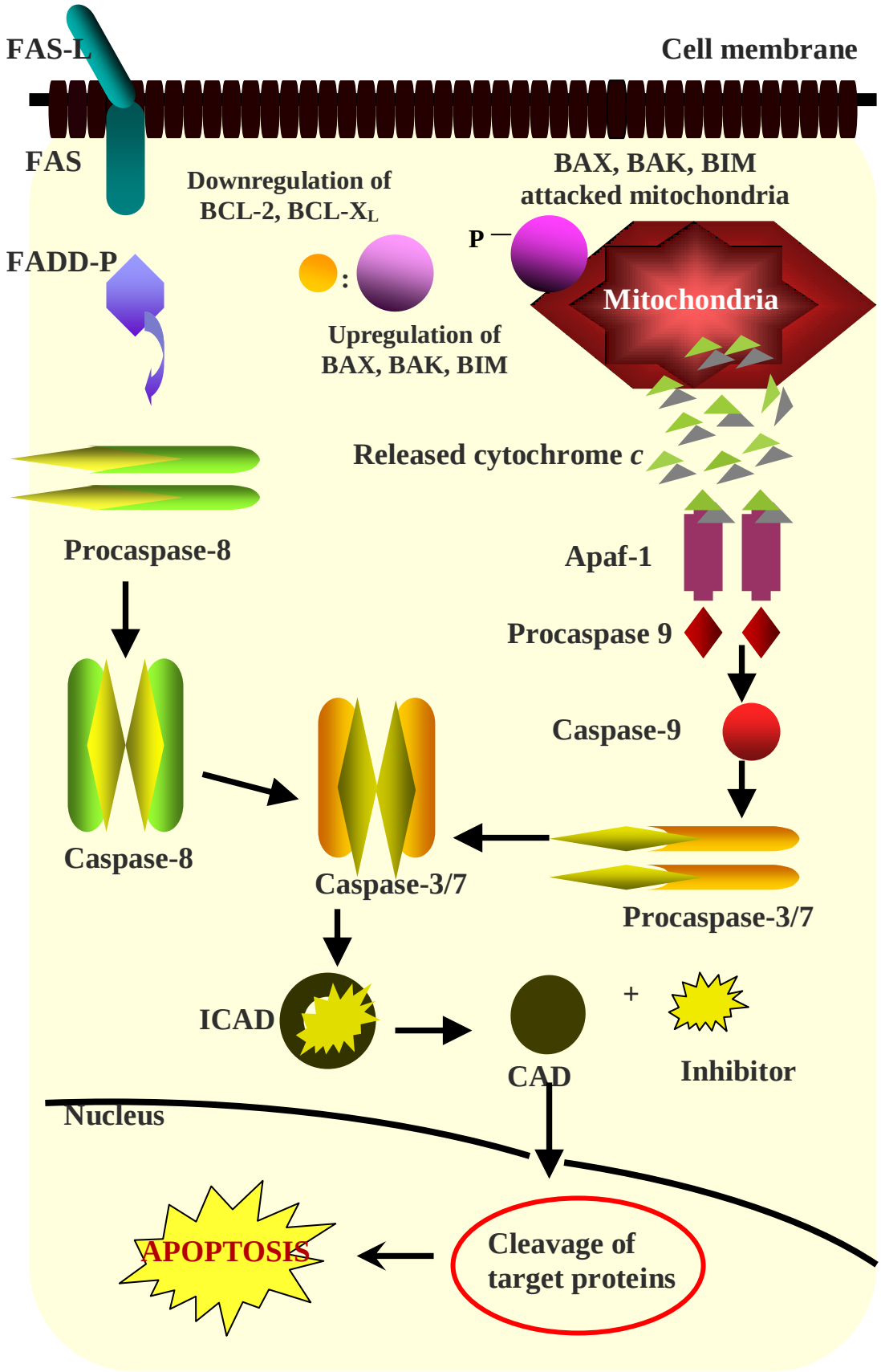


Figure 1.5. The apoptosis overview

1.4 Epidemiology of watercress

Epidemiological studies have suggested that a diet rich in cruciferous vegetables such as broccoli, cabbage and watercress is associated with reduced risk of multiple cancer types (Higdon et al 2007). The potential anticancer effect of high cruciferous vegetable intake has been linked to the presence of glucosinolates within these foods. The release of the plant enzyme myrosinase by chewing or cutting, myrosinase interacts with glucosinolates forming hydrolysis products including isothiocyanates (ITCs), indoles, thiocyanates and nitriles (Zhang et al. 2006; Wu et al. 2009). Over 100 glucosinolates have been identified which give rise to chemically distinct hydrolysis products. Following absorption, ITCs are rapidly conjugated to glutathione (GSH) via the action of glutathione-*S*-transferase (GST) enzymes and metabolized predominantly via the mercapturic acid pathway (Zhang et al. 2006; Zhang 2004).

Several studies have indicated that the potential cancer protective effects of a high cruciferous vegetable diet are modulated by sequence variations within GST enzymes, most notably GSTM1 and GSTT1 (Brennan et al. 2005; London et al. 2000; Zhao et al 2001; Spitz et al. 2000). These variants may be linked to enhanced protective effects of a high cruciferous vegetable diet via potential effects on ITC metabolism (Syed Alwi et al. 2011). The mechanisms of action of the potential anti-cancer activity of ITCs are complex and at present incompletely understood. However, alterations of carcinogen metabolism via inhibition of phase I and induction of phase II enzymes, as well as direct modulation of pathways controlling key cancer hallmarks, such as proliferation, resistance to apoptosis and angiogenesis, are thought to be involved (Syed Alwi et al. 2011).

1.4.1 Diet of Cruciferous vegetables versus cancer risk

Cruciferous vegetables are good sources of nutrients and phytochemicals and the consumption of these vegetables may decrease the risk of cancer though the protective effect might depend on the individual's genetic variation (Liu, 2004; Higdon et al. 2007). It is suggested that high dietary intake of cruciferous vegetables offer some protection against several cancers in human populations due to the present of ITCs (Gill et al. 2007). There are limited numbers of studies to evaluate the effects of isothiocyanates in human. In bladder cancer, decreased of 29% bladder cancer risk was related to the highest consumption of cruciferous vegetables (Zhao et al. 2007). However, previous

epidemiologic studies reported the intake of cruciferous vegetables prior to reducing bladder cancer risk is inconsistent. It was reported the inconsistency of the result could be due to the damage of ITCs while cooking which affected the study inconsistencies. Thus it was suggested that, by consuming raw vegetables the risk of getting bladder cancer is reduced with odds ratios of 0.64 (95% CI = 0.42-0.97) (Tang et al. 2008).

In lung cancer, several epidemiology studies have shown higher dietary intakes of cruciferous vegetables which is more than three weekly servings significantly reduced lung cancer risk in Dutch men and women (Voorrips et al. 2000), U.S women with relative risk of 0.74 (95% CI = 0.68–1.20) for more than 4 servings (Feskanich et al. 2000) and Finnish men with 32% lower risk of lung cancer compared to a population that ate one-half servings or less (Neuhouser et al. 2003). High CV intake was also reported to decrease colorectal cancer risk (Walters et al. 2004). In 1990's case-control studies, people who were diagnosed with colorectal cancer were associated with the lower intakes of various cruciferous vegetables than those without colorectal cancer risk though cohort studies have not found significant converse associations between the intake of cruciferous vegetables and the risk of developing colorectal cancer over time (Kojima et al. 2004; McCullough et al. 2003; Michels et al. 2000; Steinmetz et al. 1994). However, another prospective study on Dutch adults demonstrated significantly less developing colon cancer in both gender with highest cruciferous intake (consumption of more than 3 servings) compared to those with the lowest intake with rate ratios of 0.7 (95% CI 0.5–1.0) (Voorrips et al. 2000).

In breast cancer risk, a meta-analysis which has been carried out from 17 studies (14 case-control studies and 3 cohort studies) in association with cruciferous intake, suggested that vegetable consumption might reduce 25% of the breast cancer risk with relative risk of 0.75 (95% CI = 0.66–0.85) (Gandini et al. 2000). Nevertheless, total cruciferous intake was not significantly associated with the reduced risk of breast cancer in premenopausal and postmenopausal women even after more than 1000 g/month serving with odds ratios of 0.7 (95% CI = 0.5–1.2) and 0.8 (95% CI = 0.6–1.2) respectively (Ambrosone et al. 2004). Moreover, numerous studies reported no evidence found on the association between cruciferous consumption and menopausal status (Smith-Warner et al. 2001). An inconsistent result was also found in the epidemiological studies of cruciferous vegetables intake associate breast cancer risk. Although, lower intake of cruciferous vegetable was found in women with breast cancer compared to the cancer-free control group (Ambrosone et al. 2004; Fowke et al. 2003; Terry et al. 2001), the consumption of cruciferous vegetable was found not to be associated with the risk of breast cancer in the

analysis of seven large prospective cohort studies (Smith-Warner et al. 2001). In prostate cancer, a previous study reported the intake of more than 3 servings per week with participants from cases and controls could reduced the risk of prostate cancer with odds ratios of 0.54 (95% CI = 0.38-0.76) (Cohen et al. 2000).

Another study in human subjects reported after one and two weeks of watercress consumption of 56.8g at each meal for 3 days in 7 of 11 smokers shows an increased levels of urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*OD*-glucosiduronic acid (NNAL-gluc). NNAL is formed by reduction of NNK and NNAL-gluc that is the glucuronate conjugate of NNAL. The percentage of urinary NNAL and NNAL-gluc increased during days 2 and 3 of watercress consumption related to PEITC intake as measure by total urinary PEITC-NAC. Thus, the overall results show PEITC inhibited the oxidative metabolism of NNK in humans. Meanwhile, HPLC shows linearity and reproducibility with human plasma over a range of 49-3003 nmol/L of PEITC (Wu et al. 2009).

In addition to this, the adjustment for odds ratios included fat, energy, race, age, family history, body mass index, prostate-specific antigen tests in previous 5 years as well as education (Cohen et al. 2000). There were several more of case-control studies which associated with the low intake of cruciferous and the risk of getting prostate cancer compared to cancer-free control group (Jain et al. 1999; Joseph et al. 2004; Kolonel et al. 2000). However, there was similarity found in the epidemiological studies of prostate cancer where no consistent outcome were found to relate between the intakes of cruciferous vegetable with the reduction of prostate cancer in association with the prospective cohort studies. Therefore, several factors are to be taken into consideration since there are wide ranges of risk-enhancing factors that contributed to the epidemiological study in all cancer types such as metabolic genotypes, lifestyle which may lead to more refined understanding and better overview in epidemiological study.

1.5 Introduction to Isothiocyanates

1.5.1 Cruciferous vegetables

Cruciferous vegetables come from the family of Cruciferae with four equal-sized petals in the shape of a 'crucifer' cross (Higdon et al. 2007). Cruciferous vegetables contain considerable amounts of nutrients and phytochemical (Liu, 2004). A phytochemical is a natural bioactive compound found in fruits and vegetables potentially decreasing the risk of major chronic diseases and damage to genetic materials (Okwu 2004; Liu 2004). 'Phyto' derived from a Greek word meaning plant (Liu 2004) and usually related to plant pigments. Thus, fruits and vegetables with bright colours generally contain the most phytochemicals and nutrients.

Cruciferous vegetables (CVs) are a rich dietary source of β-phenylethyl isothiocyanate (PEITC). In addition, it is also a good source of caroteinoids such as lutein that contain oxygen and the unoxygenated beta-carotene give benefits to the maintenance of healthy skin and eyes. It contains vitamin A (from beta-carotene) and vitamin C as well as being a source of folate, calcium, iron and vitamin E. Numerous studies had reported the anticarcinogenic properties of CVs with a variety of underlying mechanisms. Some of the mechanisms include alterations in metabolic enzymes activities resulted in reduction of carcinogenicity of dietary or environmental carcinogens *in vivo* as well as reduction in the level oxidative DNA damage (Gill et al. 2007). Additionally, CVs not only reduced DNA damage in human lymphocytes ex vivo (Gill et al. 2004) but may also reduce the risk of several cancers such as lung (Zhao et al. 2001; Brennan et al. 2005), prostate (Cohen et al. 2000) and colon (Slattery et al. 2000).

CVs are rich source of a group of secondary metabolites known as glucosinolates (Keck & Finley 2004) which consists of sulfur that gave pungent aromas. Watercress in particular has been reported to contain the highest concentration of glucosinolates per gram weight of any vegetables (Gill et al. 2007). Several studies reported watercress has the ability to increase the level of antioxidants in the blood as well as protecting against DNA damage (Gill et al. 2007). Watercress is also suggests to lower the risk of prostate, colon and breast cancers (Pledgie-Tracey et al. 2007; Juge et al. 2007).

1.5.2 Chemistry of glucosinolates

One key potential class of anti-cancer phytochemical is the glucosinolates. Glucosinolates are β-thioglucoside N-hydroximinosulfates. They are water-soluble anions and belong to the glucosides which been classified in organic compounds. The skeleton of glucosinolates consists of a side-chain (R) group which characterized by a wide variety of chemical structure and a sulfur-linked β-D-glucopyranose group moiety (Fahey et al. 2001) (Figure 1.6). The highly variable R group is derived from amino acids and may be aliphatic (such as alkyl, alkenyl, hydroxyalkenyl, w-methylthioalkyl), aromatic (such as benzyl, substituted benzyl) or heterocyclic (such as indolyl). It is also variable in properties from lipophilic to mark hydrophilic. Meanwhilst the sulphate group imparts strongly acidic properties. Therefore, glucosinolates occur in nature as anions counterbalanced by a cation. The cation is usually potassium, one of the most abundant cations in plant tissues. Additionally, both sulphate group and thioglucose moiety impart nonvolatile and hydrophilic properties to all glucosinolates. Glucosinolates have a large number of homologues and ß-hydroxylated analogues.

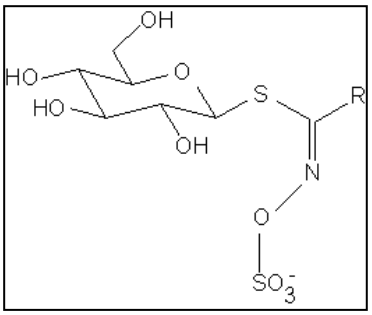


Figure 1.6 The general structure of glucosinolates

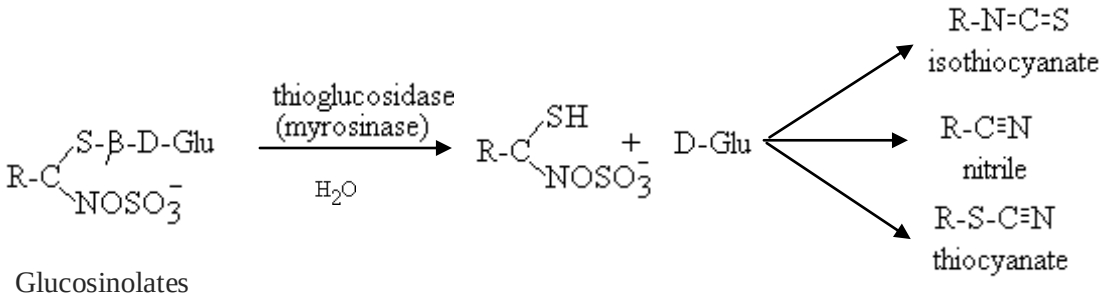


Figure 1.7 Normal products of glucosinolates hydrolysis upon tissue disruption.

Glucosinolates are chemically stable and remain in the cytoplasm. However, upon tissue disruption, glucosinolates are catalyzed by the plant enzyme myrosinase (Hayes et al. 2008) (Figure 1.7) resulting in the formation of various bioactive compounds including isothiocyanates and indoles. Myrosinase catalyse the cleavage of thioglycoside linkage, giving D-glucose and an unstable thiohydroximate-O-sulphonate (aglycone) (Hayes et al. 2008). Although indoles and isothiocyanates are derived from the degradation of glucosinolates, these two compounds appear to be structurally unrelated.

1.5.3 Isothiocyanates

Isothiocyanates are electrophiles and characterized by the presence of –N=C=S group. The biological activities of ITCs may be primarily mediated through the reaction of the electrophilic central C of –N=C=S that react with various cellular nucleophilic targets such as those which are S-based. The strong reaction of ITC with cellular thiols especially glutathione (GSH) which is the most abundant intracellular thiols causing glutathione depletion in cells (Zhang et al. 2006; Yuan et al. 2009).

ITCs are metabolized in mammals by conjugation with glutathione followed by conversion via mercapturic acid. ITCs penetrate a cell via diffusion which later conjugated rapidly with glutathione and other thiols in the cells. Conjugation of ITCs and GSH will form glutathione dithiocarbamate with or without enzymatic reaction (GST catalyze). The ITCs and GSH conjugates later undergo further enzymatic modification forming cysteinylglycine, cysteine and N-acetylcysteine conjugates which are then removed from the cells by membrane efflux pump (Zhang & Callaway 2002; Callaway et al. 2004) and

excreted in the urine. The hydrolysis of these conjugates may release ITCs that leads to the re-accumulation in cells (Zhang & Callaway 2002; Wang et al. 2009).

There are four common isothiocyanates use for cancer chemopreventive agents; phenethyl ITC (PEITC), allyl ITC (AITC), benzyl ITC (BITC) and sulforaphane. ITCs are suggested to inhibit carcinogen-induced carcinogenesis at three stages, firstly by modulating carcinogen metabolism via inhibiting the activity of phase I cytochrome P450 (CYP) enzymes. CYP involved in the activation of many environmental carcinogens and the inhibition of these enzymes depending on the isoforms such as CYPs 1A1, 1A2, 2B1 and 2B6 (Thornalley 2002). Secondly, ITCs inducing the phase II detoxifying and antioxidant gene expression such as glutathione-S-transferases, NAD(P)H:quinine oxidoreductase, UDP-glucuronosyl transferase and thioredoxin reductase resulting in the excretion of the potential carcinogens. Induction of phase II gene expression is mediated by the Nrf2 transcription factor (Itoh et al. 1997; Kwak et al. 2001; Motohashi et al. 2004). Thirdly, ITCs induce apoptosis via activation of the stress-activated protein kinase pathway mediated by sustained activation of JNK I (Chen et al. 1998; Huang et al. 1998; Rose et al. 2000). Though ITCs are known for these effects, it is not known whether they can inhibit carcinogen-induced carcinogenesis at all three stages. However, PEITC (Structure of PEITC; Figure 1.8) has been reported to have the three abilities of inhibiting cytochrome P450s, inducing apoptosis as well as inducing phase II enzymes (Wallig et al. 1998: Rose et al. 2000).

It has been reported that, ITCs contributed in the anticarcinogenic effect and significantly more potent towards cancer cells in comparison to normal cells (Gamet-Payrastre et al. 2000; Xiao et al. 2003; Musk et al. 1993). ITCs and their conjugates also have been shown to inhibit the proliferation of cultured cells (Bonnesen et al. 2001; Chen et al. 1998; Gamet-Payrastre et al. 2000). However, its effectiveness as the chemopreventive agents depends on the specific structure of the ITCs (Rose et al. 2000). Moreover, certain isothiocyanates had high potential to become chemopreventive agents for human cancers (London et al. 2000; Conaway et al. 2000; Liebes et al. 2001).

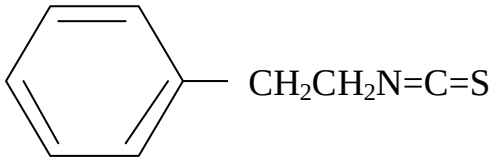


Figure 1.8 Structure of PEITC

1.5.4 Indole-3 carbinol

Indole-3-carbinol is one of the breakdown products of indole glucosinolate such as glucobrassicin at neutral pH (Plate & Gallaher 2006) (Structure of indole-3-carbinol; Figure 1.9). Similarly, upon tissue disruption, myrosinase enzyme cleaves the thioglucose bond results in the formation of a very unstable isothiocyanate. This isothiocyanate will degrade spontaneously to form alcohol indole-3-carbinol. In an acidic condition of the stomach, indole-3-carbinol can undergo several condensations and produce at least 15 different oligomeric products (Anderton et al. 2003) including indolo[3,2-b]carbazole and 3,3'-diindolylmethane (DIM) that can form dimers, trimers and tetramers. I3C also can react with ascorbic acid to form ascorbigen (Piironen & Virtanen 1962).

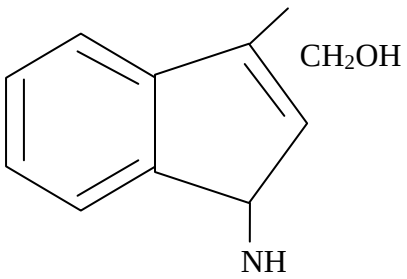


Figure 1.9 Structure of Indole-3-carbinol

1.5.5 Quercetin

Quercetin is a small molecule with slightly lipophilic (Paliwal et al. 2005) (Structure of Quercetin; Figure 1.10). It is a major bioflavanoid in common human dietary. Quercetin is reported to cause DNA damage, cell cycle arrest and has been demonstrated as a chemopreventive agent for various cancer treatment including breast cancer (Singhal et al. 1995; Choi et al. 2001), colon cancer (Salucci et al. 2002), ovarian cancer (Chan et al. 2003) and prostate cancer (Knowles et al. 2006; Nakanoma et al. 2001; Kobayashi et al. 2002). It has also been reported to induce type II estrogen receptor (ERII) expression in

both type I ER positive and ER negative. The induction of ERII in ER negative cells causes greater growth inhibition by Quercetin (Scambia et al. 1993). Moreover, the concentration of which tumor cell growth was inhibited by 50% inhibitory concentration of Quercetin (IC_{50}) has been reported to range from 7nM to just 100µM (Lamson & Brignall. 2000).

Quercetin is also known to be the most mutagenic of the flavanoids. This has been demonstrated in the Ames test, in cell culture as well as in human DNA (Bjeldanes & Chang 1977; Nakayasu et al. 1986; Duthie et al. 1997; Lamson & Brignall 2000). The mutagenicity of Quercetin lies in the formation of quinine or semiquinone. Due to the nucleophilicity of the thiol group, protein and nonprotein thiols become a major target for quinone (Monks & Lau 1998; Bolton 2002). However, mutagenicity does not always imply carcinogenicity since most studies show no carcinogenic activity of Quercetin in *in vivo* (Lamson & Brignall 2000). Though preliminary studies in animal and human suggested that Quercetin have therapeutic activity in at least certain cancers, the exact mechanism on how Quercetin exert its effect as well as types of malignancy most likely to benefit from Quercetin are still unclear (Lamson & Brignall 2000).

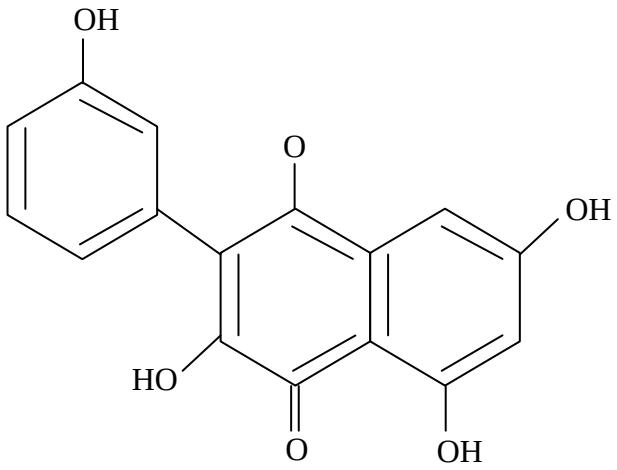


Figure 1.10 Structure of Quercetin

1.6 Importance of glutathione

1.6.1 What is glutathione

Glutathione (GSH) is a natural tripeptide with molecular weight of 307 (Sies 1999) (Structure of glutathione; Figure 1.11). GSH contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side-chain (Balendiran et al 2004). It is highly reactive with the sulfhydryl group of cysteine serving as a proton (Balendiran et al. 2004). Glutathione can be synthesized from amino acid L-cysteine, L-glutamic acid and glycine in two adenosine-triphosphate dependent steps (Figure 1.12). First step involves the synthesis of gamma-glutamylcysteine from L-glutamate and cysteine via gamma-glutamylcysteine synthetase (γ-GCL) which is also known as rate-limiting enzyme (Rahman & McNee 2000).

Second step involved the addition of glycine to the C-terminal of gamma-glutamylcysteine via glutathione synthetase enzyme (Anderson 1998; Pasternak et al 2008). Although there are several steps of control in GSH synthesis, the availability of cysteine and γGCL activity are important to be considered (Foyer & Noctor 2009). Glutathione exist in several additional forms that are reduced (GSH), oxidized form of GSH which also known as glutathione disulfide (GSSG), sulfonates which formed from further oxidation of GSSG and GSSR, a mixed type of disulfide. The free and/plus bound glutathione moieties are referred as total glutathione. The synthesized GSH will later transported across plasma membrane to be exported from the liver to the bloodstream for supply of other tissues and will be transported across the canalicular membrane for biliary excretion (Sies 1999).

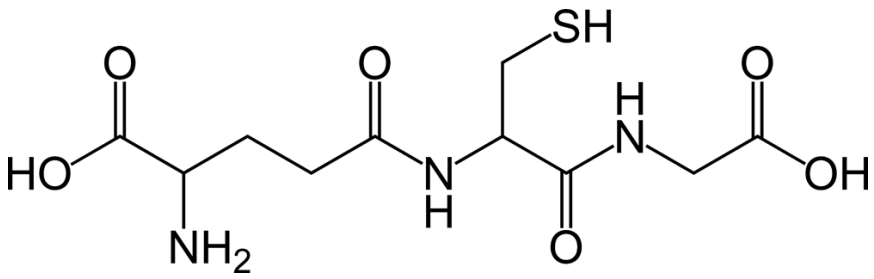


Figure 1.11. Structure of glutathione

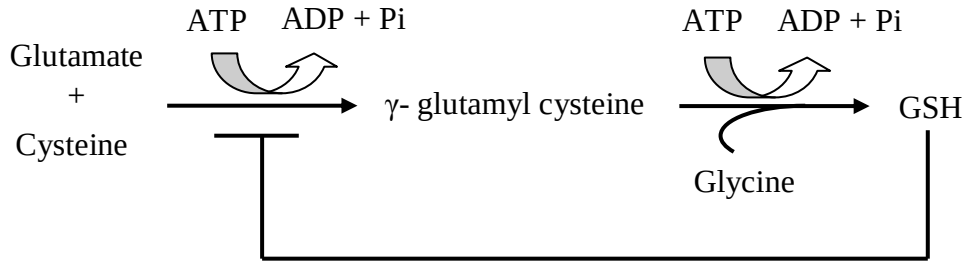


Figure 1.12. Synthesis of glutathione involved two main steps that are synthesize from L-glutamate and cysteine via γ-glutamylcysteine synthetase. Later, glycine is added to the C-terminal of γ-glutamyl cysteine via enzyme glutathione synthetase.

1.6.2 Role of glutathione in cancer

Glutathione plays critical role in cell proliferation. The increased level of GSH has been observed in various human cancer tissues such as breast (Raderer & Scheithauer 1993), colon, lung, bone marrow, ovary compared to normal tissue. It has been reported that cancer cells with higher intracellular glutathione level are resistant not only to apoptosis (Estrela et al 2006) but also to chemotherapy (Schroder et al. 1996). It is due to the important role of GSH in multi-drug resistance through spontaneous reaction or via its function as a coenzyme in glutathione-S transferase reacting with cytostatics (Meister 1991; Calvert et al. 1998; Tew et al. 1998). However, depletion of glutathione in tumor cells made them more vulnerable to the effects of anticancer drugs or agents that promote apoptosis for example, CD95 or APO-1/Fas. GSH-depletion also resulted in the reduction of cell proliferation rate which was demonstrated in human lung (Kang & Enger 1990), colon (Bernard & Balasubramanian 1997) and carcinoma as well as in rodent fibroblasts (Kang & Enger 1992; Shaw & Chou 1986).

Glutathione is also an important intracellular radical scavenger that protects cells against reactive oxygen species (ROS), redox regulation of protein thiols and maintaining redox homeostasis (Circu & Aw 2008). Cellular redox state is a crucial regulator for cell cycle (Vivancos et al. 2010) due to oxidation process that occurs in the early G1-phase of cell cycle (Menon et al. 2003). At this stage GSH intracellular level are low and the increase in total GSH is necessary for cells progression from G1 to S-phase (Kerk &

Feldman 1995). Therefore, depletion of GSH can alter the microtubule structure permitting oxidation of the sulfhydril groups in tubulin (Liebmann et al. 1993). GSH is oxidized by ROS at high rates however the relatively high concentrations of GSH dominantly contributes to cellular redox potential with more than 90% of the cellular nonprotein thiols. In oxidized state, thiol group of cysteine able to donate reducing equivalent to other unstable molecules such as reactive oxygen species. Upon donating, glutathione itself becomes reactive and reacts with other glutathione to form glutathione disulfide (GSSG). However GSSG can be converted back to GSH by glutathione reductase enzyme. Increases in GSSG relative to GSH are a useful indicator of oxidative stress or 'disulfide stress' (Vivancos et al. 2010).

The glutathione pool is maintained predominantly in reduced state due to the action of glutathione reductase (GR). GR is found in many cellular compartments and has a very high affinity for the substrates GSSG and NADPH (Vivancos et al. 2010). The NADPH comes from the oxidation of glucose-6-phosphate. The oxidation of GSH can also occur as a result of a number of enzyme-catalysed reactions that used GSH to reduce ROS for examples hydrogen peroxide to water or corresponding alcohol. GSH is also required for the regeneration of reduced ascorbate in order to maintain the exogenous antioxidant through DHA (dehydroascorbate) reductases. The reduction of DHA via GSH-dependent allows NADPH oxidation to be coupled to ROS removal via ascorbate and GSH pool (Noctor et al. 2011).

In addition to GSH's functions, the chemical reactivity of cysteine thiol group makes GSH more suitable for cells' metabolism and signaling (Foyer & Noctor 2005; Foyer & Noctor 2009). GSH also involved in the post-transcriptional protein modification via thiol-disulfide exchange as well as via S-glutathionylation. Thus, GSH exerts its protective effects towards irreversible protein modification induced by oxidation or nitrosylation by reactive nitrogen species. Moreover, GSH is important in xenobiotics detoxification via glutathione-S transferase that catalysed the conjugation to GSH and phytochelatin which contribute to heavy metal tolerance (Vivancos et al. 2010). GSH conjugates are then processed by gamma-glutamyl transpeptidase (gamma-GT) and dipeptidases to cysteine S-conjugate which later excreted in urine as corresponding mercapturic acid (Monks et al. 1990). During GSH-peroxidase-mediated detoxification of peroxides, GSSG is formed. The cellular GSH pool can be regenerated from GSSG via NADPH-dependent enzyme GSSG reductase (Figure 1.13).

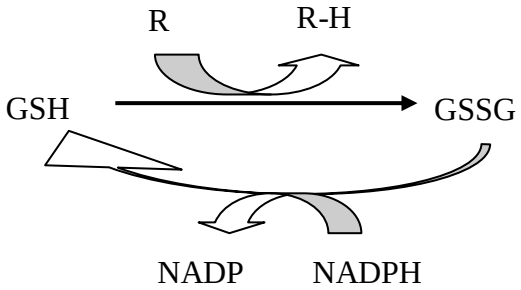


Figure 1.13. Glutathione exists in reduced and oxidized (GSSG) states. Upon oxidation of GSH which involved donation of reducing equivalent, GSH itself becomes reactive and react with other reactive GSH forming glutathione disulfide (GSSG). GSSG later converted back to reduced GSH by the constitutively active enzyme glutathione reductase.

1.6.3 Conjugation of Glutathione with Isothiocyanate

GSH is a nucleophilic and able to react with electrophilic or any oxidizing species before reacting with more critical cellular constituents such as nucleic acids and proteins. Isothiocyanates are electrophiles and can react with various nucleophiles especially GSH. Free isothiocyanate enters cells and is converted to the glutathione conjugate by glutathione S-transferases (GSTs). The glutathione conjugate is then actively secreted from cells by multidrug resistance proteins (MRPs), and metabolized in the mercapturic acid pathway. The conjugation and efflux can eventually resulted in the depletion of cellular GSH that leads to severe and impaired cellular defense that based on GSH conjugation (Figure 1.14). Electrophiles are then interacting with various critical macromolecular targets within the cell via reactive cysteine thiols (Zhang et al 2006; Mi et al 2007). Therefore, depletion of cellular glutathione and protein thiocarbamoylation activates signal transduction for cancer chemoprevention (Thornalley 2002).

Isothiocyanates had been shown to decrease the levels of intracellular GSH in several cell lines via conjugation with GSH within 30 minutes of addition (Zhang 2000). However, GSH level was increased in human ARPE-19 cells and mouse papilloma cells after 24 hours exposure to sulforaphane (SFN). Although, the level of GSH was also increased by 18% in LNCaP cells after 48 hours treatment of 15 μM SFN, GSH level was observed to decrease significantly in HL-60 cells after 24 hours treatment of 5uM PEITC.

Recent study reported that SFN significantly decreased the intracellular GSH level in HepG2 cells after 4 hours treatment and increases 2.2 fold of the control at 24 hour. Effect of isothiocyanates on the *in vivo* was observed when both of the corresponding GSH and cysteine conjugates were detected by thin-layer chromatography (t.l.c) in the kidney and liver of the rat (Brusewitz et al. 1977). The increase potencies of GSH conjugates of SFN and BITC was also observed in Hepa 1c1c7 cells with rapid accumulation of the conjugates to total intracellular (Zhang 2000).

It is therefore suggested that depletion of cellular GSH increased the sensitivity of the cells towards compounds' treatment. L-Buthionine sulfoximine (BSO) has been a major tool in glutathione research, operating on the principle of inhibition of GSH synthesis (Meister & Anderson 1983; Griffith 1999). BSO depletes GSH via specifically and irreversibly inhibiting γ-glutamylcysteine synthetase, the rate-limiting enzyme that required in the first step of GSH synthesis and therefore induces oxidative stress (Griffith 1982). Glutathione pool in cytosolic compartment often depletes more rapidly than mitochondrial matrix space and nucleus (Reliene & Schiestl 2006). Addition of BSO eventually causes GSH depletion in all tissues including developing embryos. Moreover, BSO pretreatment enhances radiation toxicity and drugs (Meister 1991). It has also been reported the administration of BSO causing multi-organ failure and death in newborn rats (Martensson et al. 1991; Meister 1995). In *in vitro* experiment, BSO was reported to decrease 70% of cellular GSH level when treated with 100 μM and 500μM of BSO in HepG2 cells (Mari et al 2002). Pretreatment with 0.5 mmol/L BSO for 24 hour increased the sensitive of HCT116 cells towards apoptotic induced by 40 umol/L of PEITC treatment. BSO pretreatment also decreased cell viability by 83% and increased LDH leakage to 84.9% when treated when treated with PEITC (Rose et al. 2005).

By contrast, the presence of N-Acetylcysteine promotes the uptake of cysteine as well as being a precursor to a reduced GSH and an important antioxidant (Gregory & Kelly 1998). Therefore, NAC supports the synthesis of GSH when it is increasingly needed. These results were supported by the previous study with NAC exerted the protective effects via promoting the synthesis and metabolism of glutathione as well as restricting the biotransformation of carcinogenic compounds into more toxic compounds (DeFlora et al. 1985). In the present of isothiocyanate, the NCS residue of ITCs reacts with nucleophilic centers of thiols such as in GSH and NAC leads to the formation of N-substituted mono-dithiocarbamate conjugates. These interactions alter the intracellular redox state and activate phase II detoxification enzymes or apoptotic signaling pathways (Rose et al.

2005). In the *in vitro* experiment for example, SFN-NAC conjugate had little effect on the GSH level in HepG2 cells. The difference in the changes of GSH cellular level by SFN and SFN-NAC may be related.

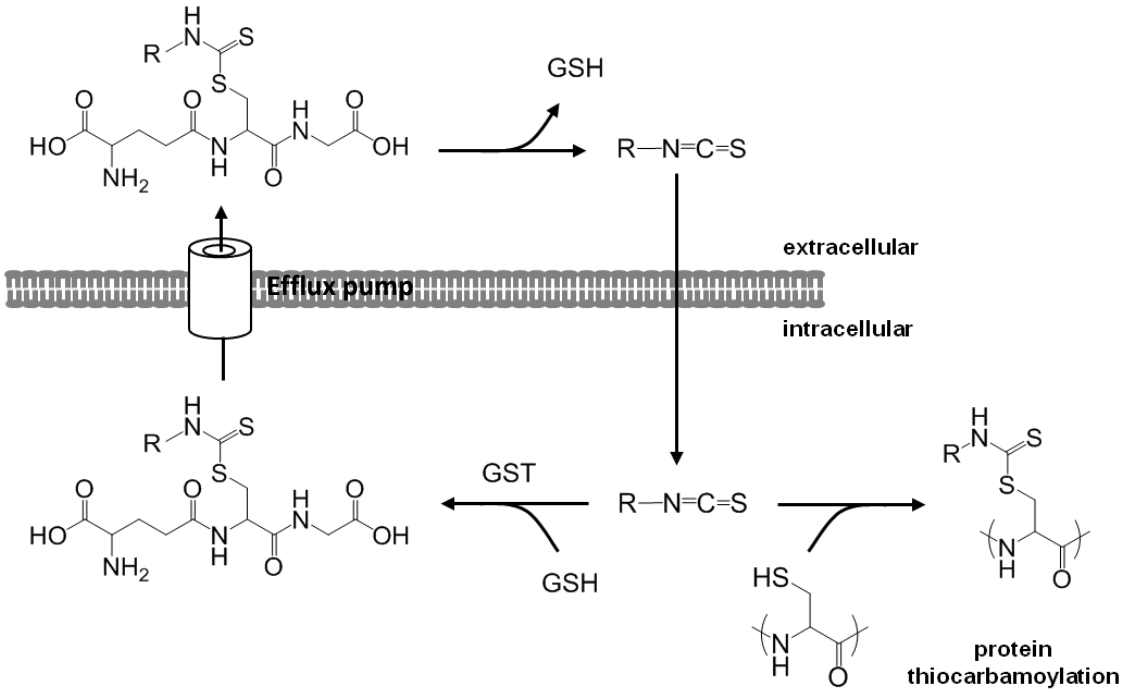


Figure 1.14. PEITC penetrates a cell via diffusion and conjugated rapidly with intracellular thiols via GST enzyme. The ITCs conjugates later undergo enzymatic modification and removed from the cells via membrane efflux pump. Hydrolysis of these conjugates may release ITCs that leads to the re-accumulation of ITCs in cells.

1.7 Anti-cancer effect of ITCs

1.7.1 Evidences of Isothiocyanate exert anticancer activity

1.7.1.1 *In vitro* Effect

ITCs had been reported to have high potential to become chemopreventive agents for human cancers (London et al. 2000; Conaway et al. 2000; Liebes et al. 2001). Therefore, rapid accumulation of ITCs in all human cultured and animal cells enables ITCs and their conjugates to inhibit the proliferation of cultured cells and tumorigenesis in lung, stomach, colon, liver, esophagus, bladder and mammary glands (Zhang & Talalay 1994; Hecht 1995, 1999; Conaway et al. 2002). However, ITCs were found to be more potent and sensitive towards blood cancer cells compared to epithelial cancer cells and far less potent towards normal cells (Gamet-Payrastre et al. 2000; Xiao et al. 2003; Musk et al. 1993).

It has also been reported that exposure of cells to ITCs such as BITC, AITC and PEITC for only 3 hours is long enough to inhibit cell growth with irreversible interaction of these ITCs (Zhang et al. 2003; Tang & Zhang 2004). In addition to this, treatment of human leukemia HL60/S cells with 10 μM of either AITC or BITC for 3 hours able to activate caspase-8 and -9 at the end of 3 hours treatment and caspase-3 activation was observed after another 3 hours which relies on the activation of caspase-8 or -9. BITC also was found to activate caspase-8 and -9 as well as caspase-3 in MDA-MB-231 cells at concentration of 2.5 μM. However, PEITC was not activating caspase-8 in human ovarian cancer OVCAR cells, instead PEITC activated caspase-9 and caspase-3 which certainly suggested the induction of apoptosis was via the intrinsic pathway. Meanwhile in human breast cancer MCF-7 cells, PEITC was found to activate caspase-7 and -9 which later lead to apoptosis (Lee & Cho 2008).

The activation of caspase-9 which was due to the leaking of cytochrome *c* will bind to Apaf-1 to form apoptosome complex. The whole intrinsic pathway will later focus on the cascade caspases. Caspase -9 will eventually activating caspase-3 and caspase -7 and lead to the digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA and phagocytosis of the cell. However, apoptosis induction in human bladder cancer UM-UC-3 cells by PEITC at 15 μM concentration with 24 hours treatment was not only via caspase-9 but also involved caspase-8. Thus, this indicates that PEITC able to induce

apoptosis via both pathways and this might depends on the cancer cell types (Satyan et al. 2006).

Moreover, induction of apoptosis imposed by ITCs was not only time and concentrations dependent but also depends on the sensitivity of the cells towards ITCs treatment. In HL60/S cells there were no increases in the number of apoptotic cells at 2 or 10 μM exposures to ITC within 3 hours. Nevertheless, at longer hours treatment (24 hours), a significant increase of apoptotic cells up to 4.9- and 3.2-fold of 2 or 10 μM of BITC and 0.8- and 4.4-fold of AITC respectively was observed (Zhang et al. 2003). In contrast, at 24 hours exposure to 12 μM AITC, human colon cancer HT29 cells displayed less apoptotic cells with 90% of cells remained healthy and only less than 10% of cells undergoing early apoptosis (Smith et al. 2004) while, morphologically in HT29 cells treated 15 μM sulforaphane at longer hours (48 hours) showed more cells undergoing apoptosis with the binding of annexin V fluorescent to the plasma (Gamet-Payrastre et al. 2000). In human non-small cell lung carcinoma A549 cells, induction of apoptosis was increased prior to PEITC's dose dependent manner. Though no percentage recorded, higher intensity of annexin V observed in A549 cells after treated with 10 μM of PEITC suggesting that cells had undergone apoptosis.

However, at 25 μM of PEITC, the intensity of PI staining was more apparent indicating A549 cells were in the late apoptosis or necrotic (Kuang & Chen 2004).

It was observed that, inhibition of cancer cell growth is due to the high potential of ITCs not only in inducing apoptosis but also in causing cell cycle arrest (Zhang et al. 2006). Current study demonstrated 48 hours treatment of BITC and PEITC strongly inhibited the growth of human adenocarcinoma Caco-2 by causing the cells to accumulate gradually in late G2/M phase (Visanji et al. 2004). Similarly, sulforaphane, AITC and BITC were also found to induce cell cycle arrest at G2/M phase in human colon cancer HT29 cell line, prostate cancer and both human breast cancer, MCF-7 and MDA-MB-231 cells respectively (Gamet-Payrastre et al. 2000; Xiao et al. 2003; Xiao et al. 2006). In human prostate cancer PC3 cells, sulforaphane was found to significantly increase the G2/M phase arrest at 20 μM concentration and reduced the G2/M arrest at 40 μM with an increase observed in G0/G1 phase indicating the cells undergoing apoptosis and irreversible effect of sulforaphane (Singh et al. 2004). Thus, this shows the cell cycle arrest in certain types of cancer cells might depend in a dose and time-dependent manner.

Table 1.4. The effects of ITCs in *in vitro*

ITCs	**Cell type**	**IC_{50} (μM)**	**Assay/Notes**	**Refferences**
PEITC	-human promyelocytic acute leukemia HL60/S cells -human myeloma 8226/S cells -human breast cancer MCF-7 cells -human hepatoma HepG2 cells -human colon cancer HT29 cells -transformed human epidermal HaCat keratinocytes	3.6 ± 0.4 3.1 ± 0.3 11.0 ± 1.2 11.2 ± 2.7 9.6 ± 1.1 6.1 ± 1.0	MTT assay -PEITC and BITC able to exert its anti-proliferative effect in these panel of 6 cancer cells at earlier time point after only 72 hours exposure with IC_{50} less than 20 μM -AITC and BITC appear to be more potent towards the HL60/S after 3 hours treatment compared to other tested ITCs (IC_{50} = 3.3 ± 1.1 μM and 2.0 ± 0.3 μM respectively).	Zhang et al. 2003
BITC	-human promyelocytic acute leukemia HL60/S cells -human myeloma 8226/S cells -human breast cancer MCF-7 cells -human hepatoma HepG2 cells -human colon cancer HT29 cells -transformed human epidermal HaCat keratinocytes	1.8 ± 2.3 2.2 ± 0.6 4.6 ± 0.5 7.3 ± 0.6 5.1 ± 1.4 4.3 ± 0.7		
BITC	-human breast cancer MCF-7 cells	4.11 ± 0.26	Sulforhodamine B assay	Tseng et al. 2004

PEITC		6.51 ± 0.86	-all of these isothiocyanates were found to be less potent towards normal MCF-12A cells. -These are the IC_{50} values of BITC, PEITC, NITC and sulforaphane treated MCF-12A: 8.07 ± 0.29, 7.71 ± 0.07, 33.6 ± 1.69 and 40.5 ± 1.25 μM respectively	
NITC		78.4 ± 5.26		
Sulforaphane		27.9 ± 5.30		
Alkyl carbon chain; -PMITC, PEITC and PBITC	-human cervical squamous carcinoma HeLa cells	-Similar IC_{50} value ~7.5 μM	MTS assay -PITC which has no alkyl carbon showed far less potent with IC_{50} greater than 40 μM - PHITC was more potent with IC_{50} less than 5 μM	Yu et al. 1998
Sulforaphane	-human colon carcinoma HT29	15 μM	MTT assay	Gamet-Payrastre et al. 2000
AITC	-human prostate cancer PC3 -human prostate cancer LNCaP	Both cancer cell lines were inhibited in dose dependent	Sulforhodamine B assay -AITC was found to be less effective towards normal human prostate epithelial PrEC cells with 80% survival at almost	Xiao et al. 2003

		manner with IC_{50} ~15-17 μM	highest concentration 40 μM	
BITC; PEITC	-human breast cancer MDA-MB-231 cells -human breast cancer MCF-7 cells	Both BITC and PEITC have the same IC_{50} value <2.5 μM BITC = < 5 μM PEITC = <10 μM	Sulforhodamine B assay -although BITC and PEITC displayed the same IC_{50} value in MDA-MB-231 cells, BITC was relatively more potent than PEITC since at <2.5 μM, BITC able to kill 97% of cells population while PEITC decreased 76% of cell viability. -however, normal MCF10A was significantly more resistant towards BITC treatment since >50% cells survival was recorded even at higher concentration of BITC (20 μM).	Xiao et al. 2006
PEITC	-human ovarian cancer OVCAR cells -human cervical cancer HeLa cells	23.2 μM 18.0 μM	MTS assay -PEITC inhibited the growth of these cell line in concentration dependent manner after 48 hours incubation	Satyan et al. 2006

1.7.1.2 *In Vivo* Effect

The anticarcinogenic activity of ITCs depends at least on their accumulation in cells. ITCs have been shown able to act as anti-carcinogenic inhibiting tumorigenesis induced by a variety of chemical carcinogens such as nitrosamine and polycyclic aromatic hydrocarbons (Zhang & Talalay 1994; Hecht 1995) and animal studies offer more evidence of cancer chemopreventive effect of ITCs (Visanji et al. 2004).

Previous research reported BITC and PEITC at concentrations of 5 to 12 μM were also able to induce both chromosome aberrations and sister chromatic exchanges in Chinese hamster ovary cells (Musk & Johnson 1993; Musk et al. 1995). ITCs were also observed to either stimulate the tumorigenesis in the intestine of male rats after given in the diet at 640 or 320 mg/kg for 2 weeks or promote carcinogenesis by continue with the treatment of 15 mg/kg of azoxymethane via injection in the subcutaneous once a week for 2 weeks (Rao et al. 1995). Moreover, when rodents were fed with 1000 mg/kg of PEITC or BITC without involvement of any carcinogens increased the formation of papillary or nodular hyperplasia in the bladder of Fisher 344 rats to 100%. However, the high administration of these ITCs did not increase the number of foci in the liver (Hirose et al. 1998). Similar phenomenon occurred with the administration of 500 or 1000 mg/kg PEITC in the rats' diet (Ogawa et al. 2001) while BITC showed more potent effect towards the increased of epithelial hyperplasia in bladder with less administration which range from 100 or 1000 mg/kg (Okazaki et al. 2002).

Table 1.5. The effects of ITCs in *in vivo*

ITC	Model	Carcinogen	Notes	Reference
PEITC; PHITC	Lung mice tumorigenesis	Anticarcinogenic	-Potent inhibitors of 4-(methylnitrosamino)-1-(3-pyridil)-1-buta-none (NNK)	Hudson et al. 2005
PEITC; BITC	Lung tumorigenesis in A/J mice	Anticarcinogenic	-A dietary of PEITC and a mixture of PEITC and BITC successfully inhibited tumorigenesis in A/J mice which induced by a mixture of the tobacco smoke carcinogens benzo[*a*]pyrene (B[*a*]P) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).	Sticha et al. 2002
PEITC	Syrian golden hamster	Anticarcinogenic	-Metabolisme of 4-(methylnitrosamino)-1-(3-pyridil)-1-buta-none (NNK) was reduced by PEITC.	Richter & Tricker 2002
SFN	Mammary tumors in Sprague-Dawley rats	Anticarcinogenic	-Tumor in the rats were induced with single doses of 9,10-dimethyl-1,2-benzanthracene -Treated with 75 or 150 mμmol per day for 5 days. -After treatment, the weight of mammary tumors was significantly reduced.	Zhang et al. 1994
PEITC	F344 rats esophageal tumors	Anticarcinogenic	-Tumors that were induced by *N*-nitrosobenzylmethylamine (NBMA) was inhibited by PEITC at almost 99%-100% inhibition at concentration of 3 and 6 μmol/g.	Stoner et al. 1991

PEITC	Pulmonary neoplasia in mice	Anticarcinogenic	-PEITC inhibited 4-(methylnitrosamino)-1-(3-pyridil)-1-buta-none	Morse et al. 1989
PEITC	PC-3, prostate cancer in athymic mice	Xenograft	-PEITC significantly reduced the tumor volume in the mice at the concentration of 12 μmol thus significantly inhibited PC-3 xenograft growth without causing any side effects to the mice.	Xiao et al. 2006
AITC; SFN	PC-3, prostate cancer in nude mice	Xenograft	-The growth of PC-3 xenograft was significantly inhibited by the administration of 10 μmol of AITC via injection for 3 times/week -Histological analysis showed increased number of apoptotic bodies -Decreased in the level of anti-apoptotic protein, Bcl-2 in AITC treated compared to control. -Exhibited cleavage of Bid	Srivastava et al. 2003; Singh et al. 2004

SFN	PANC-1 human pancreatic cancer cells in mice	Xenograft	-The activities of mice were decreased with 500 μmol/kg body weight of sulforaphane. -Some mice were found dead at higher concentration of sulforaphane. -The final mean tumor volume for sulforaphane-treated animals was 40% less than the control group.	Pham et al. 2004
PEITC	PC3 prostate cancer in mice	Xenograft	-A dietary of PEITC-NAC (8 μmol/g) able to suppress the tumor growth in mice compared to the control for 9 weeks of treatment. -The weight of tumors from autopsy confirmed that the tumors were ~50.2% smaller (P = 0.05) after given the PEITC-NAC diet. -PEITC-NAC diet reduced the expression of cyclins D and E, up regulating $p21^{WAF-1/Cip-1}$ and $p27^{Kip1}$	Chiao et al. 2004
PEITC	TRAMP-C1 prostate cancer in nude mice	Spontaneous tumor	-Orally ingested PEITC at 9 μM and 12 μM -Increased apopototic bodies without significant changes in xenograft size -Associate with the induction of proapoptotic Bcl-2 family members	Xiao et al. 2005

BITC	ADJ/PC6 plasmacytoma subcutaneous tumor		-The dose of administration was 200 mg/kg although no reduction in tumor mass	Pintao et al. 1995
PEITC	$Apc^{Min/+}$ mice		-$Apc^{Min/+}$ mice were fed with diet supplemented with 0.05% of PEITC for 3 weeks. -PEITC treated mice showed smaller and significantly reduced polyps (31.7% reduction). -PEITC treatment led to the cleavage of caspase-3, -7 and PARP, inhibition of cyclins and activation of p21.	Khor et al. 2008

1.7.2 Mechanistic effect of Isothiocyanates

Further researches reported there are various underlying mechanisms to the anticarcinogenic properties including alterations in the activities of metabolic enzymes (Nijhoff et al. 1995) and reduction of DNA damage in human lymphocytes *ex vivo* prior to increased oxidative stress after supplementation with cruciferous (Gill et al. 2004). Oxidative stress can damage other molecules, cell structures and able to induce several kinds of DNA damage (Halliwell & Aruoma 1993). 7, 8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) is one of the major oxidatively modified DNA *in vivo* (Halliwell & Aruoma 1993; Toyokuni et al. 1994).

Previous research reported cruciferous consumption also able to reduce oxidative DNA damage associate cancer risk. This involved 10 healthy male volunteers who have been taken on diet free of cruciferous vegetable for the first period. However, during 3 weeks intervention period, the first group (consist of 5 male volunteers) continued with the diet free while the other 5 volunteers start consuming 300g of cooked cruciferous per day. The urine that was collected after 12 and 33 days showed there was a 28% decreased in the level of 8-oxodG in a group of five volunteers that change the diet from non-cruciferous to cruciferous-only compared to the other group which continue the diet free of cruciferous (Verhagen et al. 1995). In addition, there was also significant change in smokers due to higher exposure of toxins which lead to significantly lower total antioxidant status compared to nonsmokers (Gill et al. 2007).

The ability of ITCs to concurrently elevate the levels of many enzymes and nonenzymes proteins which have antioxidative or anticarcinogenic effects in a cell makes these compounds potent and versatile as antistress agents although ITCs might not contribute in any unswerving oxidation or reduction reactions in cells. A recent study reported that, ITC was found to penetrate cells via diffusion and rapidly conjugated with intracellular thiols. Glutathione was found to be the major driving force for ITC accumulation (Zhang 2004). Depletion in the pool of cellular thiols is a consequence of the ITCs-thiols conjugation (Zhang 2004; Xu & Thornally 2001) being removed rapidly by membrane efflux pumps (Zhang & Callaway 2002; Callaway et al. 2004), regeneration of ITCs by extracellular hydrolysis and reuptake of ITCs that leads to the ITCs accumulation within cells. This eventually causes more depletion in the thiols level and the cells to be more susceptible to ROS that play important

role in suppressing the growth and survival of transformed cells (Trachootham et al. 2006) and stress-induced damage.

Further accumulation of intracellular ITCs upon GSH depletion leads to conjugation with various cellular proteins thiol for example Keap1. Nrf2 normally sequestered in cytoplasms by Kelch-like-ECH associated protein 1 (Keap1). Keap 1 has been observed to act as a molecular sensor for cellular redox changes in response to exogenous stimuli (Wakabayashi et al. 2004; Dinkova-Kostova et al. 2002). This was further confirmed by the role of two of the 15 cysteine residues (Cys^{273} and Cys^{288}) of Keap 1 in sensing the intracellular redox conditions. Upon exposure to antioxidant response elements (ARE) mediated inducers such as ITCs, they will interact directly to the –SH groups of cysteine of Keap1 which later change the conformation of Keap 1 via formation of an intermolecular disulfide bridge (Wakabayashi et al. 2004). As a consequence, Keap 1 no longer able to bind to Nrf2 causing the activation of Nrf2 and translocation to the nucleus. This gives respond to the stimulation of Nrf2-ARE signaling pathway. Thus, Keap1-mediated ubiquitination and degradation play a central role in regulating the level of Nrf2 and subsequent activation of protective cellular antioxidant protective (Rushworth & McEwan 2011).

Nrf2 is the key transcriptional factor that mediated the major cellular antioxidant and transmits the inducer signal to ARE. There are multiple kinase signaling pathways that involved in the transcriptional activation of ARE in Nrf2-dependent manner. For example, extracellular signal-regulated kinase2 and 5 (ERK2 and ERK5) and c-Jun NH2-terminal kinase1 (JNK1) that upregulate ARE (Keum et al. 2003; Shen et al. 2004; Yu et al. 1999). Moreover, protein kinase C can directly phosphorylate Nrf2 potentially at Ser40 (Huang et al. 2002; Cullinan et al. 2003; 2004) as well as being phosphorylated directly by PERK, a transmembrane transcription factor. However, phase II inducers which are a strong electrophiles can directly induced Nrf2 activation without involved any phosphorylation (Xu et al. 2006). ITCs also able to inhibit the formation of a cancer cell and eliminating an existing one via multiple mechanisms including modulating carcinogen-metabolizing enzymes to protect DNA, maintaining cellular antioxidants and reducing oxidative stress and inhibiting cell proliferation and cell cycle progression.

Some of the ARE mediated inducers such as AITC and sulforaphane have been reported able to increase the Nrf2 accumulation (Jeong et al. 2005). A previous study reported

that PEITC strongly increased the accumulation of Nrf2 in time dependent manner (30 minutes to 4 hours) at concentration of 7.5 μM in PC3 cells. ARE levels was also increased in a dose dependent manner with concentrations varied from 7.5 to 10 μM of PEITC after 24 hours incubation. However, at concentration of 20 μM, the induction of ARE was lessened which probably due to the cellular toxicity. There is also a condition where the doses of ITCs which cellular stress is induced might be overlapping with those needed for enhancement of cellular antioxidant defense.

High cruciferous vegetable intake was also reported to decrease colorectal risk via alteration of the metabolism and eliminating PhIP and related dietary heterocyclic amine carcinogens (Walters et al. 2004). This is due to the close correlation between consumption of red meat and the increased colorectal cancer. It was reported that, heterocyclic amine and PhIP were derived from cooked meat and fish and PhIP able to enhance the growth of tumors in mammary gland, prostate and large intestine in rat (Ito et al. 1991; Shirai et al. 1997). However, the consumption of cruciferous vegetables significantly increased the excretion of PhIP in 0 hour to 48 hours urine samples thus reduced the risk of developing cancer (Walters et al. 2004). Therefore, it was suggested that the effects of cruciferous vegetable on cancer risk were more susceptible to the genetic polymorphism which affected the capability of the individuals to metabolize and eliminate the glucosinolate hydrolysis products (Zhao et al. 2001; Lewis et al. 2001; Spitz et al. 2000; London et al. 2000).

In a small clinical trial on healthy postmenopausal women for breast cancer disease, higher intake of cruciferous vegetables for 4 weeks can shift the estrogen metabolism lead to increase urinary ratios of 2OHE1: 16αOHE1. It has been hypothesized the shifting of 17β-estradiol towards 2-hydroxyestrone (2OHE1) could decrease the risk of estrogen-sensitive cancers such as breast cancer. In contrast, there was a significant effect when 17β-estradiol shifted to 16α-hydroxyesterone since 16OHE1 is highly estrogenic that enhanced the proliferation of estrogen-sensitive breast cancer in culture (Telang et al. 1992; Yuan et al. 1999). In other case-control studies, women with lower urinary 2OHE1: 16αOHE1 ratios had found to be diagnosed with breast cancer disease. However, the association between urinary 2OHE1: 16αOHE1 ratios and breast cancer is still unclear and is not significant in the prospective of cohort studies (Higdon et al. 2007).

In human breast cancer MCF-7 cells, PEITC was reported to decrease the expression of Bcl-2 and increase the Bax level although no changes in the expression level of p53 and p21 observed (Lee & Cho 2008). The integrity of mitochondrial membrane is regulated by members of Bcl-2 family consisting of pro-apoptotic proteins such as Bax, Bak and Bad and antiapoptotic proteins including Bcl-2 and Bcl-X_L. Similarly, Bcl-2 was also phosphorylated leading to the increase in the Bak translocation to the mitochondria in human bladder cancer UM-UC-3. In this research, BITC and PEITC were found able to cause damage to both outer and inner mitochondrial membranes which lead to the release of cytochrome *c* from mitochondrial intermembrane space (Tang & Zhang 2005) while malate dehydrogenase which is normally residing in the mitochondrial matrix are detected in the cytosolic fraction (Nakamura et al. 2002; Tang & Zhang 2005). In contrast, PEITC and BITC were not affecting the expression of Bax but upregulating the expression of p53 and p21 in A549 cells in a dose dependent manner (Kuang & Chen 2004).

In addition to the growth inhibitory effect of ITCs, anti angiogenesis and anti metastatic also contribute more to the inhibiting and blocking the proliferation of cancer cells as wells as chromatin remodeling and gene expression . ITCs also cause mitotic arrest via conjugation of ITC with cysteine residue in alpha-tubulin. The growth and progression of solid tumors beyond 2 to 3 mm is often preceded by an increase in the formation of angiogenesis that is essential for nutrients and oxygen delivery (Carmeliet 2000, 2003; Dhanabal et al. 2005). Thus, angiogenesis allows the cancer cells to invade and metastasize to distant organ (Folkman 1971; Carmeliet 2000). Therefore, by eliminating and blocking the formation of angiogenesis might prevent and control the growth and invasiveness of the tumors. Current studies demonstrated that PEITC, AITC and PITC able to inhibit the angiogenic features human umbilical vein endothelial cells (HUVEC) (Xiao & Singh 2007; Thejass & Kuttan 2007) and significantly inhibited neovascularization *ex vivo*.

The exposure of HUVEC to less than 1 μmol/L of PEITC leads to the suppression of VEGF secretion to the medium. Moreover, the viability of HUVEC decreased significantly upon SFN and BITC treatment, however the IC_{50} for both of these ITCs was 3 and 10 fold higher compared to PEITC. Interestingly, the ability of PEITC was not only limited in angiogenesis inhibition, but also in blocking the migration potential of HUVEC by about 58%,

72%, 82% and 99% at 0.5, 1, 2 and 4 μmol/L of PEITC exposure respectively (Xiao & Singh 2007).

Endothelial cells play a major role in angiogenesis. Thus, most of the angiogenesis studies have focused on this cell type. The stimulation of mitogenic by proangiogenic signal, will lead to the formation of capillary-like tube (Unger et al. 2002). Endothelial cells also produced type IV collagenase, other members of matrix metalloproteinase (MMP) and serine protease which are important for angiogenesis, tumor cell invasion and metastasis (Egeblad and Werb 2002; Rundhaug, 2003). Another member of ITCs that is sulforaphane was also found able to inhibit all the essential steps of neovascularization from proangiogenic signaling and basement membrane integrity to endothelial cell proliferation, migration and tube formation in the human microvascular endothelial cells (HMEC-1) (Bertl et al. 2006) and HUVEC cells (Asakage et al. 2006). Sulforaphane was proved able to reduce the hypoxia-mediated induction of VEGF mRNA under hypoxia condition, reduced the levels of MMP2 mRNA, inhibit the growth of HMEC-1 and blocking the complete capillary-like tubular formation in a dose dependent manner (Bertl et al. 2006) thus preventing from migrating.

Vascular endothelial growth factor (VEGF) is known as a proangiogenic growth factor which provides prosurvival signals (Xiao & Singh 2007) by stimulating endothelial mitogen cell survival, migration, differentiation and self assembly (Affara and Robertson, 2004). The signal transduction is mediated by VEGF receptors including VEGF-R1, VEGF-R2 and VEGF-R3. VEGF mRNA expression is regulated by several transcription factors including hypoxia-inducible factor-1α (HIF-1α), c-Myc and NF-κB. Meanwhile, VEGF transcription involved tumor necrosis factor-α, nitric oxide, prostaglandin and polyamines (Josko and Marzurek 2004; Xie et al. 2004).

Instead of angiogenesis inhibition in the endothelial cells, ITCs also inhibited the formation of angiogenesis in cancer cell line. It is supported by the findings that PEITC suppressed the expression of VEGF, EGF and G-CSF in PC-3 prostate cancer cells though PEITC did not affect the expression of IL-17, MMP-2, or MMP-9 (Xiao and Singh 2007). Moreover, PEITC inhibited the migration of PC-3 cells via inhibiting the Ser473 phosphorylation of Akt in a dose dependent manner. Akt or protein kinase B play role in promoting the endothelial cells and VEGF-stimulated endothelial cell migration (Fujio & Walsh 1999; Morales-Ruiz et al. 2000; Shiojima & Walsh 2002; Jiang et al. 2000).

1.8 AIMS

Ingestion of diets rich in CV have been linked to potential anti-cancer effects, presumably mediated by compounds such as isothiocyanates, indole-3-carbinal and quercetin. The goal of this thesis is to investigate the potential on *in vitro* anti-cancer effects of phytochemicals derived from watercress.

The aims of this study are:

1. To investigate the ability of watercress phytochemicals to inhibit the growth of human breast cancer cell lines, alone and in combination.
2. To investigate mechanism by which these chemicals inhibit human breast cancer cell line growth and determinants of differential responses.
3. To determine whether ingestion of watercress might exert similar effects *in vivo.*

Chapter 2

Materials and Methods

2.1 Methodology

2.2 Chemicals and Reagents

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, Trypsin-EDTA, antibiotics (penicillin and streptomycin) and L-glutamine were from Lonza. Fetal calf serum (FCS) was from PAA, Austria. Phenethyl isothiocyanate, indole-3-carbinol, quercetin dihydrate, LY294002, N-acetyl-L-cysteine (NAC) and buthionine sulfoximine (BSO) were from Sigma. Benzyl isothiocyanate was from Aldrich. The watercress packages for the feeding study were obtained from Vitacress Salads Ltd (Andover, UK). For NAC/BSO pretreatment, cells were pretreated with NAC for 6 hours and with BSO for 24 hours prior to addition of test agents.

2.3 Cell lines

Breast cancer cell lines (MDA-MB-231, SKBr3, BT549, MCF7, T47D and ZR75.1) were obtained from the American Type Culture Collection (ATCC). Normal Human Dermal Fibroblast (NHDF) were obtained from Lonza. Cells were cultured in complete DMEM medium (i.e. supplemented with 10% (v/v) FCS, antibiotics and L-glutamate). Cells were incubated at 37^{o}C in a 5% CO_2 containing atmosphere. All culture work was performed in sterile conditions. Cells were subculture by splitting 1:2 or 1:3 depended on the confluency. This was done by trypsinization with trypsin-EDTA. Cell numbers/viability were determined using trypan blue and a haemocytometer. Cells were cryopreserved in freezing mix (70% (v/v) DMEM, 20% (v/v) FCS and 10% (v/v) DMSO), stored in liquid nitrogen and recovered using standard techniques.

2.4 Growth inhibition assay

Cells at 70%-80% confluence were harvested with trypsin-EDTA. $2x10^3$ cells were plated in wells of a 96 well plate in 0.05 ml complete DMEM medium. After overnight incubation to allow cell attachment, compounds that were freshly prepared were added to the culture

medium. The compounds were first dissolved in DMSO, diluted in complete DMEM medium (at 2x final desired concentration) and 0.05 ml were added to each well. Each concentration of the compound was assayed in triplicate.

The assay was terminated at day 6 and "relative cell growth" was measured using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega). The principle of this assay is that MTS tetrazolium is reduced in cells to a coloured formazan which is soluble in cell culture medium (Malich et al. 1997). This conversion occurs in the present of mitochondrial dehydrogenase enzymes found in metabolically active cells. After 6 days incubation, the plate was drained and 0.1 ml of RPMI medium containing 5µl MTS reagent was added to each well. The plate was incubated for 1 hour 30 minutes at 37^{o}C with 5% CO_2 and the optical densities were measured using an ELISA plate reader at 492 nm. Relative cell growth was determined by the following calculation:

Relative cell growth

= ((OD treated – OD blank) / (OD untreated – OD blank)) x 100%

2.5 Data Analysis

For growth inhibition assay, all data was analysed using the software package Prism GraphPad Programme (GraphPad Software, USA). Meanwhile, to calculate the synergistic effect of the compound, all data was analysed via CalcuSyn (Biosoft, UK).

All mean percentage data values were normalized to an untreated control to remove variability in cell numbers allowing comparisons of the ratio between experimental conditions. Error bars represent ± the standard error of the mean (SEM) for that data set and thus control values have an SEM of zero. Comparisons within groups of data were analysed using T-test and differences between means of $P<0.05$ were considered to be significant. Asterisks on graphs are used to denote the significant differences within groups.

2.6 Annexin V staining

Annexin V staining was carried out using the FITC-Annexin V Apoptosis Detection Kits II (BD Biosciences) following the manufacturer's instructions. The principle of Annexin V staining is to detect the exposure of phosphatidylserine (PS) on the external surface of the cell that binds to the negatively charged PS in the presence of calcium (Vermes et al. 1995).

Three samples required for the Fluorescence Activated Cell Sorted (FACS) setting were unstained cells, stained with Annexin V only and propidum iodide only. Cells were seeded at $1x10^5$ cells per well in 6 wells plate and incubated as required. Cells were then harvested and transferred into a 5 ml FACS tube. Cells were washed twice with cold PBS and centrifuged at 486 x g 5 minutes. Master Mix that contained 300 µl binding buffer, 2.5 µl of 50 µg/ml PI and 1.25 µl AnnexinV- FITC for each sample were prepared in the dark and added 300 µl into each sample. Cells were then incubated for 15 minutes at room temperature in the dark prior to flow cyometry using FL1 channel on a BD Biosciences FACSCalibur.

2.7 Cell cycle Analysis

The principle of cell cycle analysis is the stained cell that has incorporated with an amount of dye proportional to the amount of DNA is measured by FACSCalibur. Cells were seeded at $1x10^5$ cells per well in 6 wells plate and incubated as required. Cells were then harvested and transferred into a 5 ml FACS tube. The samples were washed with phosphate buffered saline (PBS) and 1ml of 70% (v/v) of ice-cold ethanol was added dropwise onto the dissociated cell pellet. Samples were stored at 4 ^oC for at least 24 hour. Supernatants were removed and pellets were washed twice in ice cold PBS. The cell pellet was resuspended in PBS containing 1µl of 100 µg/ml RNAse A and 3 µl of 50 µg/ml propidium iodide (both Sigma), incubated at 37^oC for 15 minutes and analysed by flow cytometry. The flow cytometry analysis was performed using FL2 channel on a BD Biosciences FACSCalibur.

2.8 ROS Measurement

Human breast cancer cell lines, MCF7 and MDA-MB-231 cells were plated in 6 wells plate with 3 x 10^5 cells/ml per well and incubated overnight. On the next day, cells were treated with PEITC at required concentration and incubated at 3, 4 and 5 hours. The method for ROS detection was performed as per manufacturer's instructions. After PEITC treatment, MDA-MB-231 and MCF7 cells were incubated for another 45 minutes with 2 µM dihydrorhodamine 123 or 2 µM dichlorodihydrofluorescein diacetate (H_2DCFDA; both from Invitrogen). These ROS-dependent fluorescent probes are converted to hydrorhodamine or 2',7'-dichlorofluorescein (DCF) in the presence of superoxide radicals or H_2O_2. After incubation, cells were harvested using cell scrapers and washed with PBS. The ROS intracellular levels were then detected by flow cytometry using BD Biosciences FACSCalibur FL1 channel.

2.9 Western Blotting

2.9.1 SDS-PAGE and Western blot

2.9.1.1 Preparation of cell lysates

Cells were cultured in 100 mm dishes to 70% confluency in complete DMEM and treated as required. At the end of the incubation period, both floating cells and attached cells were collected using a cell scraper and centrifuged at 311 x g for 3 minutes. The supernatant was discarded and cells were washed twice with ice-cold PBS. 1 ml of 1x Radioimmunoprecipitationassay (RIPA) buffer (750 mM sodium chloride, 1% Tween 20, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris, pH 8) containing protease and/or phosphatase inhibitors as required (Sigma) was added to the cell lysates and left on ice for 15 minutes. Samples were then centrifuged at 4^{o}C for 2 minutes at 13,000 x g microcentrifuge and the clear supernatant collected. The supernatant was aliquoted and stored at -80^{o}C. Total protein content was quantified on a Lambda 25 UV/VIS (Perkin Elmer, Fremont,CA, USA) spectrophotometer at 750 nm using the BSA protein assay (Sigma, Aldrich).

2.9.1.2 SDS-Polyacrylamide Gels

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described in reducing conditions according to Laemmli (1970) with a Biorad Miniprotean II protein separation apparatus. The gel plates were assembled according to the manufacturer's instructions. Ammonium persulfate and TEMED (both Sigma) were added just prior to pouring the gels as these reagents promote and catalyze the polymerization of the acrylamide. After mixing thoroughly, the resolving gel mix was poured into the assembled gel plates, leaving sufficient space at the top for addition of the stacking gel later. The gel mix was allowed to polymerize for 30 minutes.

After polymerization, the surface of the resolving gel was rinsed with deionized water to remove any unpolymerized acrylamide. The remaining space was then filled with the stacking gel and a clean comb was immediately inserted. After the stacking gel has polymerized, the comb was gently removed and the wells were rinsed with water to removed unpolymerized acrylmide. The gel cassette was then mounted unto the electrophoresis apparatus. Electrophoresis buffer was added at the top and bottom of the reservoirs. Protein aliquots of 20 μg from samples were then loaded into the wells. Molecular weight standards were also loaded into the wells. Electrophoresis was then run at 200 V for 45 minutes until the separation was completed.

2.9.1.3 Western Blot Analysis

A piece of nitrocellulose transfer membrane was first cut according to the dimensions of the gel. The corner of the membrane was labeled. Meanwhile, the acrylamide gel was removed from the cassette and placed in a transfer filter paper and membrane 'sandwich' consisting of 2 sponges, 2 filter papers and 1 nitrocellulose blotting membrane in a blotting cassette as described by the manufacturer. Approximately, 500 ml of transfer buffer was added into the blotting cassette and was connected to a power pack and run at 100V for 60 minutes.

The membrane was removed and stained with 0.1% Ponceau S solution (Sigma-Aldrich, Dorset, UK) to visualise total protein bands and to confirm equal loading of protein. The membrane was blocked with 5% milk (dried semi-skimmed Marvel) made in 0.05% TBS-

Tween 20x (Sigma-Aldrich, Dorset, UK) (as required) for 1 hour at room temperature. The membrane was stained with primary antibody overnight at 4°C on an automatic roller. The membrane was washed three times for 5 minutes each with 0.05% TBS-Tween and stained with the relevant secondary horseradish peroxidase (HRP)-conjugated antibody (GE Healthcare, Little Chalfont, UK) for 1 hour at room temperature on an automatic roller. The membrane was rewashed before incubation with SuperSignal West Femto substrate (Thermo Fisher Scientific Inc, USA). Images were collected using Fluor-S MultiImager (Bio-Rad Labarotaries) and all the data were quantified using Fluor-S software Quantity One Version 4.6.3 (Bio-Rad Laboratories Inc.).

Table 2.1: List of primary antibodies

Primary antibodies	**Companies**
Caspase-3	Cell Signaling
Caspase-7	Cell Signaling
Caspase-9	Cell Signaling
Caspase-8	BD Pharmingen
Bax	Santa Cruz
Bcl2	Santa Cruz
Nrf2 and Keap1	Santa Cruz
Akt Thr 308, Ser 473 and Akt total	Cell Signaling
4EBP1 antibody (Thr 37/46 and Thr 70)	BD phospho-specific (BD Biosciences)

2.9.1.4 Stripping of membranes

Membranes were stripped of antibody complexes using Re-Blot plus (mild) solution (Chemicon, Hampshire, UK) diluted 1:10 n distilled water for 10 to 15 minutes on a belly shaker and rinse in TBS-Tween. Blots were reblocked in 5% milk in TBS-Tween for 1 hour at room temperature before re-probing.

2.10 Glutathione Measurement

GSH/GSSG assays were performed using kits from Cayman Chemical Company. Cells were collected by using a cell scraper, centrifuged and the cell pellet was sonicated in 1-2ml of GSH MES cold buffer (0.4M 2-(N-morpholino)ethanesulphonic acid, 0.1M phosphate and 2mM EDTA, pH 6.0) and centrifuged at 10000 x g for 15 minute at 4^{o}C. The supernatant was transferred and deproteinated by adding an equal volume of metaphosphoric acid (VWR International Ltd.) to the sample and vortexing. The mixture was incubated at room temperature for 5 minute and centrifuged at 2000 x g for 2 minutes. The supernatant was collected and stored at -20 ^{o}C. When the samples are ready to be analysed, 50 µl of 4M triethanolamine reagent (VWR International Ltd.) was added per ml of supernatant and vortexed immediately. To quantify the GSSG, 10 µl of 2-vinylpyridine (Sigma-Aldrich) per ml was added to the deproteinated sample, vortexed and incubated at room temperature for 60 minute.

To measure the level of GSH and GSSG, 50 µl of samples were added into each well. As a standard, 50 µl of GSSG Standard was used and added into each designated well. The Assay cocktail was prepared by mixing the GSH MES buffer, GSH Cofactor mixture ($NADP^{+}$ and glucose-6-phosphate), GSH enzyme mixture (glutathione reductase and glucose-6-phosphate dehydrogenase in 0.2 ml buffer) and GSH 5,5'-Dithiobis(2-nitrobenzoic acid). The plate cover was removed and 150 µl of Assay Cocktail was added to the wells containing standard and samples and incubated in the dark on an orbital shaker. The absorbance was measured using plate reader at 405-414nm at five minutes intervals for 30 minutes (a total of 6 measurements). The concentration of GSH and GSSG were later determined by the End Point Method according to manufacturer's instructions.

2.11 Peripheral Blood Mononuclear Cells (PBMCs) preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Lymphoprep. 5 ml of Lymphoprep was first added into 15ml falcon tube and was overlaid with 5 ml of whole blood on top of Lymphoprep and centrifuged at 800 x g for 20 minutes using brake 1 at room temperature. PBMCs were then carefully collected from the thin layer between the plasma and the red blood cells and transferred into new 50ml falcon tube. RPMI medium was added up to 30 ml and centrifuged again at 800 x g for 8 minutes using brake 3 at room temperature.

2.12 ROS measurement in PBMCs

Upon watercress consumption, PBMCs were collected (method as previously discussed). PBMCs cells were counted and 3 x 10^5 cells were incubated with 2 µM dihydrorhodamine 123 or 2 µM dichlorodihydrofluorescein diacetate (H_2DCFDA; both from Invitrogen) for 45 minutes in the dark at 37^{o}C. After incubation, PBMCs were washed with PBS and the level of intracellular ROS were detected by flow cytometry (FL2 and FL1 respectively).

The balance of PBMCs were used to analysed the level of Keap1 after watercress consumption. Cell lysates were prepared for western blot analysis (method as previously discussed) and chemiluminescence was detected by exposure to the imager (Imager J) for an appropriate length of time.

2.13 PhosFlow analysis - 4EBP1 phosphorylation

4EBP1 phosphorylation was analysed by single cell flow cytometry using reagents from BD Biosciences. PBMCs were washed in ice-cold RPMI 1640 medium and resuspended in 1 ml RPMI 1640. CytoFix buffer was added and cells were incubated for 10 minutes at 37 ^{o}C prior to storage at -80 ^{o}C. On the day of analysis, samples were thawed and cells washed with flow cytimetry buffer. Cells were resuspended in 1ml of Phosflow Permeabilisation buffer III and incubated on ice for 30 minutes. Cells were then washed twice with Stain Buffer, collected by centrifugation and resuspended in 1ml of Stain Buffer containing 100 µl of phycoerythrin-

conjugated anti-4EBP1 antibody (Thr37/46 phospho-specific; from BD Phosflow). Unstained cells were analysed as controls. Cells were incubated in the dark room at temperature for 30 minutes, washed with Stain Buffer and resuspended in 500µl of the same buffer prior to flow cytometry. For the 4EBP1 phosphorylation analysis forwards scatter (FSC)/side scatter (SSC) profiles were used to separately gate on lymphocyte and monocytes. Flow cytometry was performed using FL2 (585nm) on a BD Bioscience FACSCalibur.

2.14 *In vivo* study

The watercress feeding study was based on the previous work of Ji et al. who studied the plasma pharmacokinetics of PEITC following ingestion of 100 g of watercress in 4 normal participants (Ji & Morris 2003). Our study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by the Southampton and South West Research Ethics Committee (Ref 08/H0504/86). Written informed consent was obtained from all participants. Potential participants were identified using the Winchester and Andover Breast Unit database of patients where clinical and diagnostic data is routinely compiled for audit purposes using criteria defined by the British Association for Surgical Oncology. Although the participants had previously been treated for breast cancer, they were considered free of clinically detectable disease for a minimum of 2 years and were otherwise healthy. None of the women were taking any pharmacological medications or herbal supplements.

Participants were requested to avoid the following foods for 3 days before the study day to exclude known sources of glucosinolates from the diet; cabbage, brussels sprouts, broccoli, calabrese, cauliflower, turnip, swede, rutabaga, kohlrabi, kale, chinese kale, sea kale, curly kale, collard, pak choi, radish, horseradish, mustard, mustard greens, mustard leaf, wasabi, salad rocket, cress, watercress, capers, papaya (pawpaw) and nasturtium (Indian cress). The work of McNaughton and Marks (2003) 186 informed the compilation of the list of food items to be excluded from the diet (Krutzik et al. 2004). The participants fasted from midnight on the day of the study. Approximately 8 hours later the volunteers were cannulated and a baseline blood sample (5 ml) obtained. The participants then ate 80 g of watercress. Blood samples were then obtained at approximately 7.5 minutes, 15 minutes, 30 minutes, 45

minutes, 1 hr, 1.5 hr, 2 hr, 3hr, 4 hr, 6 hr and 8 hr following completion of the watercress meal. A 24 hour blood sample was obtained by venupuncture. Blood samples were collected in heparinised glass tubes and stored on ice prior to processing. Participants were free to take water throughout the study and allowed to eat and drink freely from 3 hours post-watercress meal, although glucosinolate-containing foods were eliminated until end of study.

We enrolled 12 women on the study and analysed plasma PEITC concentrations and/or 4E-BP1 phosphorylation in samples from 9 of these (mean age 58 years, median age 56 years, range 48-82). For 8 participants, we prepared both PBMCs and plasma; samples were overlaid onto Lymphoprep and centrifuged at 800 g for 20 minutes. The plasma was recovered and stored at -80 ^{0}C. For 1 participant, we prepared just plasma; samples were centrifuged at 486 g for 15 minutes, the plasma was recovered and stored at -80 ^{0}C. Plasma PEITC concentrations were determined as previously described (Ji & Morris 2003) and 4E-BP1 phosphorylation was analysed as described above.

Ethical approval and patient recruitment were kindly carried out by Dr Barbara Parry, Clinical Dietician at Winchester Hospital. The feeding portion of the study was performed at the Winchester Hospital under the supervision of Dr Parry and with the support of NHS phlebotomists. PEITC measurements were performed by Dr Marilyn Morris (NY, USA).

Chapter 3

Growth inhibitory effect of watercress-derived compounds

3.1 INTRODUCTION

Ingestion of diets rich in watercress and other cruciferous vegetables has been suggested to provide protection from cancer development. Watercress is a rich dietary source of β-phenylethyl isothiocyanate (PEITC) (Rose et al. 2000) as well as other potential anti-cancer phytochemicals such as indole-3-carbinol and Quercetin (Kojima et al. 1994; Grubbs et al. 1995; Choi et al. 2001; Kobayashi et al. 2002). The goal of the work described in this chapter was to compare directly the effects of these compounds, alone and in combination, on the growth of breast cancer cells and normal human dermal fibroblast cells (NHDFs) in order to select compounds and cell lines for more detailed mechanistic studies.

3.2 RESULTS

3.2.1 Effect of watercress-derived compounds on growth of MDA-MB-231 cells

The relative growth inhibitory effect of chemical compounds derived from watercress was first determined using MDA-MB-231 cells. MDA-MB-231 cells were chosen since they represent aggressive, "triple-negative" tumours for which treatment options are limited. MTS assays were used to assess relative cell growth. Effects in these assays may be due to effects on cell proliferation and/or cell death and therefore provide an overall measure of effects on cell "growth".

All of the compounds induced a dose dependent decrease in the growth of MDA-MB-231 cells (Figure 3.1-4). However, there were differences in the potency of the different compounds. PEITC was the most potent watercress-derived compound tested with an IC_{50} value of 7.2 ± 1.6 μM (Table 3.1). As a control I also included BITC in these experiments. BITC is not present at high levels in watercress but like PEITC contains an isothiocyanate group. The potency of BITC was very similar to PEITC (IC_{50} = 6.7 ± 0.9 μM; Table 3.1). By contrast, I3C and Quercetin were less potent for growth inhibition of (IC_{50}s of 76.0 ± 5.1 μM and 110.8 ± 8.2 μM; Table 3.1). As expected, the growth of MDA-MB-231 cells was very effectively inhibited by staurosporine, a non-selective kinase inhibitor, but was unaffected by DMSO. Therefore, PEITC was the most potent

watercress-derived compound. Due to the similar results obtained with BITC, it is likely that the activity of PEITC stems from the presence of the ITC group.

Since PEITC was the most potent compound towards MDA-MB-231 cells, further analysis was performed using other breast cancer cell lines (Fig 3.5-7). Three other breast cancer cell lines with different characteristics were selected; BT549 (ER negative; normal HER2 status), T47D (ER positive; normal HER2 status) and ZR75.1 (ER positive; normal HER2 status). Results were also compared with data from MCF7 (ER positive, normal HER2 status) and SKBr3 cells (ER negative; amplified HER2 status), provided by Breeze Cavell and obtained using identical methods. The growth inhibition analysis demonstrated decreased growth of T47D, BT549 and ZR75.1 cells by PEITC with IC_{50} values of 9.2 ± 3.8 μM; 11.9 ± 7.3 μM; 40.4 ± 4.8 μM, respectively (Table 3.1). PEITC IC_{50} values for SKBr3 and MCF7 cells were 26.4 ± 2.1 μM and 13.7 ± 1.0 μM, respectively (Table 3.1).

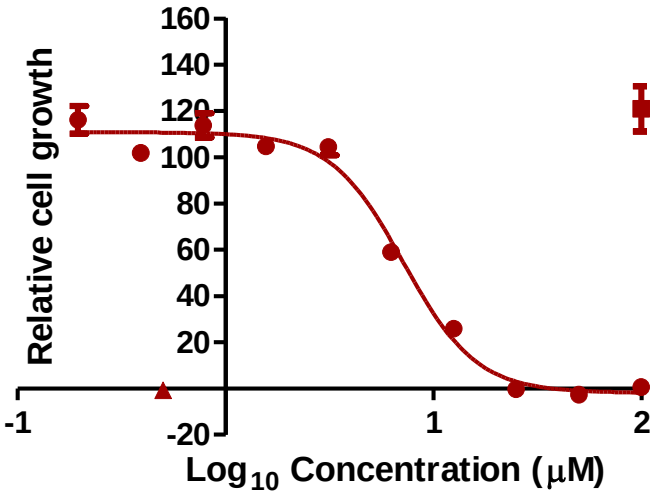


Figure 3.1. Growth inhibition of PEITC towards MDA-MB-231 cells.
Cells were treated with the indicated concentrations of PEITC (●), staurosporine (▲) or an equivalent amount of DMSO as a solvent control (■) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

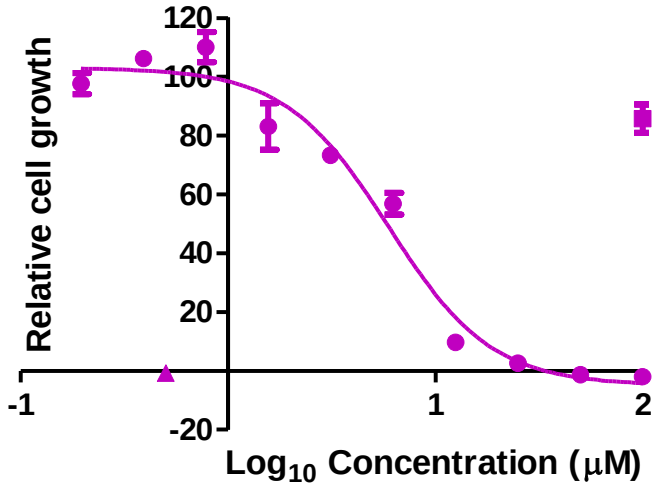


Figure 3.2. Growth inhibition of BITC towards MDA-MB-231 cells.
Cells were treated with the indicated concentrations of BITC (●), staurosporine (▲) or an equivalent amount of DMSO as a solvent control (■) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

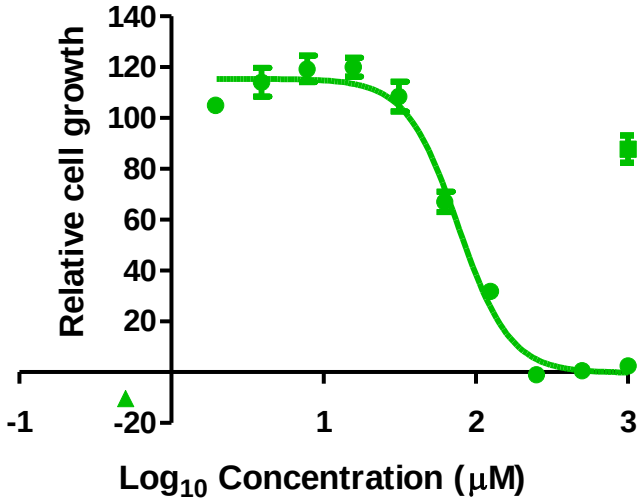


Figure 3.3. Growth inhibition of I3C towards MDA-MB-231 cells.
Cells were treated with the indicated concentrations of I3C (●), staurosporine (▲) or an equivalent amount of DMSO as a solvent control (■) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

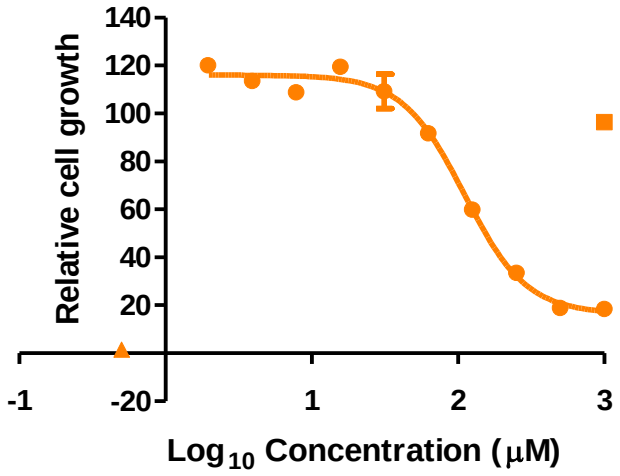


Figure 3.4. Growth inhibition of Quercetin towards MDA-MB-231 cells.
Cells were treated with the indicated concentrations of Quercetin (●), staurosporin (▲) or an equivalent amount of DMSO as a solvent control (■) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

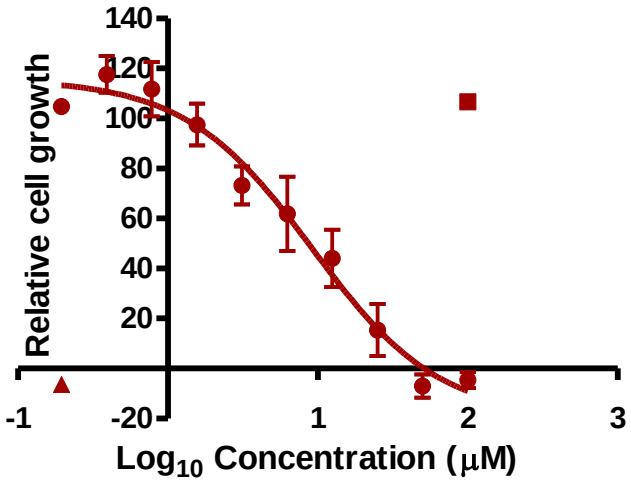


Figure 3.5. Growth inhibition of PEITC towards T47D cells.
Cells were treated at the indicated concentrations of PEITC (●), staurosporine (▲) or equivalent amount of DMSO as a solvent control (■) for 6 days incubation. Cell growth was determined by MTS. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

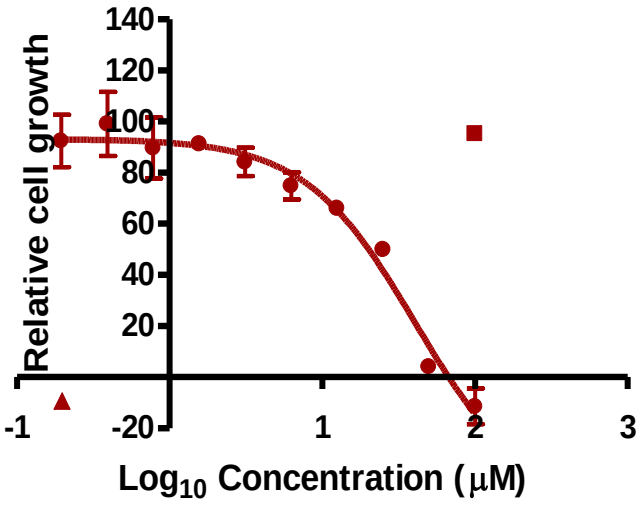


Figure 3.6. Growth inhibition of PEITC towards ZR75.1 cells.
Cells were treated at the indicated concentrations of PEITC (●), staurosporine (▲) or equivalent amount of DMSO as a solvent control (■) for 6 days incubation. Cell growth was determined by MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

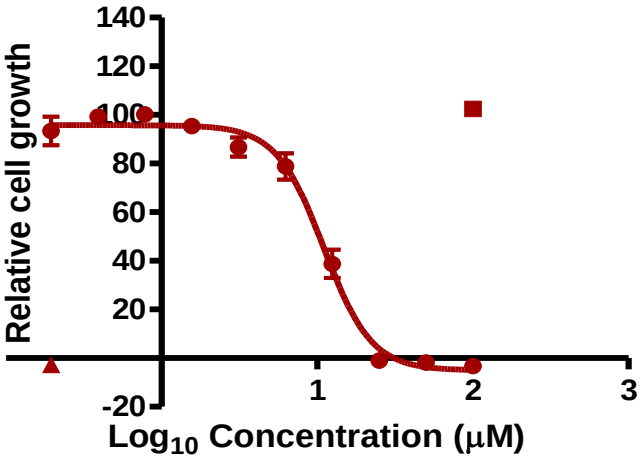


Figure 3.7. Growth inhibition of PEITC towards BT549 cells.
Cells were treated at the indicated concentrations of PEITC (●), staurosporine (▲) or equivalent amount of DMSO as a solvent control (■) for 6 days incubation. Cell growth was determined by MTS. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

3.2.2 Effect of watercress-derived compounds on growth of NHDFs

To determine whether normal cells were relatively resistant to growth inhibitory effects of these compounds, the relative potency of chemical compounds derived from watercress on growth inhibition of normal human dermal fibroblasts (NHDF) were determined. All compounds tested inhibited the growth of NHDF (Figure 3.8-11 and Table 3.1). However, for some compounds NHDFs were relatively less sensitive compared to MDA-MB-231 cells. For example, PEITC and BITC inhibited the growth of NHDFs with IC_{50}s of 27.1 ± 8.2 μM and 35.8 ± 12.2 μM and NHDFs were therefore approximately 4-fold less sensitive to these compounds compared to MDA-MB-231 cells. By contrast, NHDFs and MDA-MB-231 cells were approximately equally sensitive to I3C and NHDFs were approximately 4-fold more sensitive to Quercetin compared to MDA-MB-231 cells.

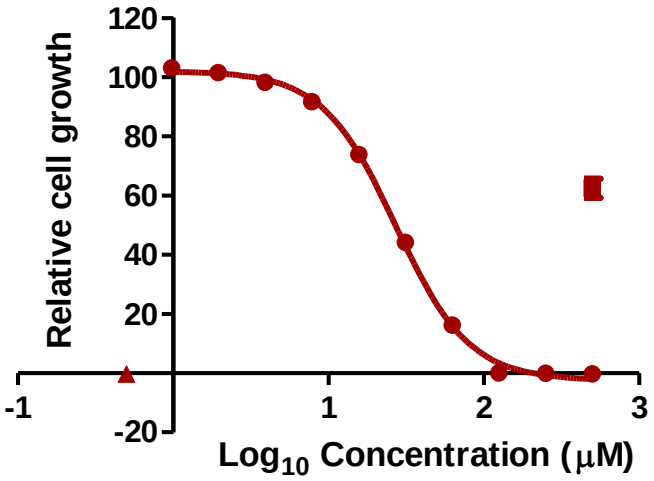


Figure 3.8. Growth inhibition of PEITC towards NHDF cells.
Cells were treated with the indicated concentrations of PEITC (●), staurosporine (▲) or an equivalent amount of DMSO as a solvent control (■) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

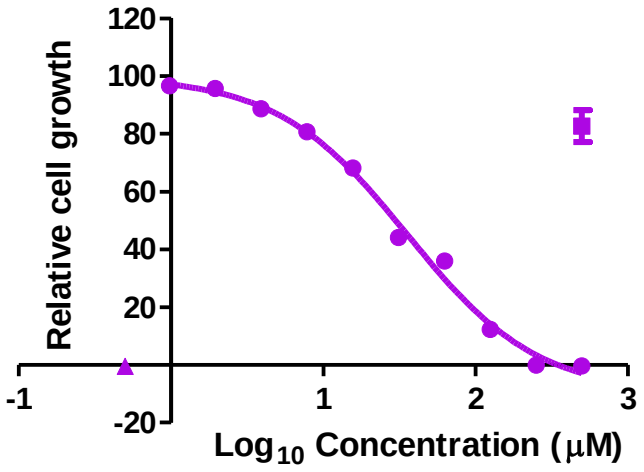


Figure 3.9. Growth inhibition of BITC towards NHDF cells.
Cells were treated with the indicated concentrations of BITC (●), staurosporine (▲) or an equivalent amount of DMSO as a solvent control (■) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

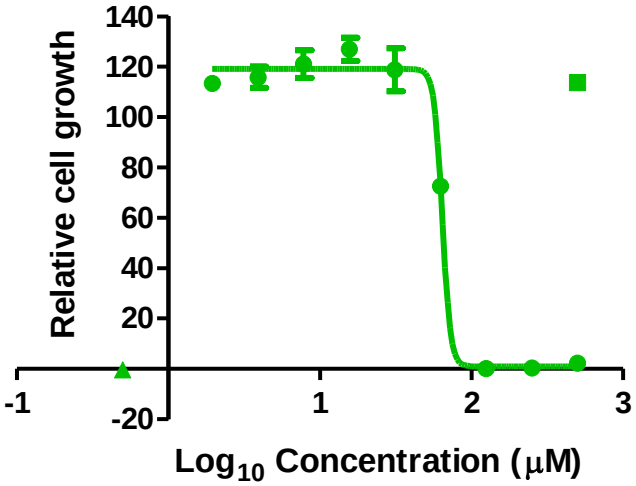


Figure 3.10. Growth inhibition of I3C towards NHDF cells.
Cells were treated with the indicated concentrations of I3C (●), staurosporine (▲) or an equivalent amount of DMSO as a solvent control (■) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

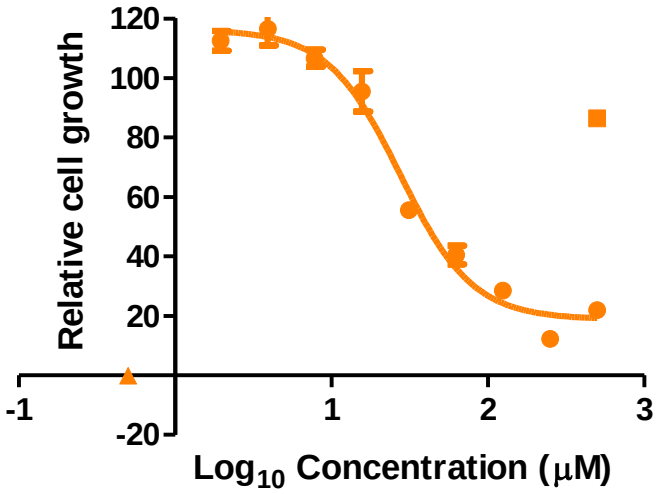


Figure 3.11. Growth inhibition of Quercetin towards NHDF cells.
Cells were treated with the indicated concentrations of Quercetin (●), staurosporine (▲) or an equivalent amount of DMSO as a solvent control (■) for 6 days. Cell growth was determined using the MTS assay. Data shown are mean (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

Table 3.1. Growth inhibitory activity of watercress-derived compounds. The table summarizes growth inhibitory effects of PEITC, I3C and BITC towards breast cancer cell lines and NHDF cells. Data shown are mean (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate. IC_{50} values were calculated and analyzed via Prism Software.

Compounds/ Cell lines	**Status**			**Watercress compounds (μM)**			
	ER	**PR**	**HER2**	**PEITC**	**Indole-3-carbinol**	**Quercetin**	**BITC**
MDA-MB-231	-ve	-ve	Normal	7.2 ± 1.6	76.6 ± 5.1	110.8 ± 8.2	6.7 ± 0.9
BT549	-ve	-ve	Normal	11.9 ± 7.3	ND	ND	ND
*SKBr3	-ve	-ve	Amplified	26.4 ± 2.1	ND	ND	ND
*MCF7	+ve	+ve	Normal	13.7 ± 1.0	88.7 ± 8.1	74.3 ± 8.0	ND
T47D	+ve	+ve	Normal	9.2 ± 3.8	ND	ND	ND
ZR75.1	+ve	-ve	Normal	40.4 ± 4.8	ND	ND	ND
NHDF	-	-	-	27.1 ± 8.2	64.2 ± 4.9	27.5 ± 2.6	35.8 ± 12.2

***** - MCF7 and SKBr3 results were obtained from Breeze Cavell and provided for comparison.

ND - Not done.

3.2.3 Effect of combinations of watercress-derived compounds on growth of MDA-MB-231 cells

It was possible that different compounds either acted cooperatively or antagonistically to inhibit cell growth. To investigate this, a series of experiments in which cells were treated with PEITC and I3C or PEITC and Quercetin were performed. Since PEITC was the most potent compound when tested alone, I focused on combinations of PEITC with the other compounds.

To assess these interactions, Calcusync software as previously described by Chou and Talalay (1984) was used in this analysis. In these experiments, cells were treated with the two compounds at a fixed ratio based upon their individual IC_{50} values. The resulting growth inhibition values are used to determine a combinational index (CI). A CI value <1 is indicative of synergistic, whereas CI values of 1 and >1 are indicative of summation and antagonistism, respectively.

For I3C, combination with PEITC gave rise to CI values of 1.0 – 1.4 (Figure 3.13 and Table 3.3), indicating that these compounds may act antagonistically. Similarly, the combination of Quercetin and PEITC gave CI values of 1.9- 4.9, indicating strong antagonism compared to PEITC and I3C (Figure 3.14 and Table 3.4). The synergistic effects of PEITC and BITC were also analyzed. This yielded CI values 0.3-0.03 indicating that these compounds may act synergistically (Figure 3.12 and Table 3.2).

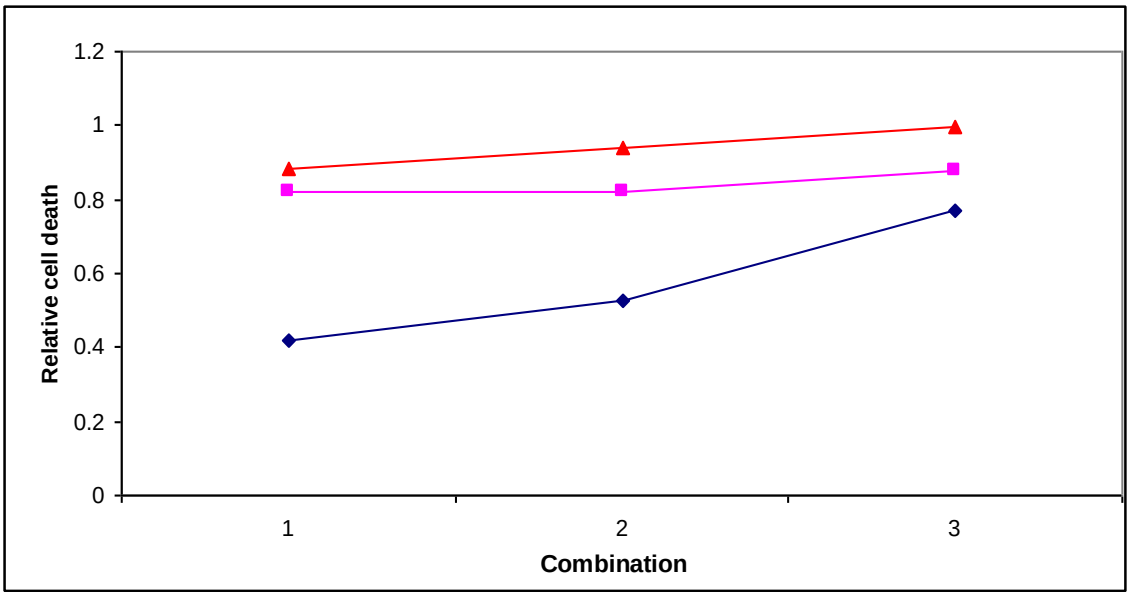


Figure 3.12. Synergistic effect of PEITC-BITC in MDA-MB-231 cells
Cells were treated with the indicated concentrations of PEITC alone (♦) or BITC alone (■) and combination of PEITC and BITC (▲) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) with relative cell death set to 1 from 3 independent experiments.

Table 3.2. Combinational index of PEITC-BITC in MDA-MB-231 cells.

Combination	PEITC (μM)	BITC (μM)	Combination index (CI)
1	**2**	**2**	**0.334**
2	**4**	**4**	**0.200**
3	**7**	**7**	**0.033**

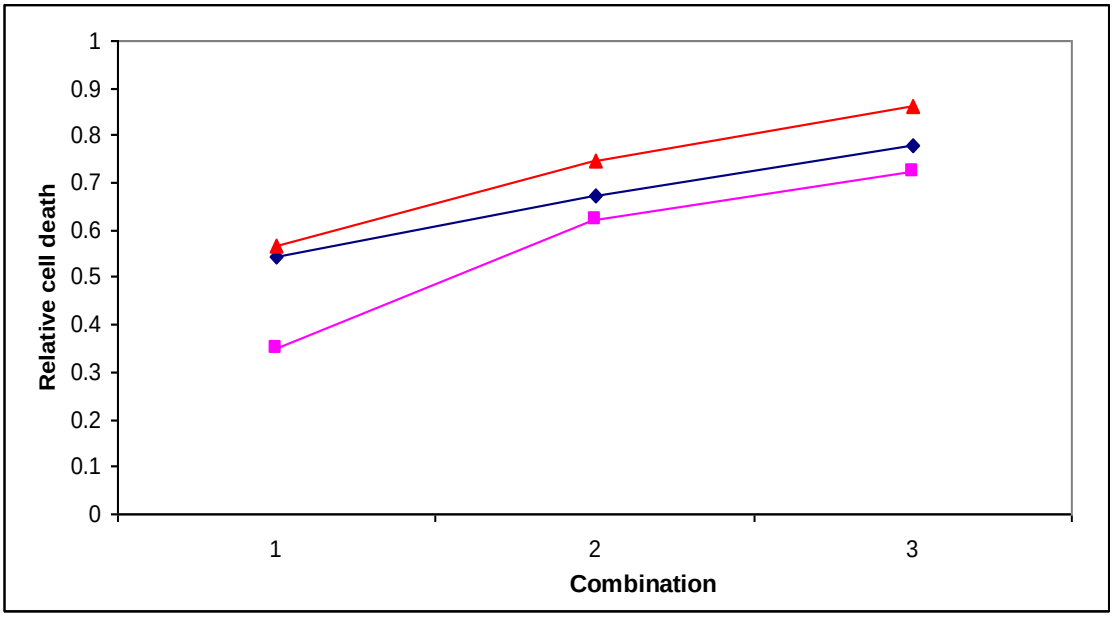


Figure 3.13. Synergistic effect of PEITC-I3C in MDA-MB-231 cells
Cells were treated with the indicated concentrations of PEITC alone (♦) or I3C alone (■) and combination of PEITC and I3C (▲) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) with relative cell death set to 1 from 3 independent experiments.

Table 3.3. Combinational index of PEITC-I3C in MDA-MB-231 cells.

Combination	PEITC (μM)	I3C (μM)	Combination index (CI)
1	**2**	**22**	**1.400**
2	**4**	**44**	**1.250**
3	**7**	**77**	**1.049**

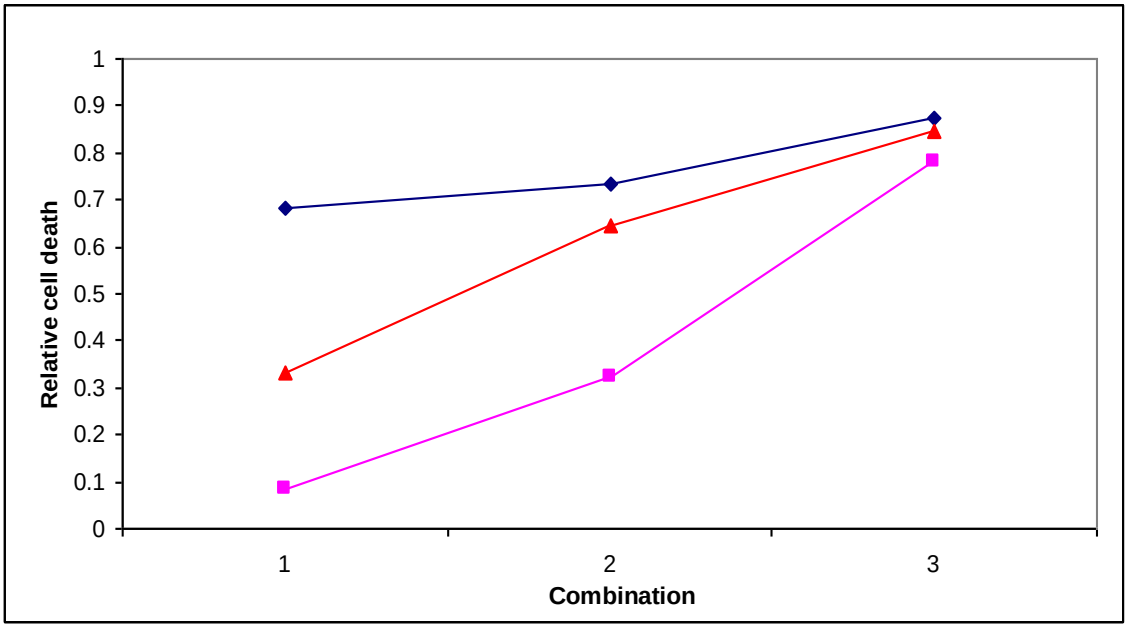


Figure 3.14. Synergistic effect of PEITC-Quercetin in MDA-MB-231 cells
Cells were treated with the indicated concentrations of PEITC alone (♦) or Quercetin alone (■) and combination of PEITC and Quercetin (▲) for 6 days. Cell growth was determined using the MTS assay. Data shown are presented as the means (±SD) with relative cell death set to 1 from 3 independent experiments

Table 3.4. Combinational index of PEITC-Quercetin in MDA-MB-231 cells.

Combination	PEITC (μM)	Quercetin (μM)	Combination index (CI)
1	**2**	**32**	**4.948**
2	**4**	**64**	**2.813**
3	**7**	**112**	**1.903**

3.3 Discussion

The aim of the work described in this chapter was to characterize the growth inhibitory activity of watercress derived compounds in human breast cancer cells prior to detailed molecular analysis. Several different breast cancer cells with different molecular features were chosen to investigate whether compound had a specific pattern of responses towards cell lines representing specific sub-types of disease. Overall, there were clear differences in the potency of different watercress derived compounds. There were also differences in the responses of different breast cancer cells and clear evidence for synergistic activity for some combinations of compounds.

3.3.1 Effect of watercress-derived compounds on growth of breast cancer cells and NHDFs

Growth inhibitory experiments were performed using several human breast cancer cell lines (MDA-MB-231, BT549, T47D, ZR75.1) and normal cell of NHDFs. Results were compared to those from a similar analysis of MCF7 cells and SKBr3 cells within the group (data provided by Breeze Cavell). Overall, PEITC was the most potent watercress- derived compound tested in MDA-MB-231 cells. BITC was approximately equipotent as PEITC, indicating that the ITC-group plays a critical role in the potent growth inhibitory activity of PEITC. This group reacts strongly with nucleophiles such as glutathione in cells (Zhang et al. 2006). Amongst the cell lines, MDA-MB-231 cells were most sensitive to PEITC, and there was a range of responses in the other cell lines so that overall sensitivity varied by more than 5-fold. Although it may be of interest that MDA-MB-231 cells represented triple-negative breast cancer, there was not an obvious relationship between potency and the molecular features of the cell lines (Table 3.1).

The sensitivity of the cancer cells and normal fibroblasts to PEITC was compared. Overall, most cancer cell lines, with the exception of ZR75.1 cells, were more sensitive to PEITC suggesting a degree of selectivity for this compound towards malignant cells. NHDFs have been used as a “normal” comparator in previous studies (Joyner et al. 2006). However, it would be important to examine this further in normal breast epithelial cells reflecting the counterparts of the malignant cells (luminal and basal) in the future.

The potency of PEITC to inhibit the growth of human breast cancer cells *in vitro* was consistent with previous studies. For example, PEITC inhibited the growth of MCF7 cells after 3, 24 and 48 hours exposure with an IC_{50} < 10 μM (Tseng et al. 2004; Xiao et al. 2006). Moreover, MDA-MB-231 cells were more sensitive to PEITC compared to MCF7 cells and PEITC was observed to be approximately 4-fold less cytotoxic towards NHDFs compared to MDA-MB-231 cells (Xiao et al. 2006).

In contrast to PEITC, other phytochemical compounds I3C and Quercetin were less potent growth inhibitory molecules. Interestingly, MCF7 and MDA-MB-231 cells were approximately equally affected by I3C and Quercetin. I3C did not differentially affect NHDF and cancer cell lines, and Quercetin was actually most potent towards NHDFs. Previous studies have demonstrated growth inhibitory effects on I3C or Quercetin in human breast cancer cell lines *in vitro* (Telang et al. 1997; Rahman et al. 2000).

3.3.2 Effect of combinations of watercress-derived compounds on growth of MDA-MB-231 cells

Although these studies have demonstrated differential effects of watercress derived compounds when used alone, when consumed as part of a diet, individuals will clearly be exposed to multiple compounds which may act together to synergistically induced growth inhibition. On the other hand, compounds may act antagonistically to reduce the overall growth inhibitory activity. It was important to investigate the effects of combinations of compounds on growth inhibition. Therefore the combinations of I3C or Quercetin with PEITC were studied in MDA-MB-231 cells, since PEITC was the most potent compound when tested alone and MDA-MB-231 cells were the most sensitive to the growth inhibitory effects of PEITC.

The results demonstrated clear interactions between the compounds. Interestingly, PEITC and Quercetin, and PEITC and I3C showed strong and modest antagonistic activity, respectively. Surprisingly, PEITC and BITC showed strong synergistic activity under some conditions. This implies that these closely structurally related ITCs must have some mechanistic differences in their effects on MDA-MB-231 cells.

This work has characterized the growth inhibitory effects of watercress-derived compounds in a small panel of cell lines. Based on these findings, future studies will

focus on understanding the molecular mechanisms by which PEITC induced growth inhibition in breast cancer cells. As models, this experiment will mainly focused on MDA-MB-231 and MCF7 cells since they represent very commonly studied cell lines. Since PEITC did not synergize with I3C or Quercetin, combinations of compounds were not studied further.

Chapter 4

Molecular analysis of human breast cancer cells treated with PEITC

4.1 INTRODUCTION

Work described in the previous chapter demonstrated that, of the compounds tested, PEITC was a relatively potent inducer of growth inhibition in breast cancer cell lines. To further understand the molecular mechanisms that mediate anti-cancer effects of PEITC, detailed studies were performed using MDA-MB-231 and MCF7 cells. These cells were approximately 2-fold differentially sensitive to PEITC and represent different sub-types of breast cancer (for example; triple negative versus ER positive). These cell lines are also widely used in studies of breast cancer. The aim of this work was to investigate the effects of PEITC on cell cycle and apoptosis in these cells, and on molecular regulators of these pathways.

4.2 RESULTS

4.2.1 PEITC induced cell cycle arrest in breast cancer cells

Propidium iodide staining was used to determine the effects of PEITC on cell cycle distribution in MCF7 and MDA-MB-231 cells.

In MDA-MB-231 cells (Figures 4.1 and 4.2), PEITC (5-20 μM) induced a time dependent increase in the proportion of cells in the G2/M phases of the cell cycle and a reduction in the proportion of cells in G1. PEITC also significantly increased the proportion of cells with a sub-G1 DNA content, indicative of cell death, especially at 48 hours. There was no clear relationship with dose for response to PEITC.

In MCF7 cells (Figures 4.3 and 4.4), PEITC induced a time dependent increase in the proportion of cells in the G1 phase of the cell cycle and a reduction in the proportion of cells in G2/M. In these experiments, PEITC was used over a higher concentration range (10-30 μM) due to reduced sensitivity of MCF7 cells compared to MDA-MB-231 cells. In contrast, to MDA-MB-231 cells, PEITC induced relatively low amounts of cells with sub-G1 DNA content. Again, there was no clear relationship with dose for response to PEITC.

Therefore, cell cycle effects of PEITC differ between MCF7 and MDA-MB-231 cells. Whereas PEITC induces a predominantly G2/M arrest in MDA-MB-231 cells, in MCF7 cells the major effect is an increase in the proportion of cells with a G1 DNA content. Analysis of sub-G1 DNA content suggests a much higher level of PEITC-induced cell death in MDA-MB-231 cells.

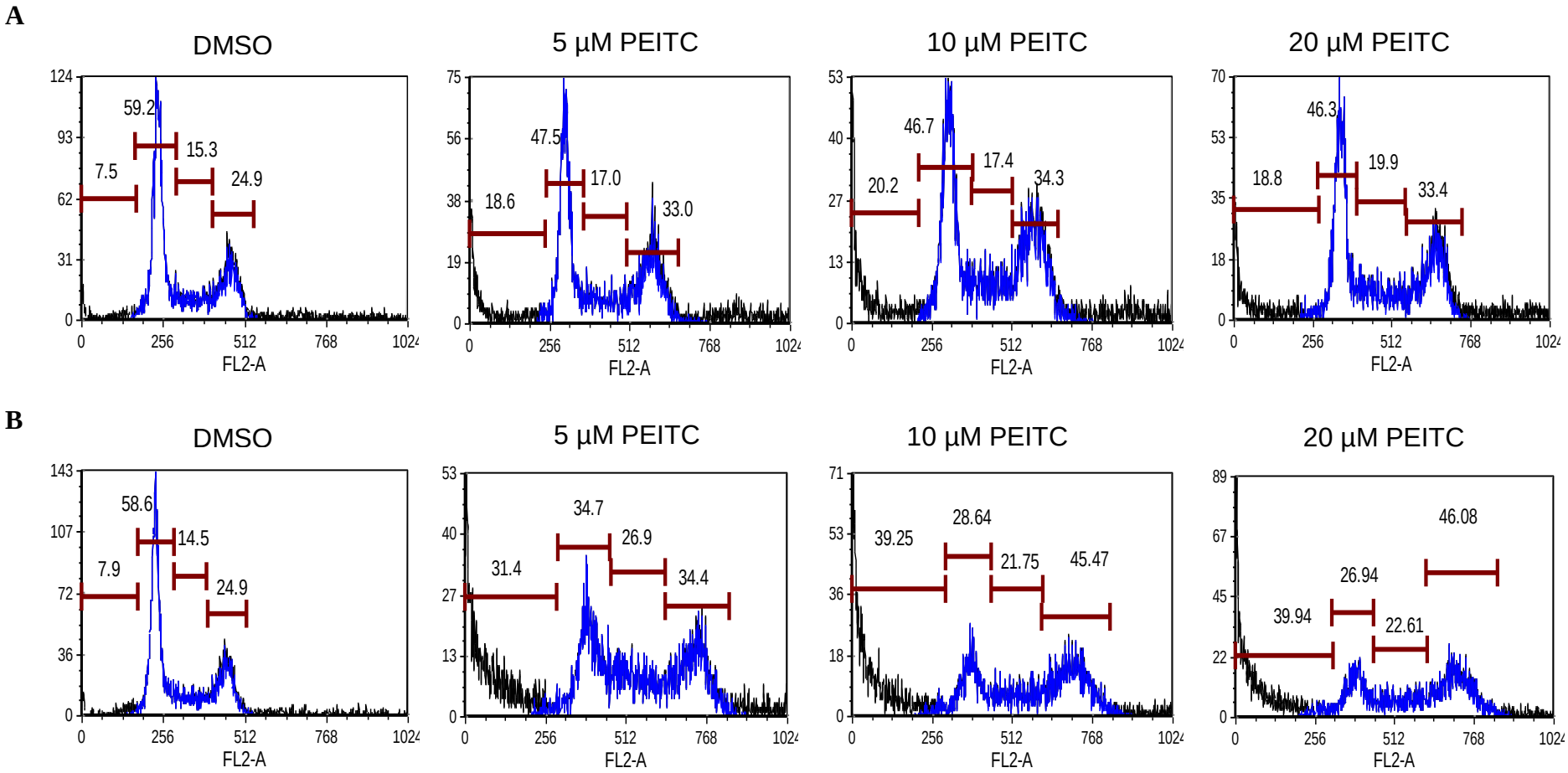


Figure 4.1. PEITC induced cell cycle arrest in MDA-MB-231 cells. MDA-MB-231 cells treated with PEITC at different concentrations and time points; 24 hours [A] and 48 hours [B]. Propidium iodide staining was determined via flow cytometry. The x axis FL2A shows red fluorochrome for PI labelling. Blue line indicates single cells population which has been set in the dot plot graph (Data not shown). Data shown above are representative of three independent experiments.

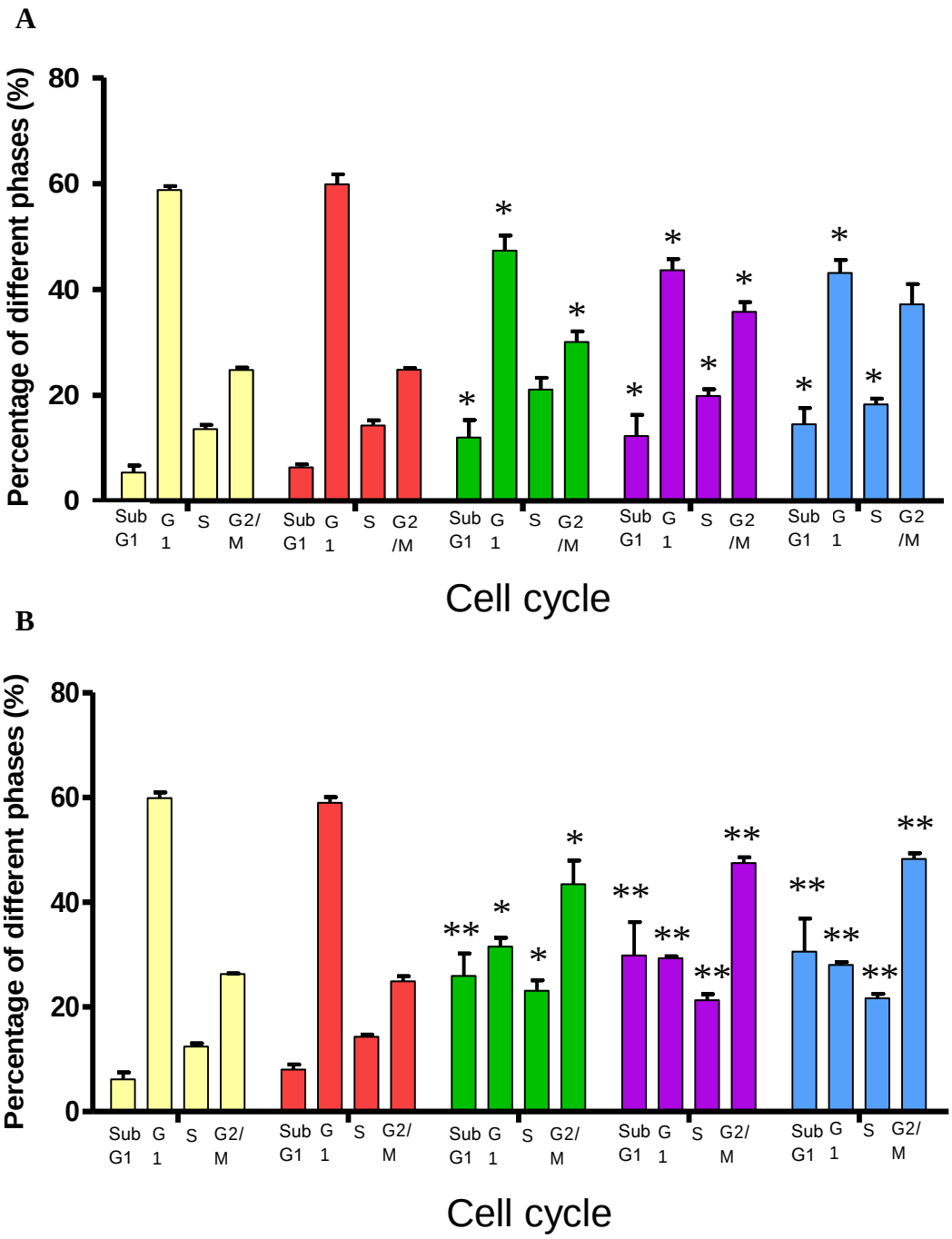


Figure 4.2. Quantitation of cell cycle arrest in MDA-MB-231 cells treated PEITC for indicated time points (24 [A] and 48 hours [B]). Untreated (), DMSO (), 5μM PEITC (), 10 μM PEITC ( ), 20 μM PEITC (). Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$). All other differences were not statistically significant. Data shown are the average of three dependent experiments.

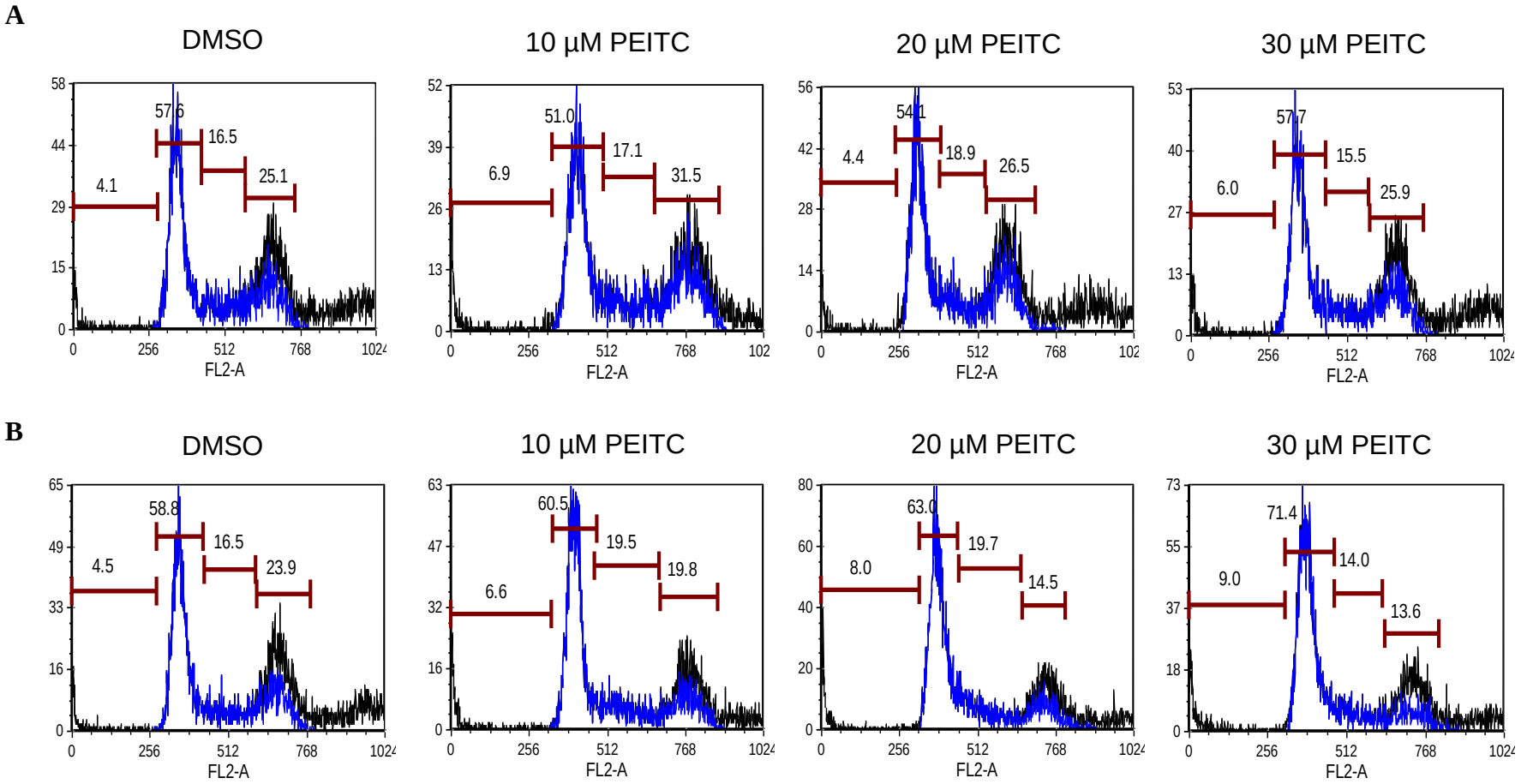


Figure 4.3. PEITC induced cell cycle arrest in MCF7 cells. MCF7 cells treated with PEITC at different concentrations and time points; 24 hours [A] and 48 hours [B]. Propidium iodide staining was determined via flow cytometry. The x axis FL2A shows red fluorochrome for PI labelling. Blue line indicates single cells population which has been set in the dot plot graph (Data not shown). Data shown above are representative of three independent experiments.

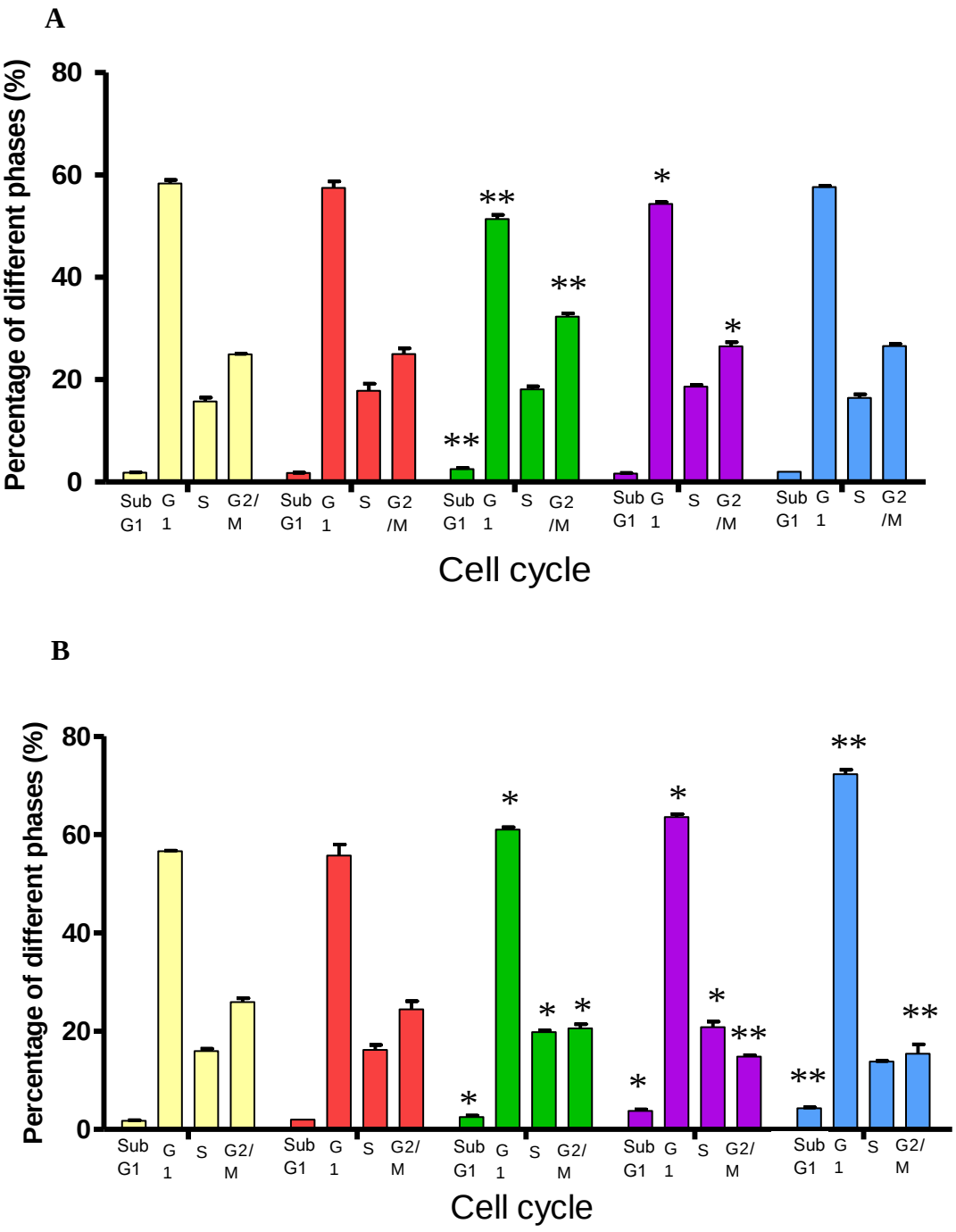


Figure 4.4. Quantitation of cell cycle arrest in MCF7 cells treated PEITC for indicated time points (24 [A] and 48 hours [B]). Untreated ( ), DMSO ( ), 10µM PEITC ( ), 20µM PEITC ( ), 30µM PEITC ( ). Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$). All other differences were not statistically significant. Data shown are the average of three independent experiments.

4.2.2 PEITC induced apoptosis in breast cancer cells

To confirm that differences in PEITC-induced sub-G1 cells were due to apoptosis, PEITC treated cells were analysed using annexin V staining. Annexin V has a high affinity for phosphatidylserine (PS) exposed on the surface of apoptotic cells. Propidium iodide (PI), which stains nucleic acids, was used to measure plasma membrane permeability (Basco et al. 2000).

In MDA-MB-231, PEITC induced statistically significant increases in the proportion of both early and later apoptotic cells, especially at 48 hours (Figures 4.5 and 4.6). Again, there was not a clear dose-responsive effect. In MCF7 cells, the levels of apoptosis induced by PEITC were very modest, at all times and concentrations (Figure 4.7 and 4.8).

Therefore, the differences in growth inhibitory effects of PEITC in MDA-MB-231 and MCF7 cells predominantly appear to reflect differences in susceptibility to PEITC-induced apoptosis, although there are also differences in the specific effect of the compound on cell cycle phases in the two cell lines.

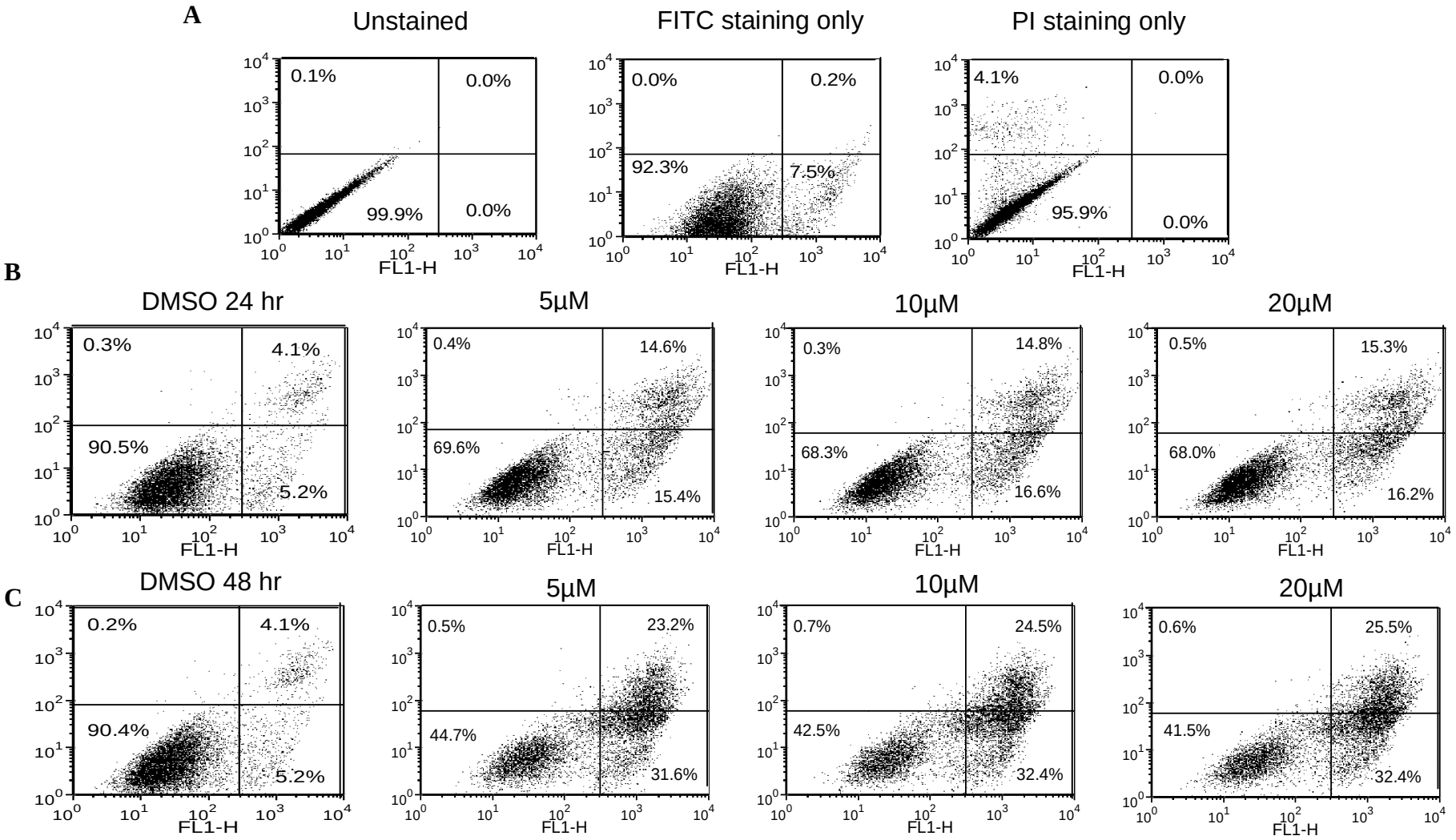


Figure 4.5. PEITC induced apoptosis in MDA-MB-231 cells. MDA-MB-231 cells treated with PEITC at different concentrations and time points; 24 hours [B] and 48 hours [C]. [A] represents the unstained, FITC staining only and PI staining only. Annexin V and propidium iodide staining were determined via flow cytometry. The x axis shows FL1-H (log) for annexin V labeling and the y axis shows FL2-H (log) with red fluorochrome for PI labeling. Data shown are representative of three independent experiments.

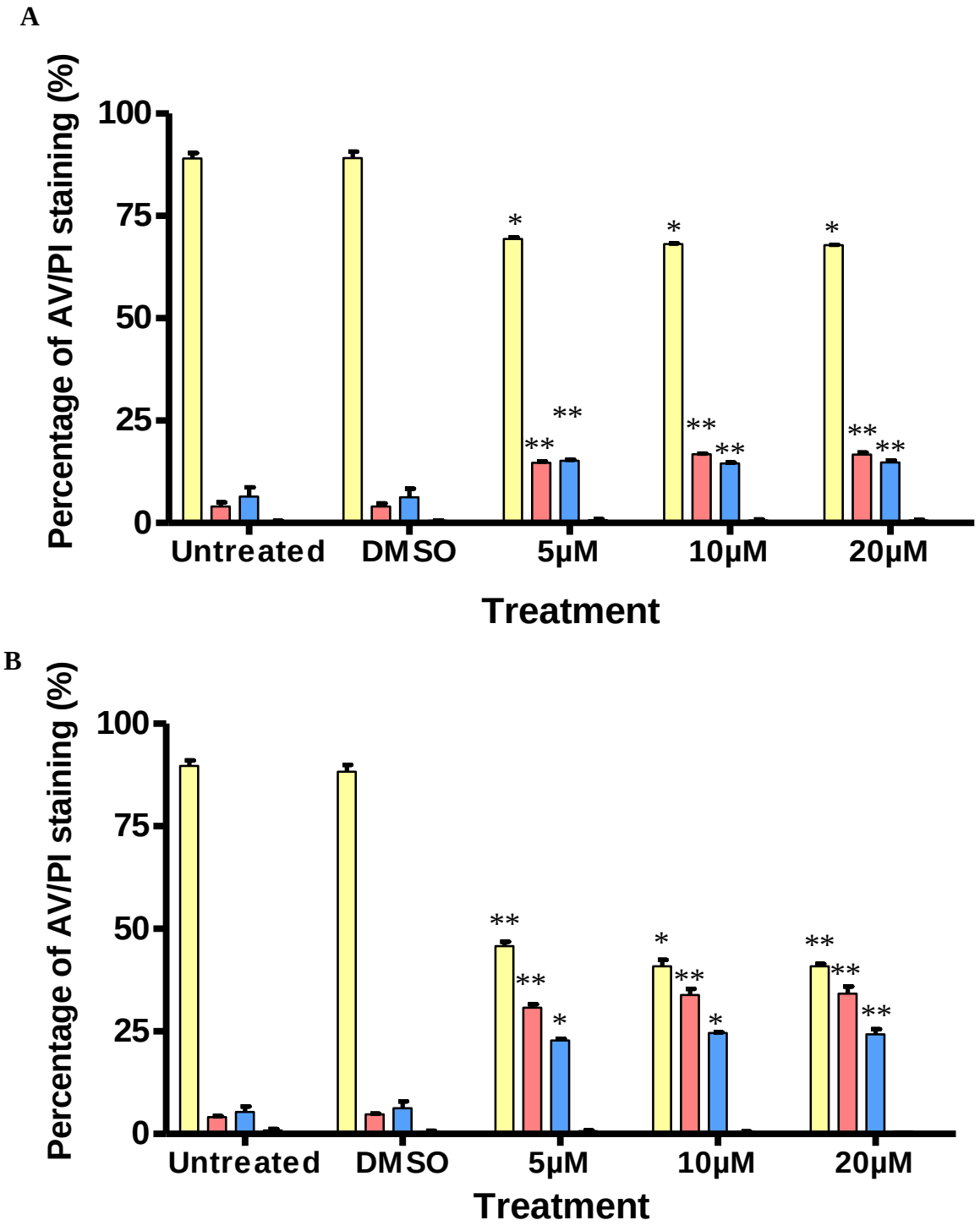


Figure 4.6. Quantitation of apoptosis in MDA-MB-231 cells treated PEITC for indicated time points (24 [A] and 48 hours [B]). AV-/PI- ( ), AV+/PI- ( ), AV+/PI+ ( ) and AV-/PI+ ( ).Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$). All other differences were not statistically significant. Data shown are the average of three independent experiments.

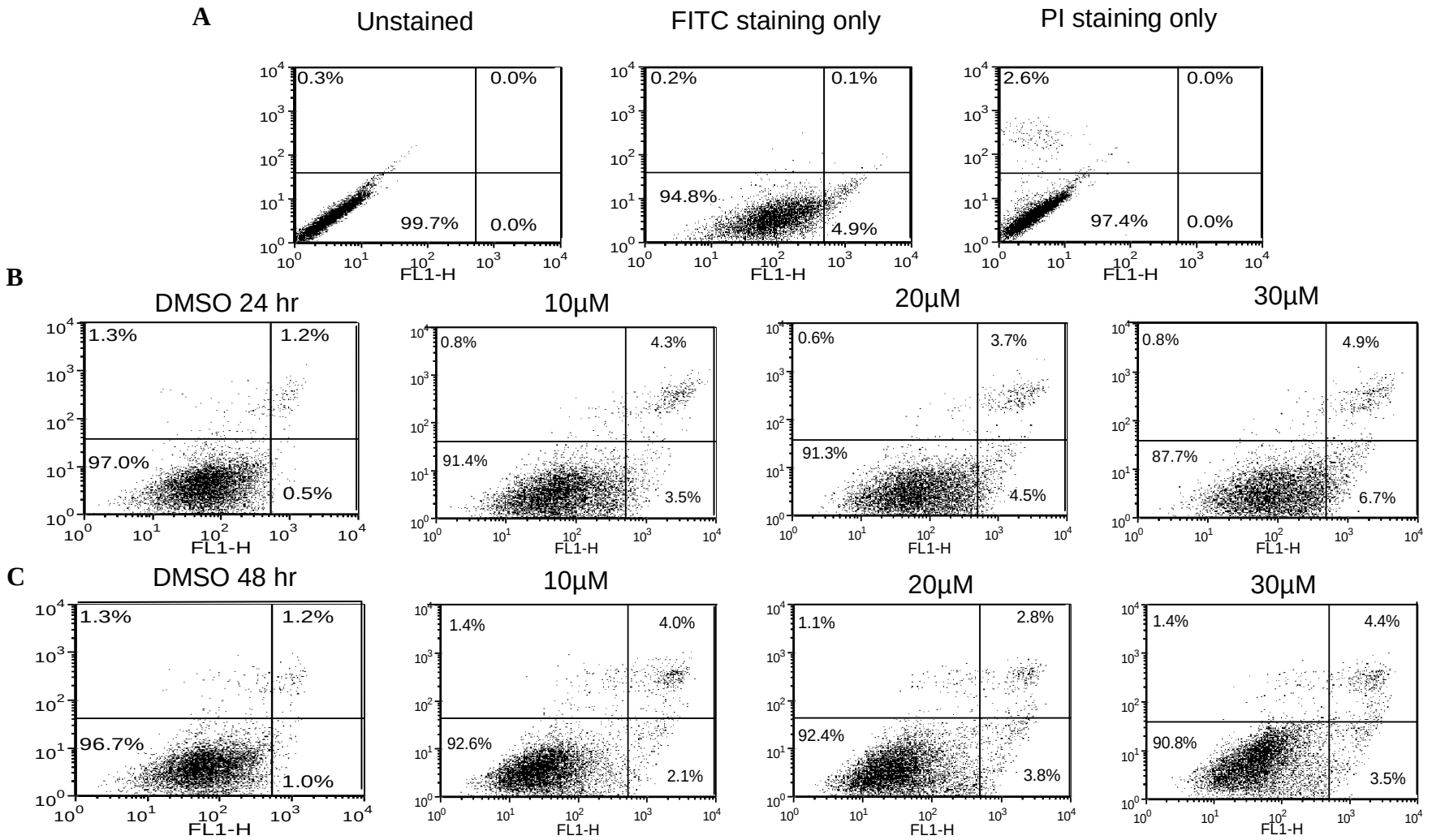


Figure 4.7. PEITC induced apoptosis in MCF7 cells. MCF7 cells treated with PEITC at different concentrations and time points; 24 hours [B] and 48 hours [C]. [A] represents the unstained, FITC staining only and PI staining only. Annexin V and propidium iodide staining were determined via flow cytometry. The x axis shows FL1-H (log) for annexin V labeling and the y axis shows FL2-H (log) with red fluorochrome for PI labeling. Data shown are representative of three independent experiments.

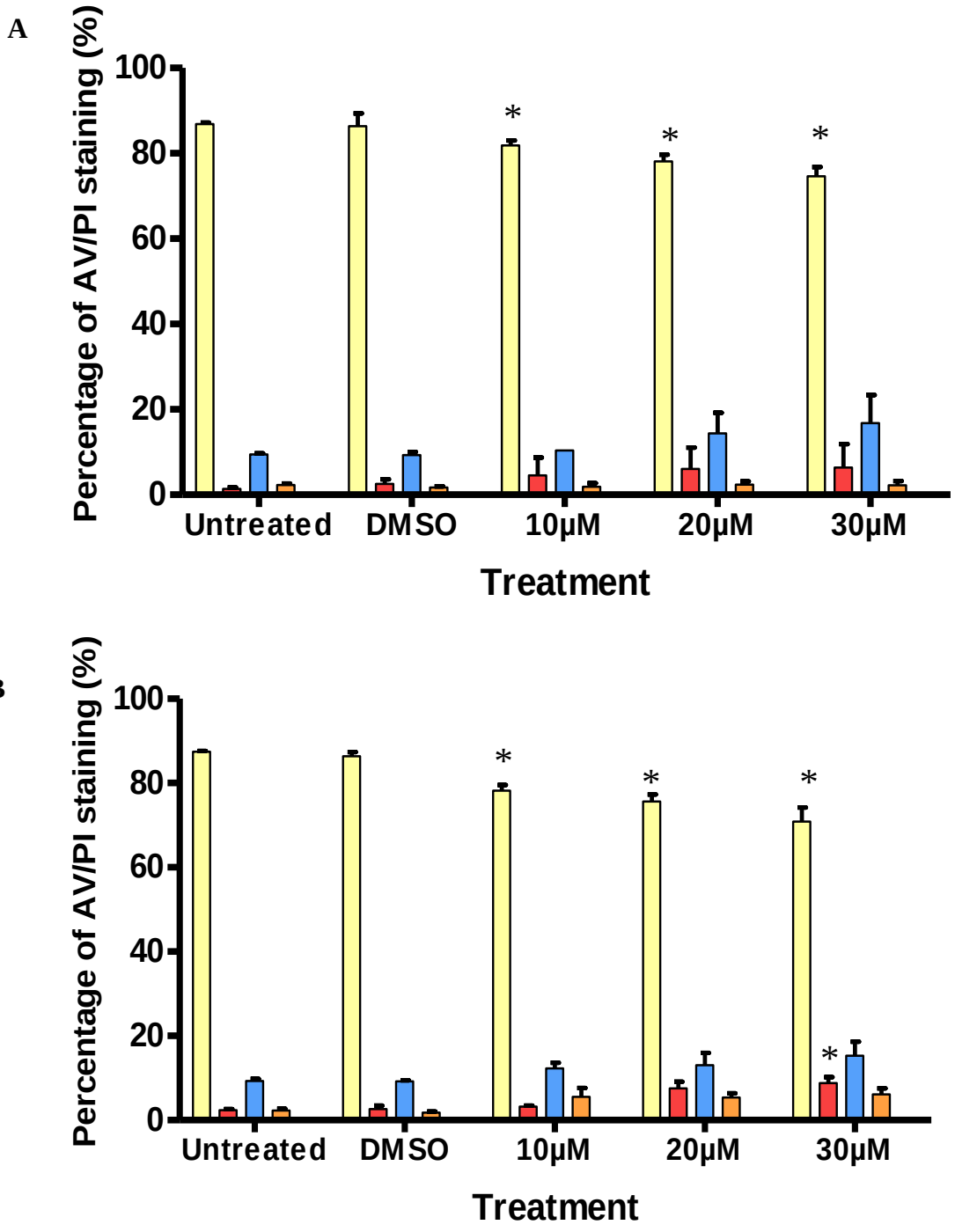


Figure 4.8. Quantitation of apoptosis in MCF7 cells treated PEITC for indicated time points (24 [A] and 48 hours [B]). AV-/PI- (□), AV+/PI- (□), AV+/PI+ (□) and AV-/PI+ (□). Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$). All other differences were not statistically significant. Data shown are the average of three independent experiments.

4.2.3 Activation of caspases by PEITC in MDA-MB-231 cells.

To further investigate pathways of which PEITC induced apoptosis in MDA-MB-231 cells, the activation of caspases, key mediators of apoptosis was analyzed (Alnemri et al. 1996; Earnshaw et al. 1999). Activation of the initiator caspases caspase-8 and caspase-9 and the effector caspase caspase-3 was measured using immunoblotting and the results quantified by image analysis (Figures 4.9). The antibodies used detected procaspase 9 and active caspase 9, active caspase 3 and procaspase 8 and active caspase 8 (http://datasheets.scbt.com/sc-5263.pdf). Studies were performed up to 24 hours, a time point that precedes maximum exposure of phosphatidylserine (PS).

Caspase 9 activation was increased significantly at 2 hours and peaked after 6 hours post-treatment after addition of PEITC (Figure 4.10 [A]). Following caspase-9 activation, active caspase 3 was detected to increase significantly over a similar time frame (Figure 4.10 [B]). By contrast, although activation of caspase 8 was not detected there was a significant decrease in expression of procaspase 8 at later hours compared to earlier activation of caspase-9 and -3 upon PEITC treatment (Figure 4.10 [D]). These results confirm that PEITC induces apoptosis in MDA-MB-231 cells. The pattern of caspases activation is indicative of activation of the intrinsic cell death pathway.

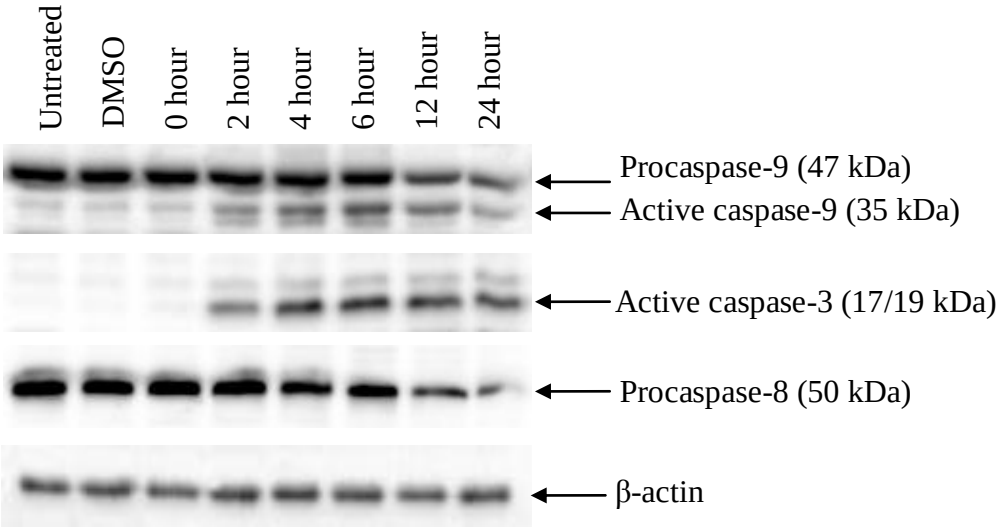
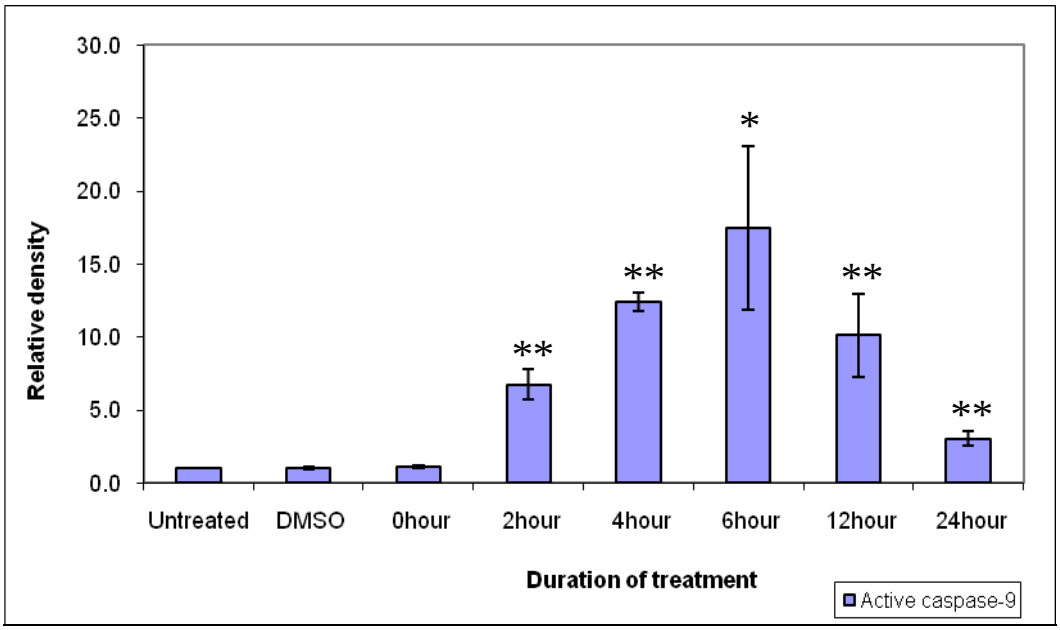


Figure 4.9. Effect of PEITC on caspase-9, -3 and -8 activation in MDA-MB-231 cells. MDA-MB-231 cells were left untreated or treated with vehicle-control (DMSO) or treated with 20 μM PEITC for indicated time points. Cell lysates were then assayed for caspase 3 and 8 activation via immunoblotting. β-actin acts as a loading control. Data shown are representative of two independent experiments.

A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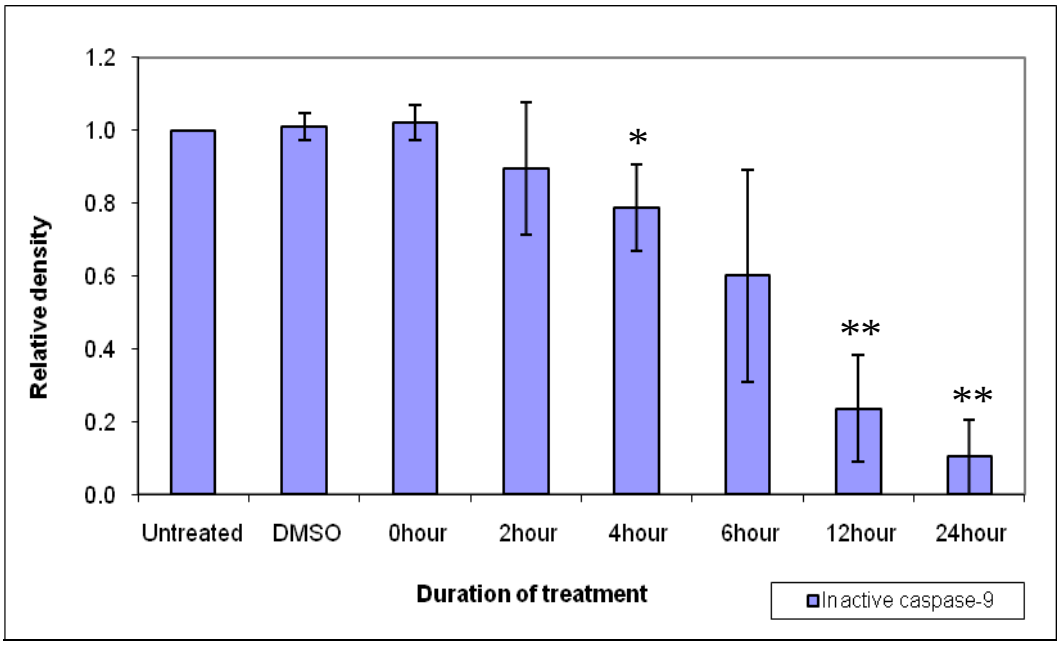
24hour
Duration of treatment
Active caspase-9

B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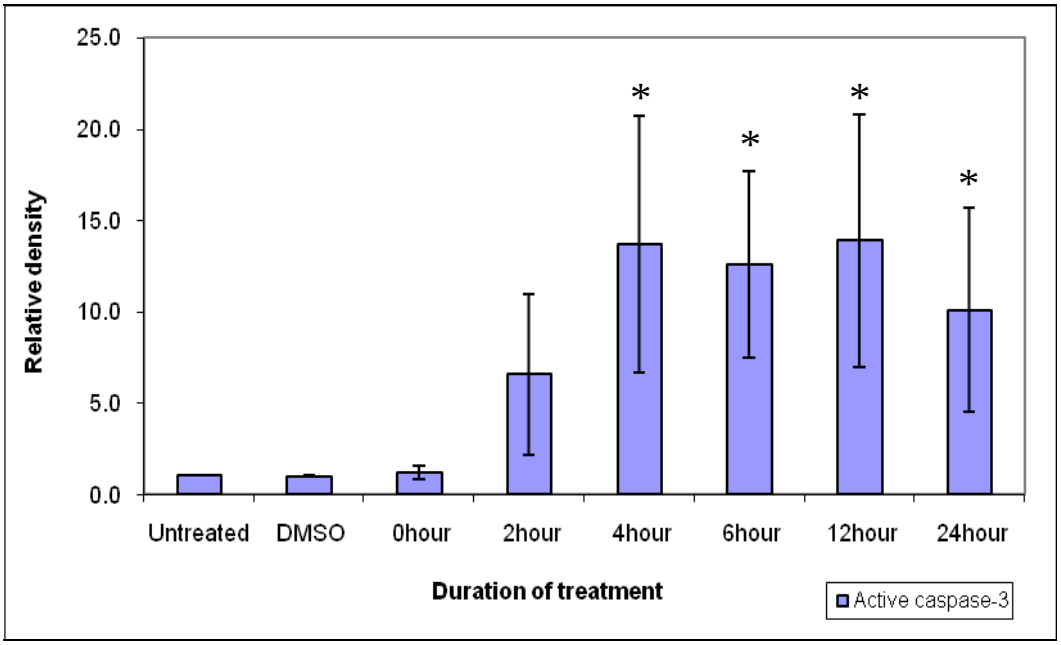
12hour
24hour
Duration of treatment
Inactive caspase-9

C



D

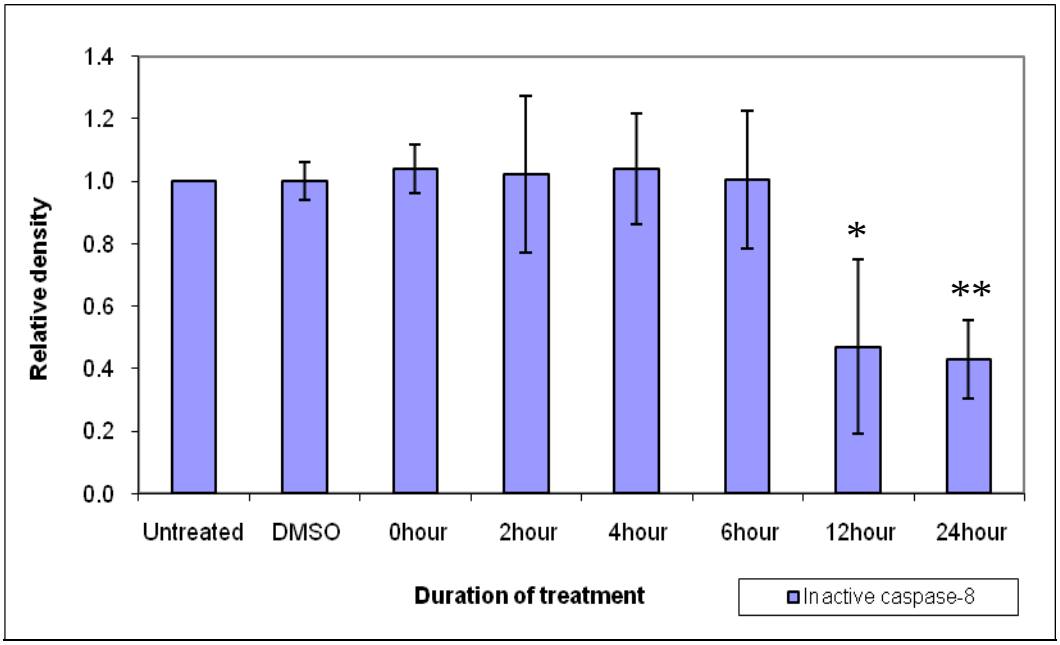


Figure 4.10. Quantitation of caspase-9 [A], procaspase-9 [B] caspase-3 [C] and procaspase-8 [D] expression in MDA-MB-231 cells treated PEITC. Data shown are means of two independent determination (±SD), normalized to expression of β-actin. Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$).

4.2.4 Activation of caspases in MCF7 cells treated PEITC

Similar experiments were performed in MCF7 cells. However, caspase 7 was analysed in place of caspase 3 since MCF7 cells lack caspase 3 expression. Overall, there was no evidence for active caspases in PEITC-treated cells. However, there was a gradual decline in the levels of procaspases that was not significant until 12 to 24 hour PEITC treatment (Figure 4.11; 4.12). These results are consistent with the Annexin V data, demonstrating low level activation of apoptosis by PEITC in MCF7 cells. The active forms of all caspases were not observed in MCF7 cells-treated PEITC.

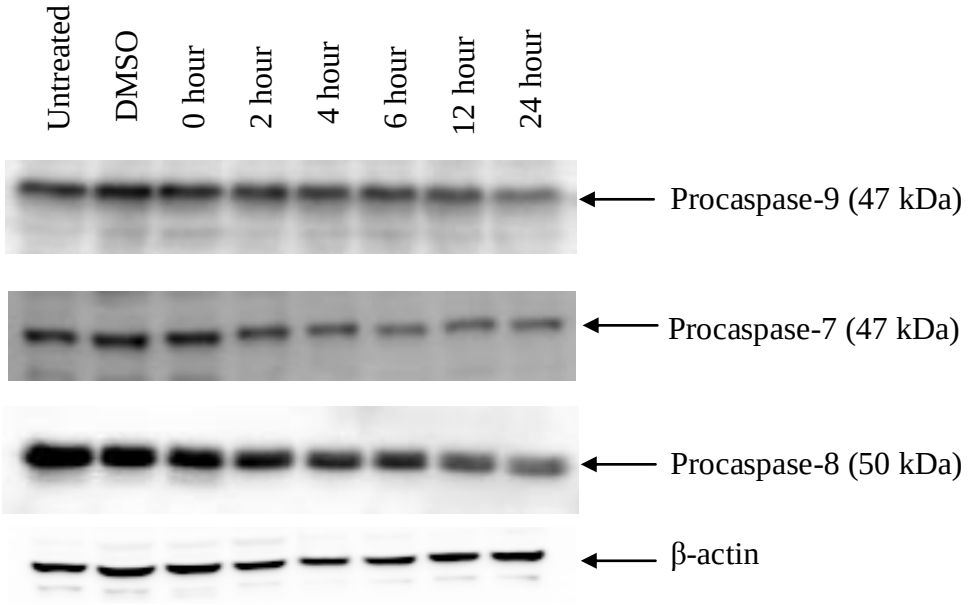
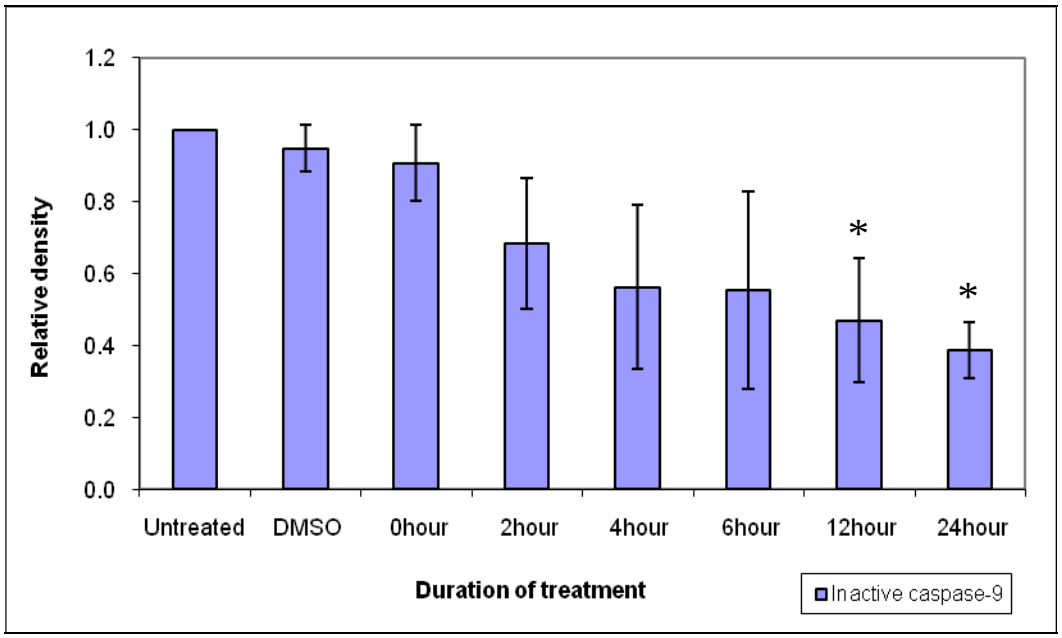


Figure 4.11. Effect of PEITC on procaspase-9,-7 and -8 in MCF7 cells. MCF7 cells were left untreated or treated with vehicle-control (DMSO) or treated with 20 μM PEITC for indicated time points. Cell lysates were then assayed for caspase 3 and 8 activation via immunoblotting. β-actin acts as a loading control. Data shown are representative of two independent experiments.

A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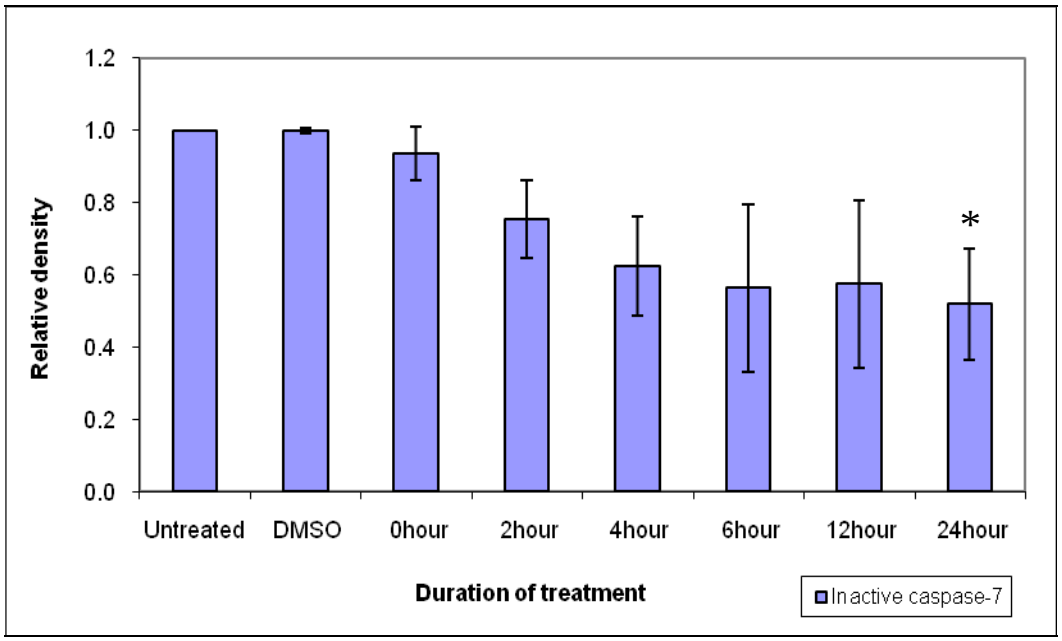
24hour
Duration of treatment
Inactive caspase-9

B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12hour
24hour
Duration of treatment
Inactive caspase-7

C

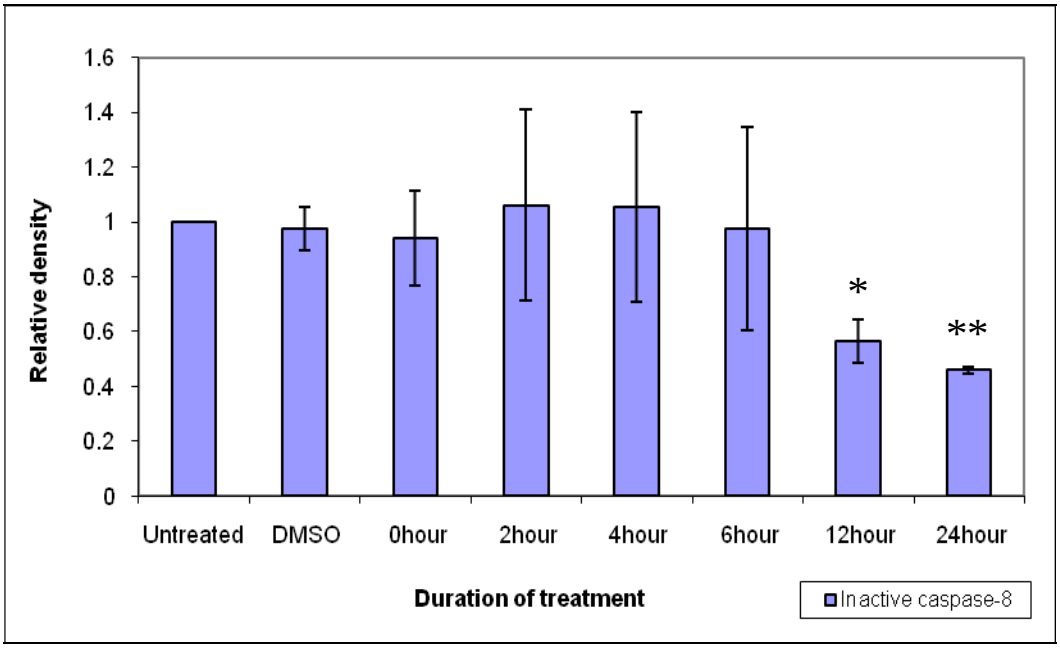


Figure 4.12. Quantitation of procaspase-9 [A], procaspase-7 [B] and procaspase-8 [C] expression in MCF7 cells treated PEITC. Data shown are means of two independent determinations (±SD), normalized to expression of β-actin. Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$)

4.2.5 Regulation of Bax in PEITC treated MDA-MB-231 and MCF7 cells

Bax is a pro-apoptotic Bcl-2 family protein that plays a role in release of cytochrome c from the mitochondria, a critical event for the activation of caspase 9 and the intrinsic apoptosis pathway. Immunoblot analysis of PEITC (20 µM) treated MDA-MB-231 cells demonstrated a modest but statistically significant increase in Bax expression at 2 - 6 hours as well as 24 hours (Figure 4.13; 4.14 [A]). By contrast, expression of the anti-apoptotic Bcl-2 protein was not altered by PEITC (Figure 4.13; 4.14 [B]). Therefore, PEITC-mediated apoptosis was associated with increased expression of Bax in MDA-MB-231 cells. There was some evidence for a biphasic response peaking at 4/6 hours, and again at 24 hours.

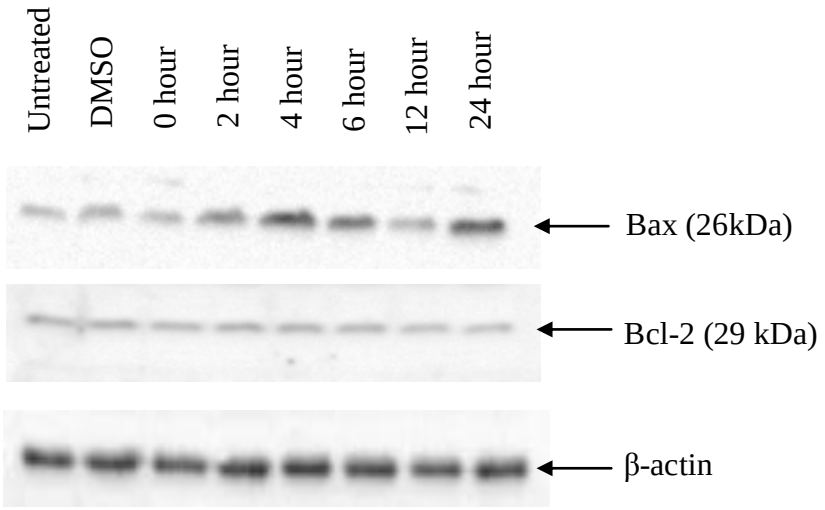
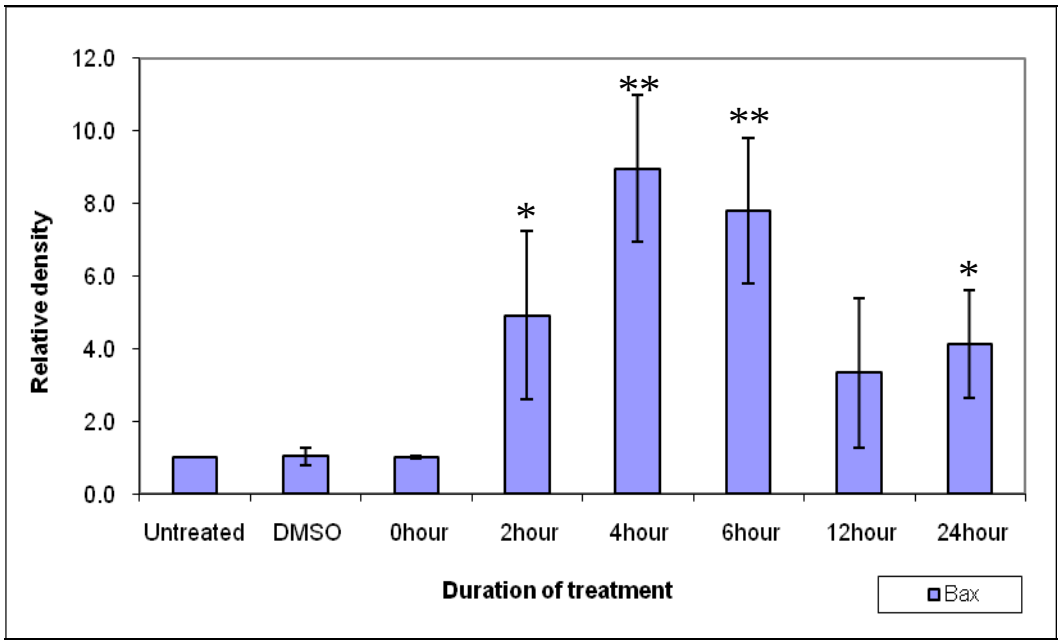


Figure 4.13. Effect of PEITC on the expression of Bax and Bcl2 in MDA-MB-231 cells. MDA-MB-231 cells were left untreated or treated with vehicle-control (DMSO) or treated with 20 µM PEITC for indicated time points. Cell lysates were then assayed for Bax and Bcl-2 regulation via immunoblotting. β -actin acts as a loading control. Data shown are representative of three independent experiments.

A



B

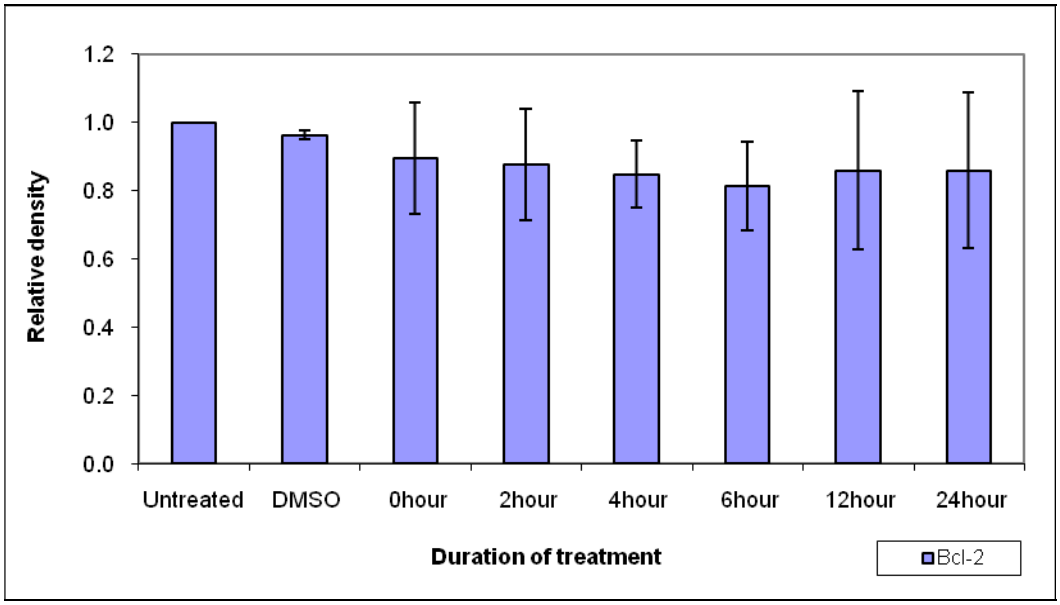


Figure 4.14. Quantitation of Bax [A] and Bcl2 [B] expression level in MDA-MB-231 cells treated PEITC. Data shown are means of three independent determination (±SD), normalized to expression of β-actin. Statistically significant differences between DMSO and PEITC treated cells are indicated (**$p < 0.05$; ** $p < 0.005$).

Similar experiments were performed in MCF7 cells. There was an increase in Bax expression in MCF7 cells, but this was observed only at 24 hours, and not at earlier time points (Figure 4.15; 4.16 [A]). PEITC did not affect the Bcl2 expression level in MCF7 cells (Figure 4.15; 4.16 [B]).

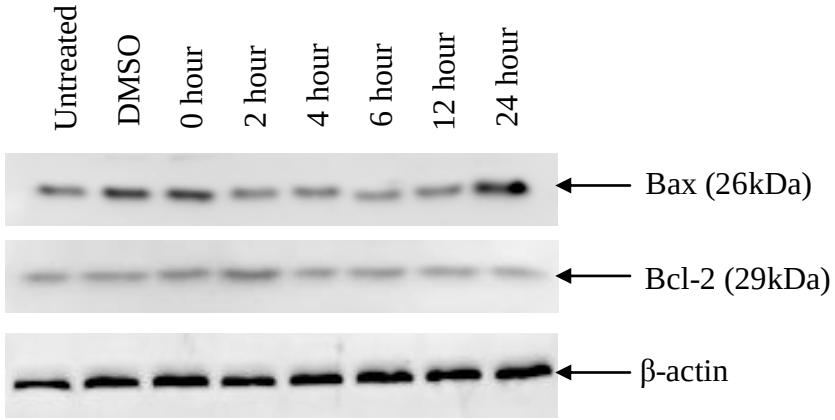


Figure 4.15. Effect of PEITC on the expression of Bax in MCF7 cells. MCF7 cells were left untreated or treated with vehicle-control (DMSO) or treated with 20 μM PEITC for indicated time points. Cell lysates were then assayed for Bax and Bcl-2 regulation via immunoblotting. β -actin acts as a loading control. Data shown are representative of three independent experiments.

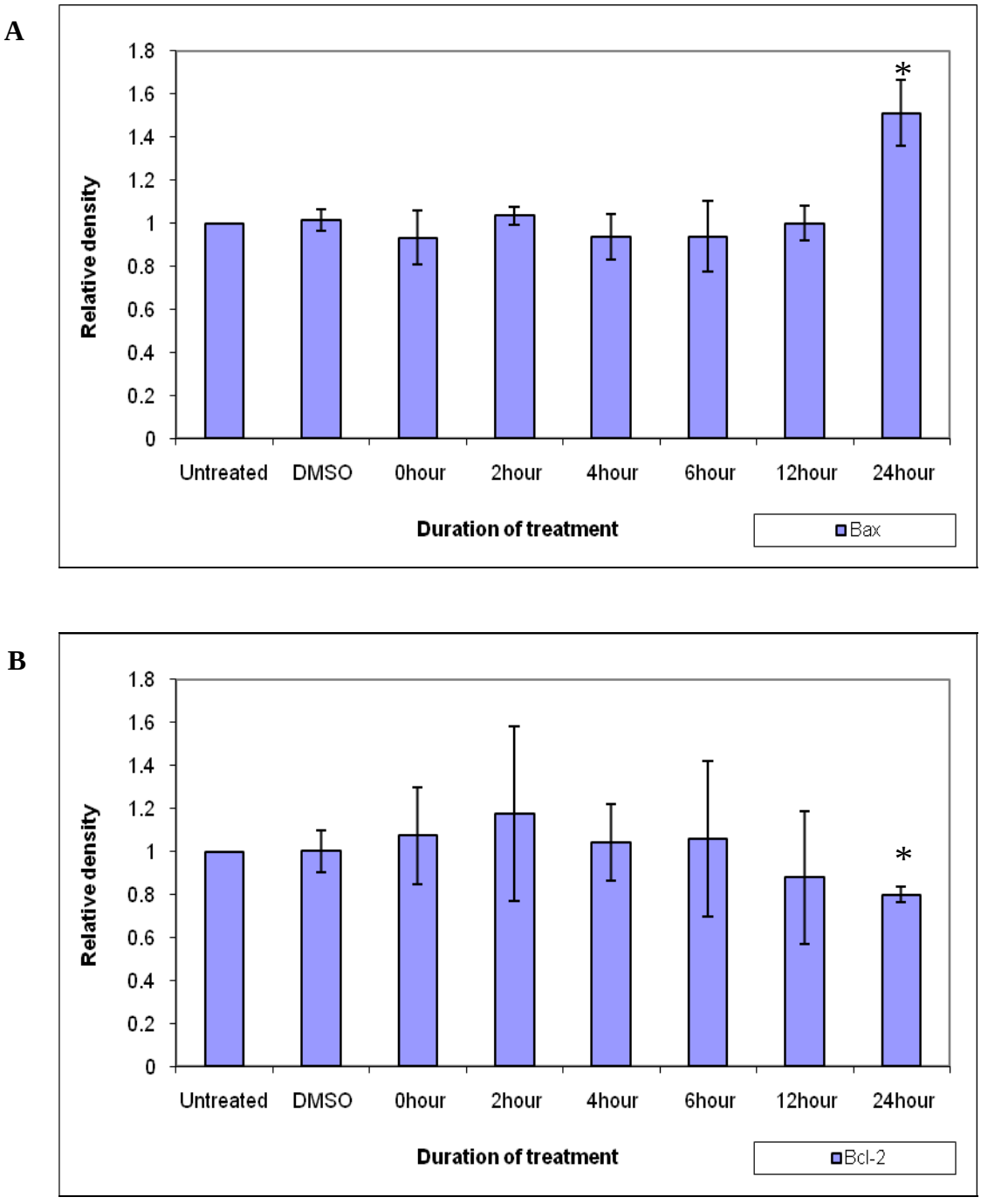


Figure 4.16. Quantitation of Bax [A] and Bcl2 [B] expression level in MCF7 cells treated PEITC. Data shown are means of three independent determination (±SD), normalized to expression of β-actin. Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$).

4.2.6 Effect of PEITC on ROS in MDA-MB-231 cells

Since it has been reported that PEITC can induce ROS in certain cancer cells, I investigated ROS levels in PEITC-treated MDA-MB-231 cells. ROS levels were analysed using the cell permeant, oxidation sensitive dye dihydrorhodamine (DHR123) which is non-fluorescent until oxidized by ROS.

A series of pilot experiments were conducted to determine the optimal PEITC (up to 30 µM) concentrations and treatment time (up to 24 hours) for subsequent detailed studies. For detailed experiments shown here, MDA-MB-231 cells were treated with different concentrations of PEITC (5 µM, 20 µM and 30 µM) and incubated at 3 different time points (3, 4, and 5 hours). The intensity of the fluorescence was determined via flow cytometry. PEITC treatment increased ROS levels in MDA-MB-231 cells. However, the effects of PEITC on ROS were complex (Figure 4.17).

Treatment of MDA-MB-231 cells with 5 µM PEITC decreased ROS levels over 3 to 5 hours (apparent from the appearance of a second cell population with decreased fluorescence). At higher concentration of PEITC (20 µM), an increase in ROS was observed at 4 hours post-treatment, which returned to background levels at 5 hours. However, at 30 µM of PEITC, no increase in ROS was observed at any time point since the ROS peak was overlapping with that of DMSO-treated cells throughout the experiment (Figure 4.17).

In summary, despite the initial reduction of ROS at lower concentration of PEITC (5 µM), the level of ROS was increased in a time and concentration dependent and peaked at concentration of 20 µM after 4 hours treatment.

4.2.7 Effect of PEITC on ROS in MCF7 cells

Similar experiments were performed using MCF7 cells. In contrast to MDA cells there were no changes in fluorescence in PEITC treated cells at any time or concentration (Figure 4.18). Therefore, PEITC does not appear to elicit alterations in ROS in MCF7 cells.

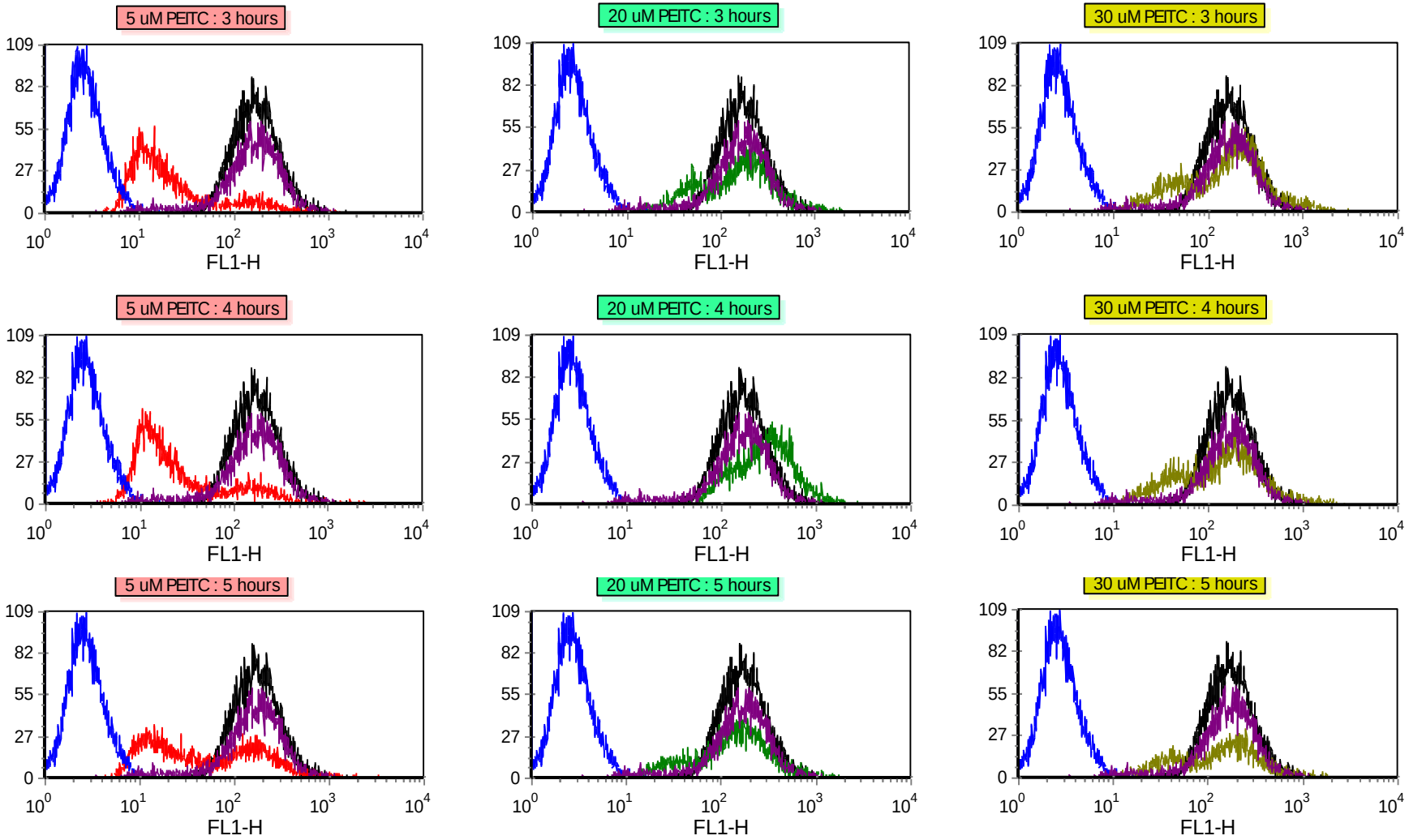


Figure 4.17. Effect of PEITC on the accumulation of ROS in MDA-MB-231 cells. MDA-MB-231 cells were treated with indicated concentrations of PEITC for 3, 4 and 5 hours. The DHR123 staining was determined by flow cytometry. Unstained cells ( ); untreated cells ( ); DMSO ( ); 5 µM PEITC ( ); 20 µM PEITC ( ); 30 µM PEITC ( ). Data shown are representative of three independent experiments.

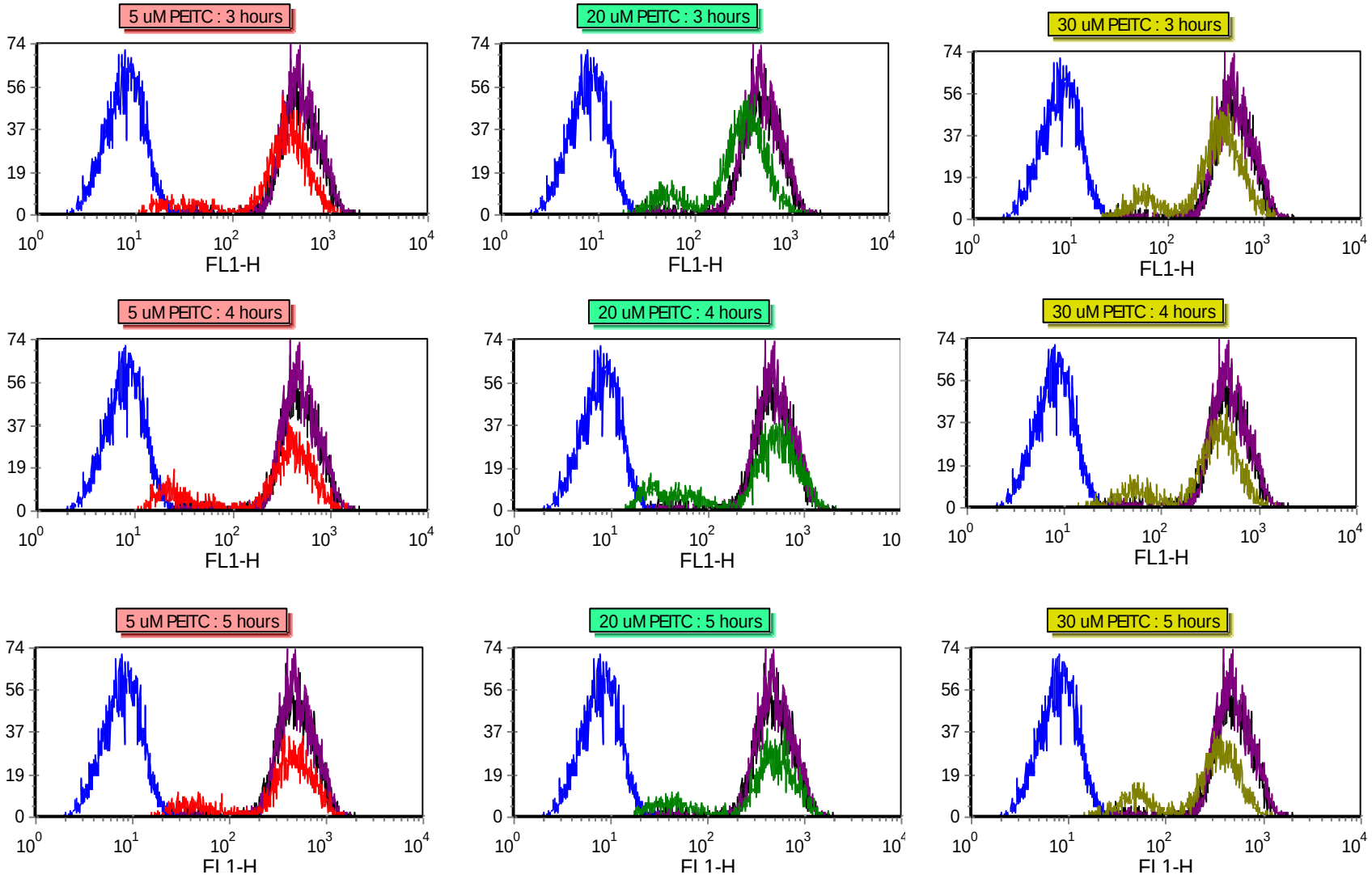


Figure 4.18. Effect of PEITC on the accumulation of ROS in MCF7 cells. MCF7 cells were treated with indicated concentrations of PEITC for 3, 4 and 5 hours. The DHR123 staining was determined by flow cytometry. Unstained cells ( ); untreated cells ( ); DMSO ( ); 5 µM PEITC ( ); 20 µM PEITC ( ); 30 µM PEITC ( ).Data shown are representative of three independent experiments

4.2.8 Effect of PEITC on the expression level of Nrf2 and Keap1 in MDA-MB-231 and MCF7 cells

PEITC can modulate the Nrf2/Keap1 signalling pathway, a major oxidant sensing pathway (Wakabayashi et al. 2004; Dinkova-Kostova et al. 2002). Since it has been demonstrated that there was increased ROS in MDA-MB-231 cells after treated with PEITC (at least at some time points and concentrations), the expression level of Nrf2 was analysed as an alternate read-out of oxidative stress. Western blot analysis of MDA-MB-231 cells showed the expression of Nrf2 was up-regulated by PEITC in MDA-MB-231 cells treated with 20 μM PEITC at 4 and 6 hours (Figure 4.19; 4.20 [A]). Further analysis demonstrated there were no obvious changes in Keap1 expression level in MDA-MB-231 cells treated with similar concentrations and time points (Figure 4.19; 4.20 [B]). Therefore, PEITC leads to increased Nrf2 expression in MDA-MB-231 cells, consistent with the increase in ROS in PEITC-treated cells under some condition

In MCF7 cells, Nrf2 and Keap1 expression levels declined following treatment with PEITC, although the changes overall were not significant. (Figure 4.21; 4.22 [A] & [B]). Comparison of Nrf2 expression levels between MDA-MB-231 and MCF7 cells shows that basal levels of Nrf2 are higher in MCF7 cells compared to MDA-MB-231 (Figure 4.23) and that PEITC treatment confirms previous figures.

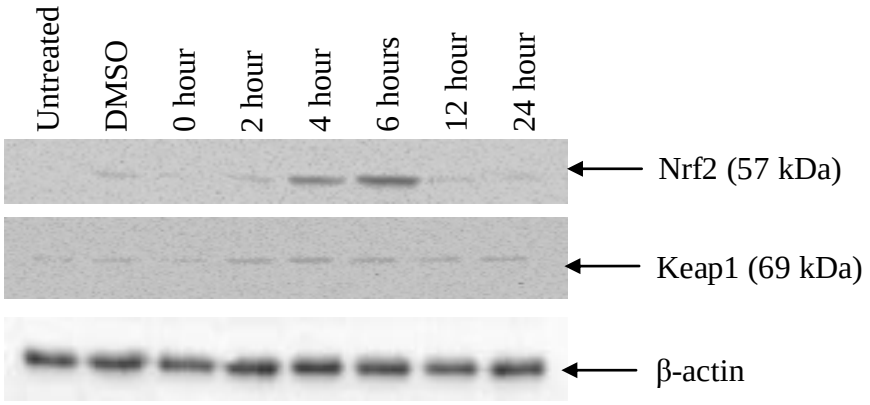
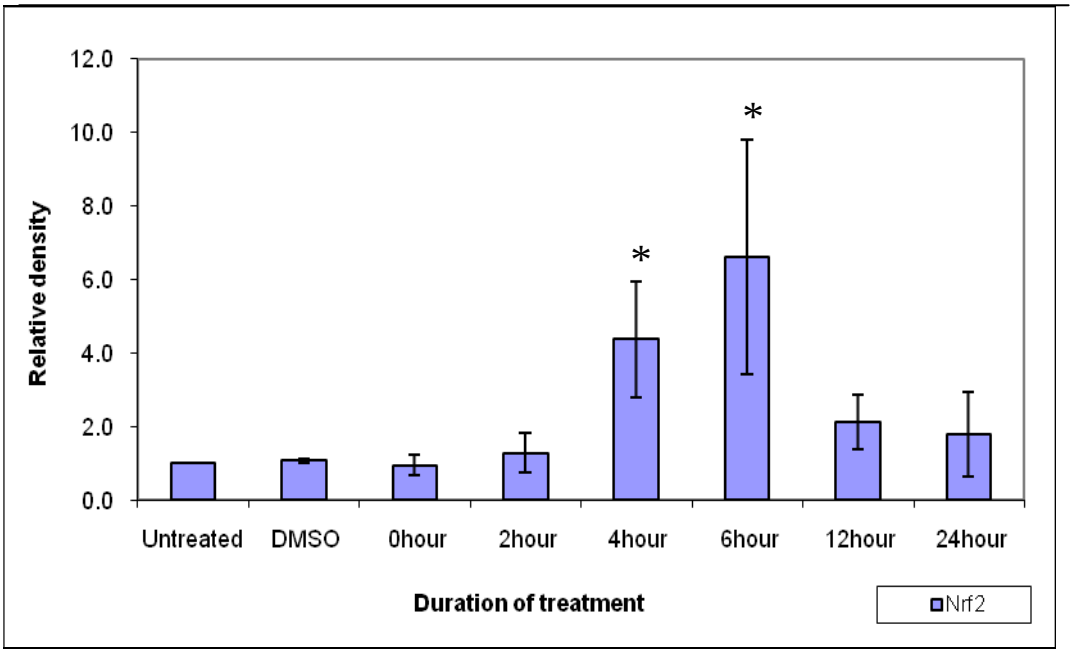


Figure 4.19. Effect of PEITC on the regulation of Nrf2 and Keap1 in MDA-MB-231 cells. MDA-MB-231 cells were left untreated or treated with vehicle-control (DMSO) or treated with 20 μM PEITC for indicated time points. Cell lysates were then assayed for Nrf2 regulation via immunoblotting. β-actin acts as a loading control. Experiments are representative of three independent experiments.

A



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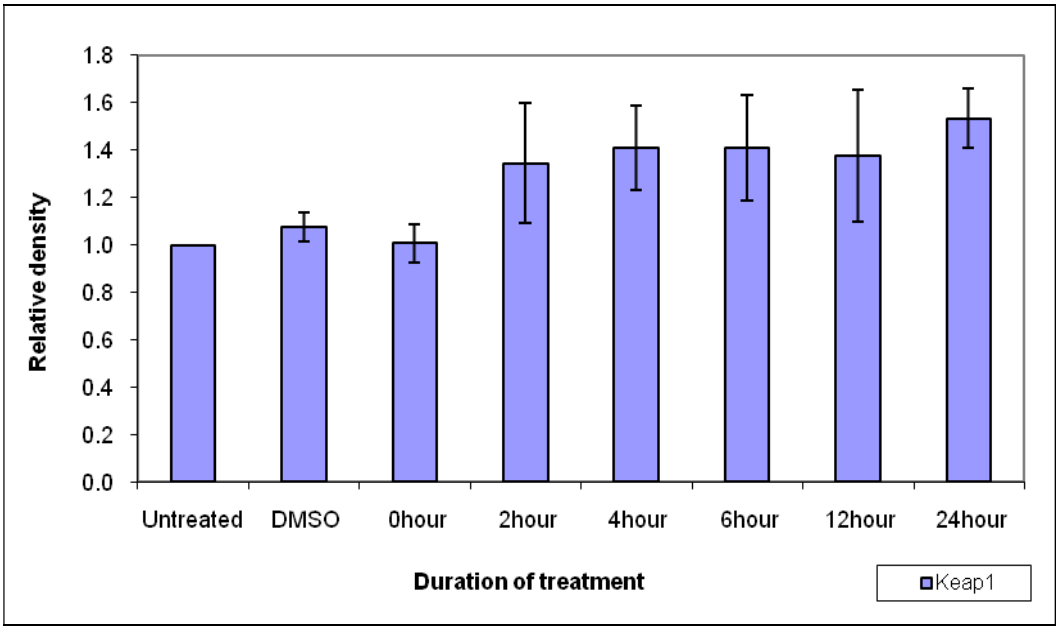


Figure 4.20. Quantitation of Nrf2 [A] and Keap1 [B] expression in MDA-MB-231 cells treated with PEITC. Data shown are means of three independent determination (±SD), normalized to expression of β-actin. Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$).

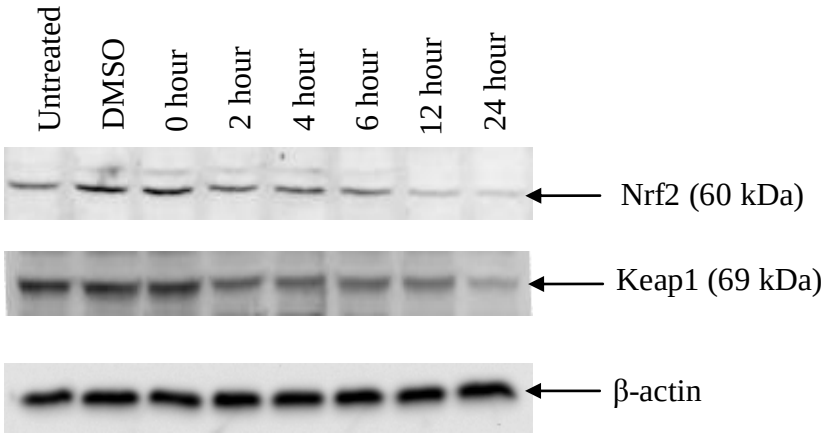
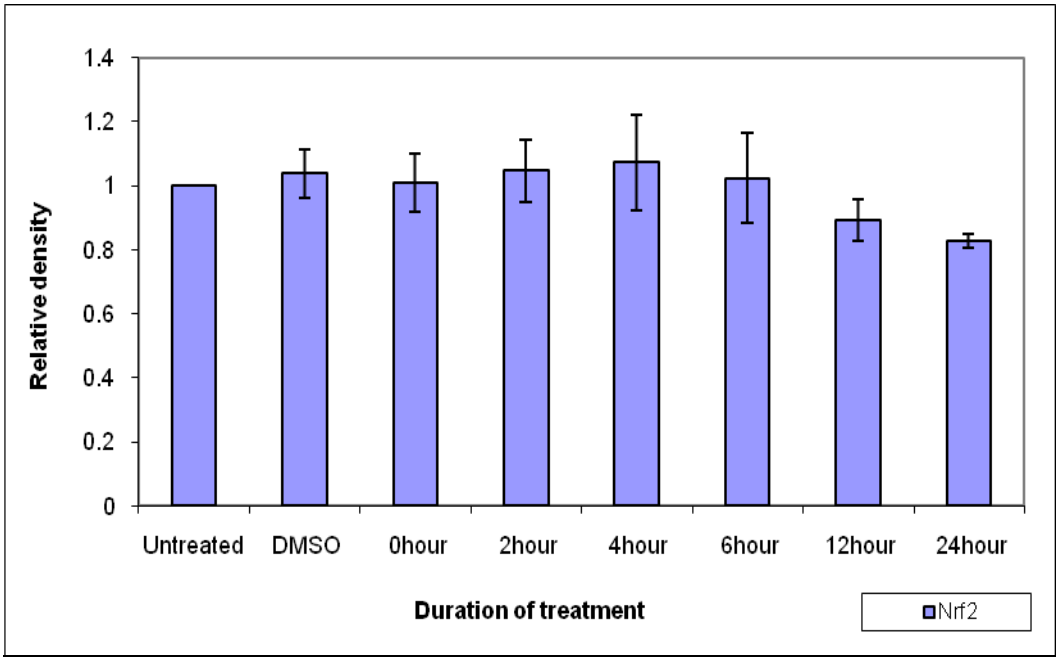


Figure 4.21. Effect of PEITC on the regulation of Nrf2 and Keap1 in MCF7cells. MCF7 cells were left untreated or treated with vehicle-control (DMSO) or treated with 20 µM PEITC for indicated time points. Cell lysates were then assayed for Nrf2 regulation via immunoblotting. β-actin acts as a loading control. Experiments are representative of three independent experiments.

A



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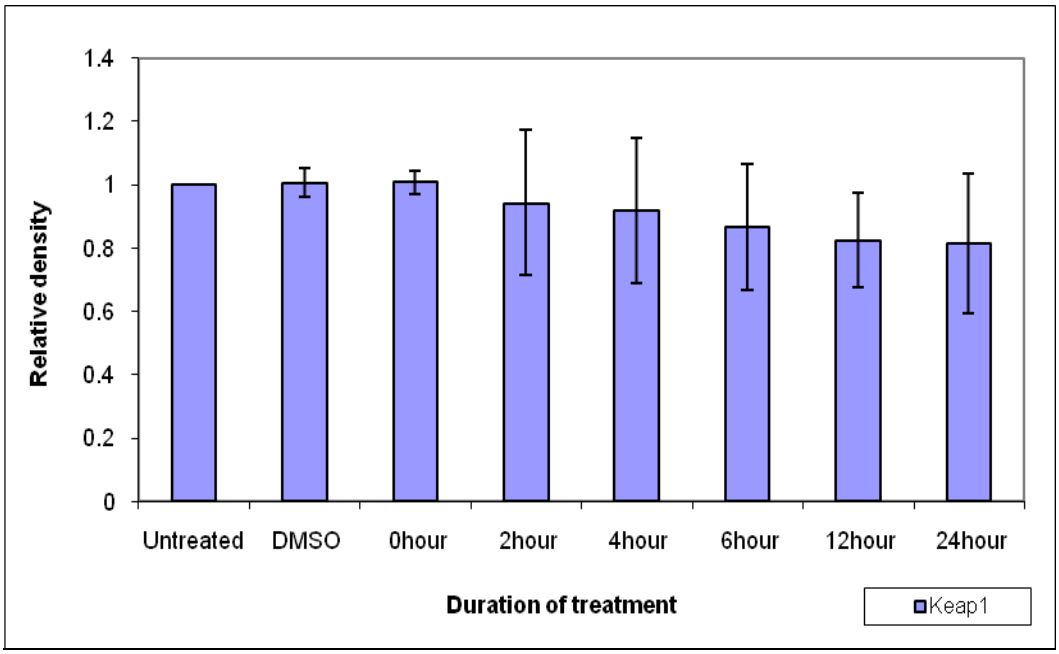


Figure 4.22. Quantitation of Nrf2 [A] and Keap1 [B] expression level in MCF7 cells treated PEITC. Data shown are means of three independent determination (±SD), normalized to expression of β-actin and no statistically significant differences between DMSO and PEITC treated cells.

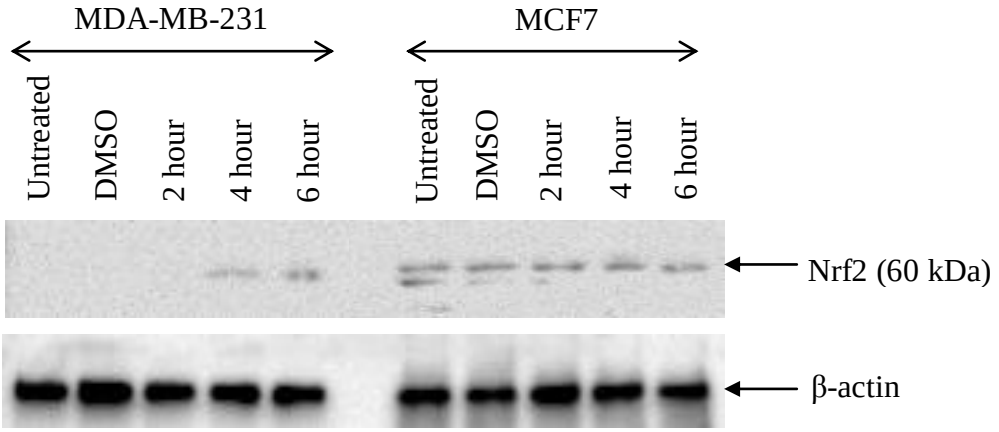


Figure 4.23. Comparison of Nrf2 expression levels in MDA-MB-231 and MCF7 cells treated with PEITC. Cell lysates were then assayed for Nrf2 regulation via immunoblotting. β-actin acts as a loading control. Experiments are representative of two independent experiments.

4.2.9 Phosphorylation of AKT (Thr308 and Ser473) in MDA-MB-231 and MCF7 cells treated with PEITC

The PI3K → AKT pathway is important in mammalian cells for enhanced cell growth, metabolism and survival. Akt activation has also been linked to increased production of ROS via increased oxidative metabolism (Robey & Hay 2010). Therefore, I investigated the effects of PEITC on Akt phosphorylation in PEITC-treated MDA-MB-231 cells. Antibodies used were specific for phosphorylation of Akt at Thr308 (downstream of PI3K, PDK1) and Ser473 (downstream of mTORC2). Phosphorylation at both sites is required for optimal Akt activation.

PEITC treatments (20 µM) lead to a transient increase in phosphorylation of Akt at both Thr308 and Ser473 in MDA-MB-231 cells (Figures 4.24, 4.25). Increased phosphorylation was observed at 2-12 hours post-treatment, and then declined to background levels at 24 hours.

Similar experiments were performed in MCF7 cells (Figures 4.26, 4.27). There was an increase in AKT phosphorylation at both sites. For Thr308 this occurred over a similar time frame as observed in MDA-MB-231 cells, whereas for Ser473 the increase was more protracted. There was a very strong decrease in total levels of Akt after 4 hours so the relative amount of phosphorylation at both sites was strongly induced.

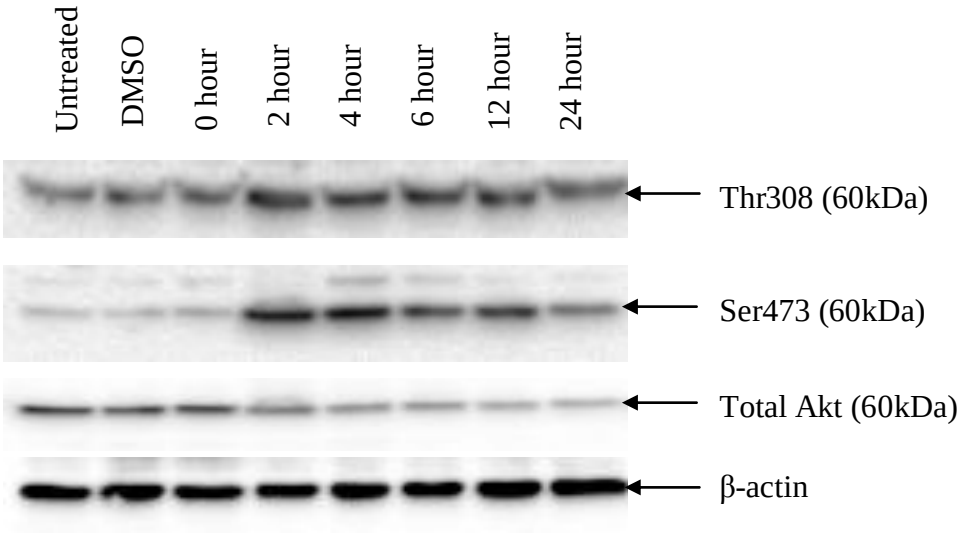
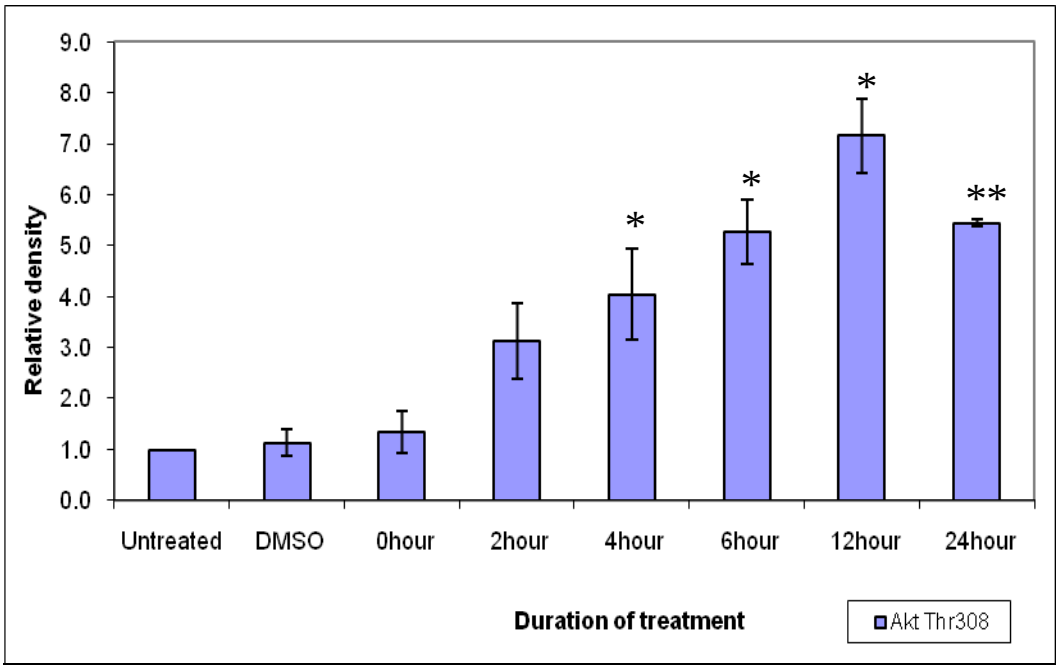


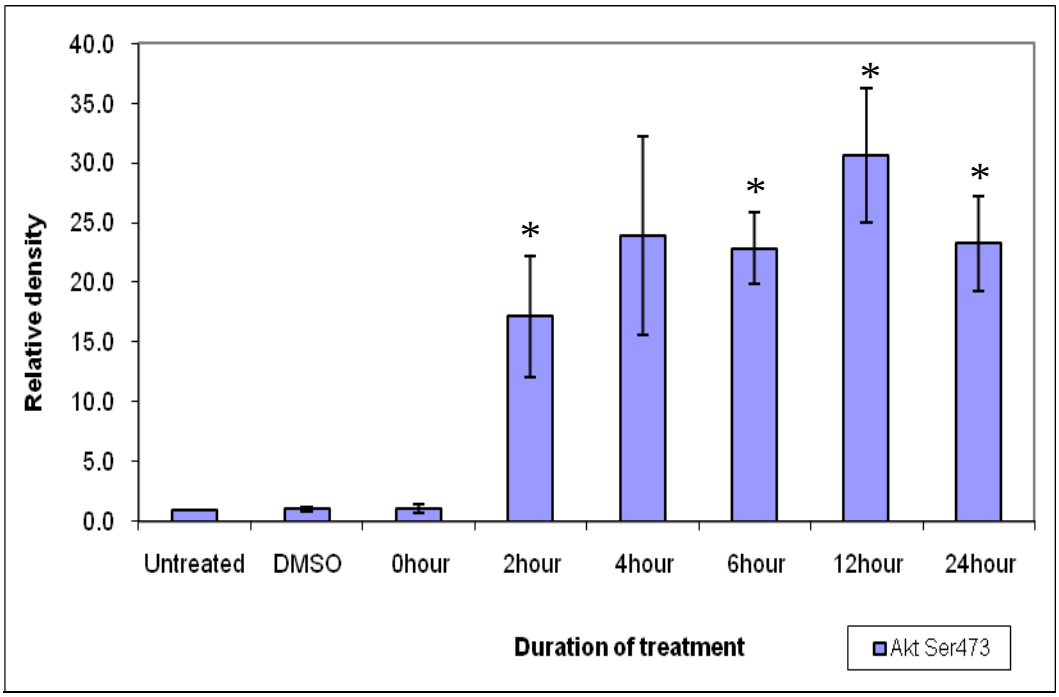
Figure 4.24. Effect of PEITC on the phosphorylation of Thr308 and Ser473 in MDA-MB-231 cells. MDA-MB-231 cells were left untreated or treated with vehicle-

control (DMSO) or treated with 20 μM PEITC for indicated time points. Cell lysates were then assayed for regulation of AKT via immunoblotting. β-actin acts as a loading control. Data shown are representative of three independent experiments.

A



B



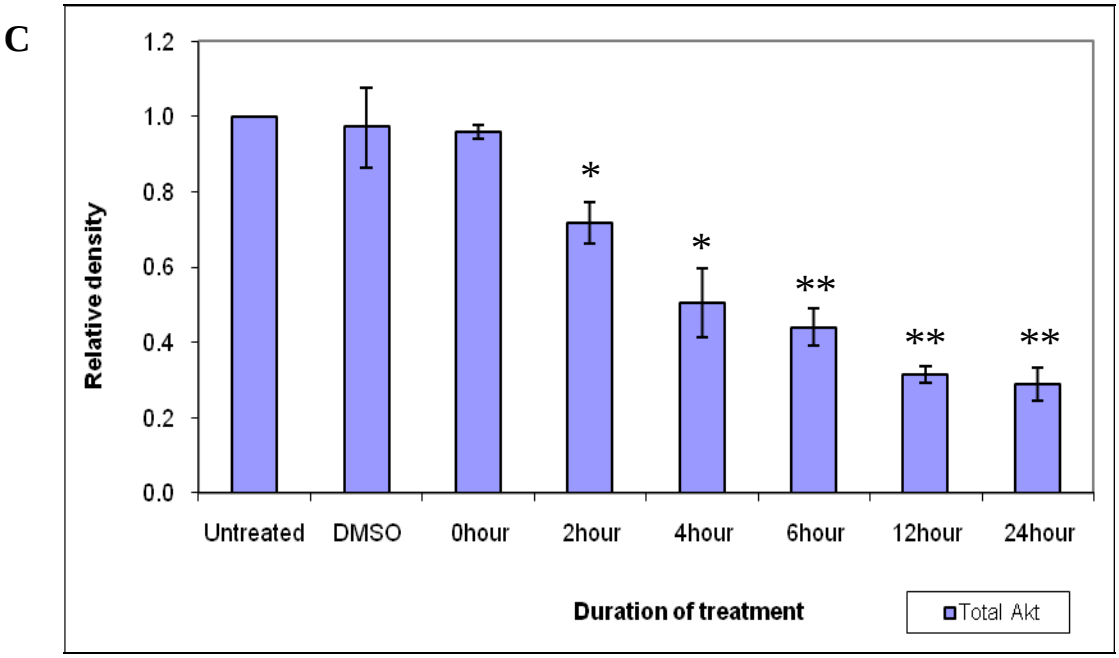


Figure 4.25. Quantitation of Akt Thr308 [A] and Ser473 [B] phosphorylation in MDA-MB-231 cells treated PEITC. Data shown are means of three independent determination (±SD), normalized to expression of β-actin and total AKT (C). Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$).

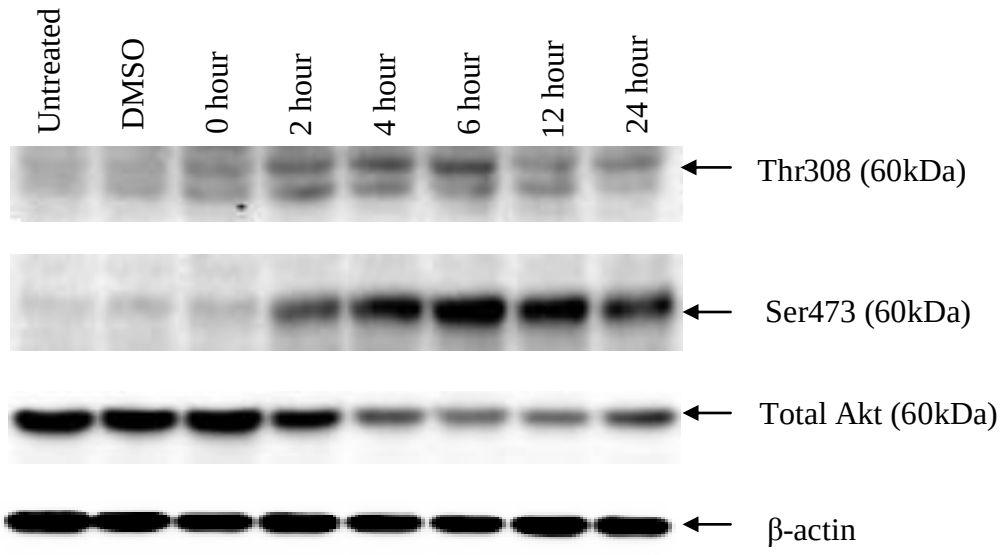
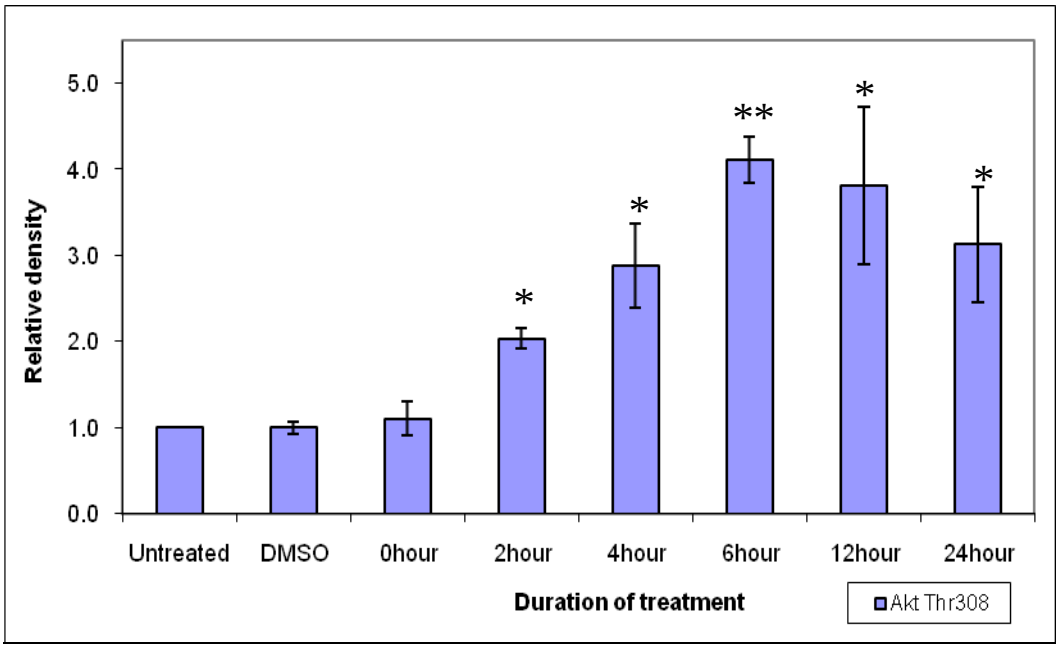


Figure 4.26. Effect of PEITC on the phosphorylation of AKT Thr308 and Ser473 in MCF7 cells. MCF7 cells were left untreated or treated with vehicle-control (DMSO) treated with 20 μM PEITC for indicated time points. Cell lysates were then assayed for regulation of AKT via immunoblotting. β-actin acts as a loading control. Data shown are representative of three independent experiments.

A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5.0
4.0
3.0
2.0
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Untreated
DMSO
0hour
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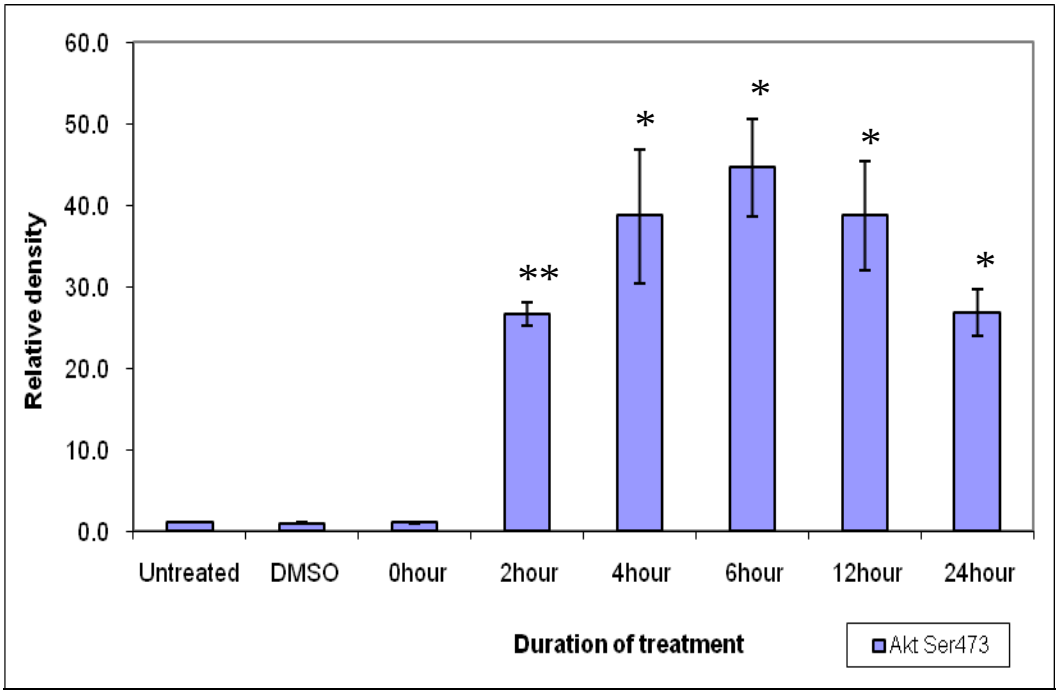
12hour
24hour
Duration of treatment
Akt Thr308

B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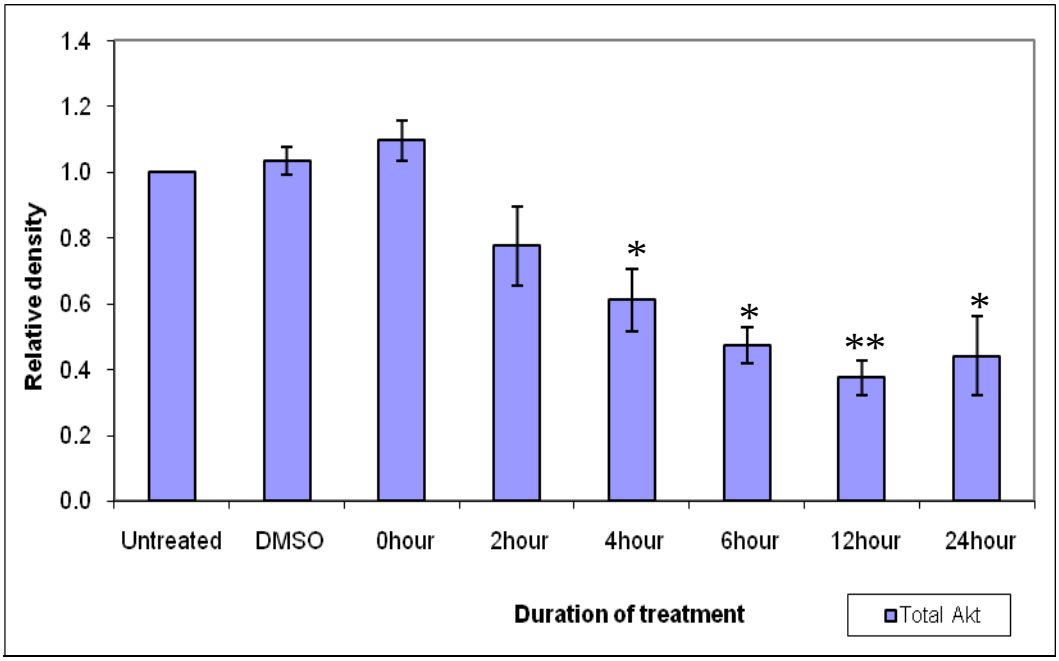


Figure 4.27. Quantitation of Akt Thr308 [A] and Akt Ser473 [B] phosphorylation in MCF7 cells treated PEITC. Data shown are means of three independent determination (±SD), normalized to expression of β-actin and total AKT (C). Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$).

4.2.10 Regulation of 4EBP1 phosphorylation in breast cancer cell line treated PEITC

Apoptosis induction is often associated with reduced eIF4E availability due to increased binding of eIF4E with 4EBP1 (Sheikh & Fornace 1999). Since PEITC has been reported to decrease 4EBP1 phosphorylation in several cancer cells such as HCT116 cells (Hu et al. 2007), the effects of PEITC on 4EBP1 phosphorylation was investigated in MDA-MB-231 and MCF7 cells.

4E-BP1 phosphorylation linked to mTOR signaling at four sites included Thr37/46 (Averous & Proud 2006) and mTORC1 is the primary kinase for Thr37/46 in 4EBP1 (Deldicque et al. 2011). The phosphorylation of 4EBP1 at this site was analysed using flow cytometry. Flow cytometry allows quantitative analysis in single cells, and was selected as a potential approach for biomarker development (see Chapter 6). Figure 4.28 shows flow cytometric analysis of unstained and PEITC-treated MDA-MB-231 and MCF7 cells, at 2 and 4 hours post-treatment. In MDA-MB-231 cells, PEITC did not alter the expression of phosphorylated 4E-BP1. By contrast, in MCF7 cells, PEITC caused a clear decrease in 4E-BP1 phosphorylation, especially at 4 hours post-treatment. As a control, cells were treated with the PI3K inhibitor LY294002. This would be expected to decrease mTORC1-dependent 4EBP1 phosphorylation by reducing upstream PI3K→AKT signaling. LY294002 decreased 4EBP1 phosphorylation in both cell lines. This confirms that basal 4EBP1 phosphorylation is dependent on PI3K activity in both cell types, and the different responses of the lines are specific for PEITC.

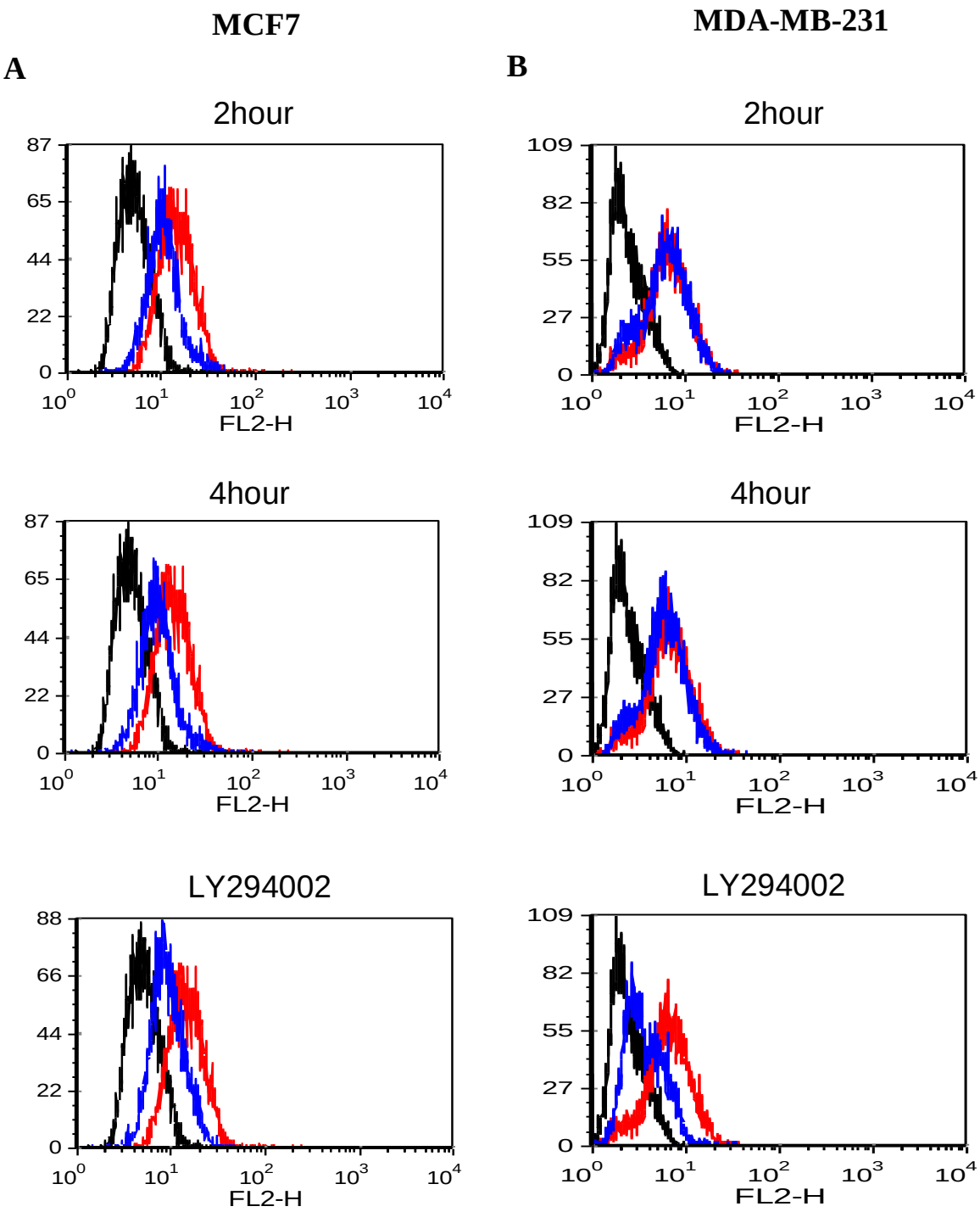


Figure 4.28. Flow cytometric analysis of 4E-BP1 phosphorylation in MCF7 [A] and MDA-MB-231cell lines [B].
MCF7 [A] and MDA-MB-231 [B] cells treated with PEITC at 20 µM for 2 and 4 hours and analysed by flow cytometry using a $Thr^{37/46}$ 4EBP1 phosphorylation specific antibody. Unstained cells are shown as a control. In [A,B] black line shows unstained control; red line shows untreated, antibody-stained cells; blue line shows PEITC (20 µM) treated, antibody stained cells. In [C] black line shows unstained control; red line shows untreated, antibody-stained cells; blue line shows LY294002 treated, antibody stained cells. All data shown are representative of 3 independent experiments.

4.3 DISCUSSION

The aim of the work described in this chapter was to investigate the mechanisms that underlie the differences in the potency of PEITC towards MDA-MB-231 and MCF7 cells. A number of differences including cell cycle and apoptosis as well as effects on Bax, Nrf2, kinase and ROS were observed (Table 4.1).

Table 4.1: Difference mechanisms between MDA-MB-231 and MCF7 cells upon PEITC treatment

Cell lines	**MDA-MB-231**	**MCF7**
Cell Cycle	G2M arrest	G1 arrest
Cell death	Significant apoptosis	Very modest apoptosis
Caspases	Activation of caspase caspase-9 and caspase-3.	Low level activation of caspase caspase-9 and caspase-7
Bax	Biphasic induction – 2/6 hours and 24 hours	Late induction only (24 hours)
ROS	ROS accumulation observed at specific time points	No ROS accumulation observed
Nrf2	Increased Nrf2 expression upon PEITC treatment	High level basal Nrf2 expression modestly increased after PEITC treatment
Akt	PEITC increased phosphorylation of Akt AT Thr308 and Ser473	PEITC increased phosphorylation of Akt Thr308 and Ser473
4EBP1	PEITC failed to inhibit 4EBP1 phosphorylation	PEITC inhibited 4EBP1 phosphorylation

4.3.1 Effect of PEITC on apoptosis and cell cycle arrest

The main difference of PEITC treatment was induction of apoptosis. Whereas MDA- MB-231 cells appeared to be readily sensitive to PEITC-induced apoptosis (measured by sub-G1 DNA content and annexin V exposure), MCF7 cells were significantly less sensitive. These differences presumably contribute to the differential sensitivity towards PEITC observed using MTS assays (Chapter 3). The potency of PEITC to induce apoptosis has been demonstrated in several cancer cells including human prostate cancer cells, human colon cancer cells, human HeLa, human leukaemia Jurkat T-cells, mouse epidermal cells and rat liver epithelial cells (Chen et al. 1998; Huang et al. 1998; Yu et al. 1998; Bonnesen et al. 2001; Nakamura et al. 2002).

Consistent with differential induction of annexin V positive cells, there was an early increase in the expression of the pro-apoptotic Bax protein in MDA-MB-231 cells, but not in MCF7 cells. Similarly, PEITC has also been reported able to induce apoptosis as well as triggered the expression of Bax in human ovarian cancer, OVCAR-3 upon PEITC treatment (Satyan et al. 2006). This could lead to increased cytochrome c release and activation of caspases. Although there was a later increase in Bax expression, this was not different between MCF7 and MDA-MB-231 cells. The mechanisms that lead to increased Bax expression require study but could also be linked to the initiation by ROS (Xiao et al. 2006). It would be important to directly investigate the role of Bax in PEITC induced apoptosis using siRNA or Bax-deficient mouse embryo fibroblasts.

The results obtained using annexin V were confirmed by analysis of specific caspases. Clear caspase activation was only observed in MDA-MB-231 cells. The principle mechanism of apoptosis appeared to be via the intrinsic pathway, since it was associated with clear activation of caspase 9, but not caspase 8. In several cancer cell lines such as human ovarian cancer OVCAR3 cells and human colon adenocarcinoma HT29 cells, caspase-8 was also observed to be affected by PEITC (Satyan et al. 2006). This is consistent, mechanistically and kinetically with the observed increase in Bax expression (early phase). The role of specific caspases could be further explored using specific peptide inhibitors.

Interestingly, PEITC caused different effects on cell cycle parameters in the two lines. In MDA-MB-231 cells, PEITC caused a G2/M arrest, whereas in MCF7 cells, G1 phase arrest predominated. Both G2/M and G1 arrest responses have been described in the literature for PEITC. For example, PEITC induced G1 arrest in human colorectal cancer

HT29 cells (Cheung et al. 2008). PEITC was also reported to induce G1 arrest in human prostate cancer cell lines LNCaP and DU-145 (Xiao et al. 2005). This was associated with an increase in p21 expression and/or decrease in cyclin D1 expression. Other studies, have shown induction of G2/M arrest in, for example, human cervical cancer HeLa cells and in multiple myeloma cells (Mi et al. 2010). This was perhaps associated with the decreasing of Cdk1, cyclin B1 and/or Cdc34 protein levels. PEITC has also been shown to directly bind to tubulin, required for mitosis (Yin et al. 2009). Presumably the differential cell cycle effects of PEITC in MDA-MB-231 and MCF7 cells reflect modulation of different cell cycle regulators, although this was not examined directly in this study.

Interestingly apoptotic and cell cycle responses to PEITC were time dependent, but were not clearly related to dose. This suggests that PEITC triggers these responses when it reaches a specific threshold, perhaps linked to depletion of GSH levels.

4.3.2 ROS in the breast cancer cells treated PEITC

Many studies have linked the growth inhibitory effects of PEITC to depletion of intracellular GSH and increased ROS (Trachootham et al. 2006; Wang et al. 2009; Zhang et al. 2006). The results of analysis of ROS in PEITC-treated MDA-MB-231 and MCF7 cells were complex, but did suggest a difference since increased ROS were only observed in MDA-MB-231 cells. By contrast, PEITC did not induce ROS at any time or concentration in MCF7 cells.

Although, the ability of PEITC to change the reaction mode of antioxidant or pro-oxidant in MDA-MB-231 cells was supported by Zhang and the colleagues since PEITC able to act as double edge sword towards oxidization, the exact mechanisms are still unclear. However, it is likely that induction of antioxidant defences may have played a role in curtailing the response at later time points. Similar complex patterns have been described previously (Zhang et al. 2005). One limitation is that the probe used, DHR123, selectively detects H_2O_2 and it is possible that other forms of ROS are involved. Alternately, production of ROS may have been limited to specific subcellular localisations. Future studies could use a range of ROS-sensitive probes, perhaps combined with microscopy. Due to these limitations, further investigation was focused on the expression of Nrf2 as an alternative read-out for oxidative stress.

4.3.3 PEITC regulated the expression of Nrf2 in breast cancer cells

Nrf2 is the major sensor of oxidative stress in cells, leading to induction of an antioxidant response (Zhang & Wang 2007). Direct analysis demonstrated significant differences in basal Nrf2 expression in MDA-MB-231 and MCF7 cells; Nrf2 expression was expressed at higher levels in MCF7 cells compared to MDA-MB-231 cells (Figure 4.23). Following treatment with PEITC, Nrf2 levels were strongly increased in MDA-MB-231. This is likely to be a response to increased ROS production since Nrf2 induction and increased ROS occurred at similar times post-treatment. By contrast, Nrf2 levels only increased by a small amount in MCF7 cells, perhaps because expression was already raised in these cells. Nrf2 induction by PEITC has been observed previously in a range of cell lines, including PC3 cells (Xu et al. 2006). It was surprising that Keap1 levels did not decrease in MDA-MB-231 cells, but it is possible that Keap1 levels may be in excess over Nrf2, so a small reduction may lead to significant Nrf2 activation. However, further studies are required to determine whether PEITC induced Nrf2 activation in MDA-MB-231 cells is Keap1-dependent or independent, perhaps linked to effects on kinase pathways.

4.3.4 Increased Akt phosphorylation (Thr308 and Ser473) in MDA-MB-231 and MCF7 cells by PEITC

Since Akt is a critical survival signalling molecule (Manning & Cantley 2007), linked to ROS, the effects of PEITC on Akt expression and phosphorylation in MDA-MB-231 and MCF7 cells was investigated. Previous studies had reported that PEITC inhibited the phosphorylation of Akt in several cancer cell lines.

For example, inhibition of Akt phosphorylation by PEITC was observed in ovarian cancer OVCAR3 cells and non-small cell lung L9981 cells with almost completely blocked of Akt phosphorylation at after 24 hours treatment at 20 μM PEITC Inhibition of Akt phosphorylation in human umbilical vein endothelial cells, HUVEC and prostate cancer PC-3 cells by PEITC was also reported to associate with suppression of cell migration in both of the cell line (Wu et al. 2009).

The phosphorylation of AKT was measured at two sites. Phosphorylation at Thr308 is mediated by the PI3K-dependent kinase 1 (PDK1) whereas phosphorylation at Ser473 is mediated by the mammalian target of rapamycin complex-2 (mTORC2) which is itself downstream of AKT (Guertin & Sabatini 2007; Mora et al. 2004). Phosphorylation at both

sites is required for full activation of Akt. In both cell lines, PEITC increased phosphorylation of Akt at Thr308 and Ser473. The functional consequences of this are not clear, but since the responses were similar, are perhaps unlikely to contribute to the differential responses of MDA-MB-231 and MCF7 cells.

4.3.5 Inhibition of 4EBP1 phosphorylation in MCF7 cells by PEITC

PEITC has been linked to inhibition of RNA translation via effects on the protein translation factor 4E binding protein 1 (4E-BP1). 4EBP1 is the most abundant member of the 4EBP family and can be phosphorylated at multiple sites. 4EBP1 phosphorylation decreases the affinity of eIF4E to form complex for the initiation of cap dependent translation. PEITC has been shown to decrease the phosphorylation and expression of the (4EBP1) at pharmacologically relevant concentrations (Cavell et al. 2010; Hu et al. 2007). Therefore the effects of PEITC on phosphorylation of 4E-BP1 in the two cell lines were examined. As previously shown (Wang et al. 2010), PEITC decreased 4E-BP1 phosphorylation in MCF7 cells, but had no effect in MDA-MB-231 cells. Over-expression of eIF4E overcomes PEITC induced growth inhibition, indicating it is likely to be important for growth inhibition in at least some cell types. The mechanism of modulation of 4E-BP1 phosphorylation in MCF7 cells is unclear, bur appears to be independent of ROS production per se. 4E-BP1 phosphorylation is induced by mTORC1 activity, so it is possible that PEITC has a selective effect on mTORC1 specifically in MCF7 cells. mTORC1 contains a redox sensitive negative regulatory domain that could be effected by PEITC, but it unclear why this did not occur in MDA-MB-231 cells.

4.3.6 Summary

Taken together, these results demonstrate clear differences in the response on MDA-MB-231 and MCF7 cells to PEITC. The pronounced induction of the intrinsic apoptosis pathway in MDA-MB-231 cells, possibly linked to an early rise in Bax expression, is likely to explain the overall increased sensitivity of these cells, relative to MCF7 cells, detected using MTS assays. Molecular analysis points to differences in ROS as a potential determinant of these responses since increased ROS was only detected in MDA-MB-231 cells. Elevated basal expression of Nrf2 may lead to constitutive production of antioxidant defences in MCF7 cells, so that these cells are relatively well protected from subsequent

PEITC challenge. By contrast, PEITC treatment of MDA-MB-231 cells does lead to increased ROS production. Despite an attempt by the cell to mount an effective response (shown by Nrf2 induction and downstream antioxidants), the resultant ROS may lead to effective induction of apoptosis. Increased AKT activation does not appear to contribute to differential responses, since it occurred similarly in both cell lines, whereas 4E-BP1 may contribute specifically in MCF7 cells.

Chapter 5

Effect of modulation of intracellular GSH on PEITC responses

5.1 INTRODUCTION

In chapter 4, PEITC was demonstrated to induce apoptosis as well as G2M arrest in MDA-MB-231 cells. By contrast, PEITC induced G1 arrest in MCF7 cells and these cells were more resistant towards PEITC induced apoptosis. Analysis of Nrf2 and ROS suggested that differential responses to oxidative stress might contribute to these different responses. PEITC is a strong electrophile and can cause oxidation of GSH (to GSSG) and depletion of intracellular GSH. Thus, the aim of the work described in this chapter was to investigate the role of GSH depletion in differential responses of MDA-MB-231 and MCF7 cells.

In these experiments, *N*-acetylcysteine (NAC) and L-buthionine sulfoximine (BSO) were used to modulate GSH levels. NAC is a direct antioxidant as well as a synthetic precursor of GSH (Morgan et al. 1983). Numerous studies had reported the potential of NAC in preventing GSH depletion thus blocking ROS accumulation and oxidative damage in mitochondria as well as suppressing apoptosis and cell death induced by PEITC (Mi et al. 2010). BSO is an inhibitor of gamma-glutamylcysteine synthetase and blocks the rate limiting step in GSH synthesis thus depleting the intracellular GSH pool (Anderson et al. 1999).

5.2 RESULTS

5.2.1 Effect of NAC on growth inhibition of human breast cancer cells by PEITC

The effects of NAC on responses to PEITC in MDA-MB-231 and MCF7 cells were first determined, initially using MTS assays. A series of pilot experiments demonstrated that 10 mM NAC and a pretreatment time of 6 hours was optimal for reversal of the growth inhibitory effects of PEITC (data not shown). Using these conditions, NAC effectively reversed the ability of PEITC to inhibit the growth of MDA-MB-231 cells indicated by a rightward shift in the dose response curve (Figure 5.1; Table 5.1). Interestingly, NAC alone appeared to enhance the MTS conversion in MDA-MB-231 cells. It is not clear whether this is a specific effect of NAC on cell growth, or reflects an artefactual effect of GSH on the assay which involves bio-reduction. Although the Prism software was not able to perform regression analysis for the data obtained from NAC/PEITC treated cells, analysis of the concentration required for 50% growth inhibition indicated that NAC-pretreated cells were approximately 6-fold less sensitive to PEITC. By contrast, NAC had

a very modest reversal effect of growth inhibition by PEITC in MCF7 and SKBr3 cells (Table 5.1; data provided by Breeze Cavell for comparison).

This analysis was extended to other breast cancer cell lines examined in Chapter 3. Using similar conditions, NAC effectively reversed the growth inhibition by PEITC in ER negative BT549 cell lines (Figure 5.2; Table 5.1) the overall reversal effects of NAC in ER positive T47D was more modest (Figure 5.4; Table 5.1). By contrast, growth inhibition by PEITC was unaffected by NAC pretreatment in ER positive ZR75.1 cells (Figure 5.3; Table 5.1).

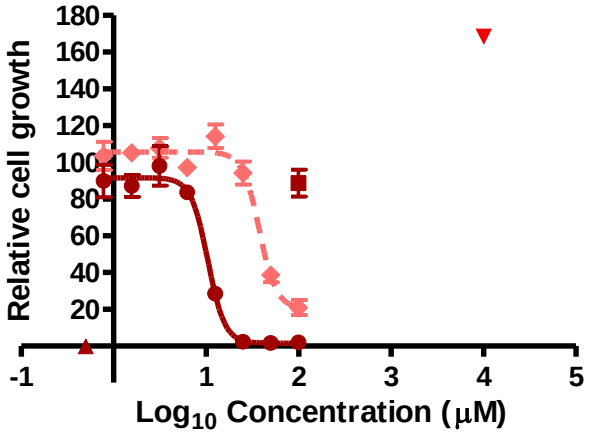


Figure 5.1. Effect of NAC on PEITC-induced growth inhibition in MDA-MB-231 cells.
Cells were pretreated with 10mM of NAC for 6 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●) or staurosporin (▲). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

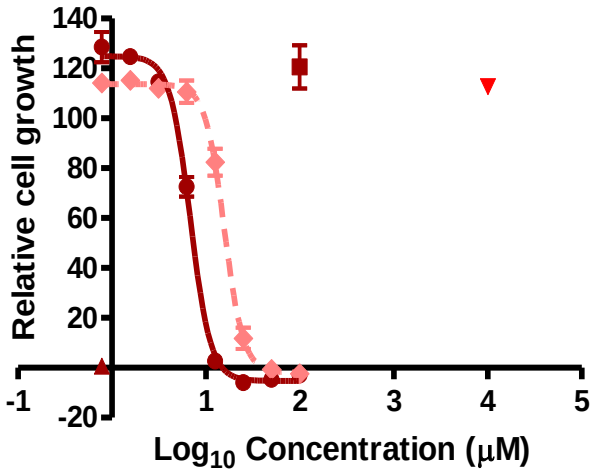


Figure 5.2. Effect of NAC on PEITC-induced growth inhibition in BT549 cells.
Cells were pretreated with 10mM of NAC for 6 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●) or staurosporin (▲). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

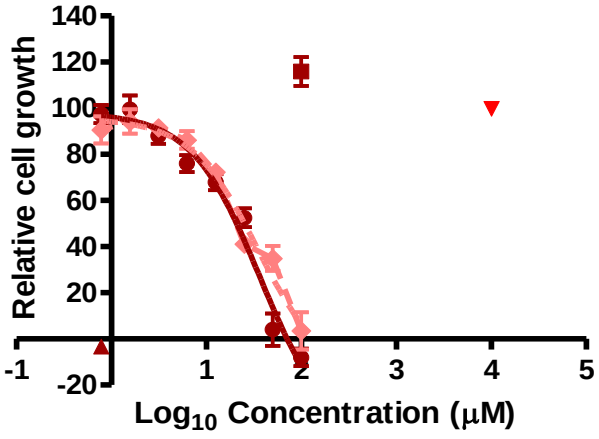


Figure 5.3. Effect of NAC on PEITC-induced growth inhibition in ZR75.1 cells.
Cells were pretreated with 10mM of NAC for 6 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●) or staurosporin (▲). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

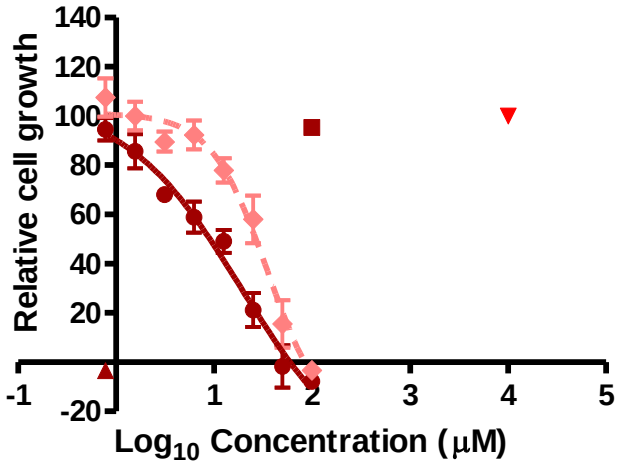


Figure 5.4. Effect of NAC on PEITC-induced growth inhibition in T47D cells.
Cells were pretreated with 10mM of NAC for 6 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●) or staurosporin (▲). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

5.2.2 Effect of BSO on growth inhibition of human breast cancer cells by PEITC

The effects of BSO to deplete intracellular GSH, on responses to PEITC were also tested. Pilot experiments demonstrated that a pretreatment of 300 µM BSO for 24 hour was optimal to sensitize MCF7 cells towards PEITC (Figure 5.8; Table 5.1). BSO increased sensitivity of MCF7 cells to PEITC by ~2-fold.

In contrast to MCF7 cells, BSO alone was highly toxic towards MDA-MB-231 cells. At concentrations >50 µM, BSO alone caused high levels of cell death. In combination experiments, a lower concentration of 10 µM BSO was used which did not alone cause significant cell killing. Upon BSO pretreatment at this concentration in MDA-MB-231 cells, growth inhibitory effect of PEITC was increased by approximately 3-fold (Figure 5.5; Table 5.1).

The effect of BSO was further investigated in 3 other breast cancer cells (all at 10 µM). BSO pretreatment effectively increased the potency of PEITC induced growth inhibition in ER negative BT549 breast cancer cells by 3 folds compared to PEITC alone (Figure 5.6; Table 5.1). However, in SKBr3 cells, BSO pretreatment affected the cell viability at rather higher concentration of PEITC (Figure 5.7; Table 5.1). By contrast, growth inhibitory effect of PEITC was relatively unaffected by BSO pretreatment in ER positive, T47D breast cancer cells (Figure 5.9; Table 5.1).

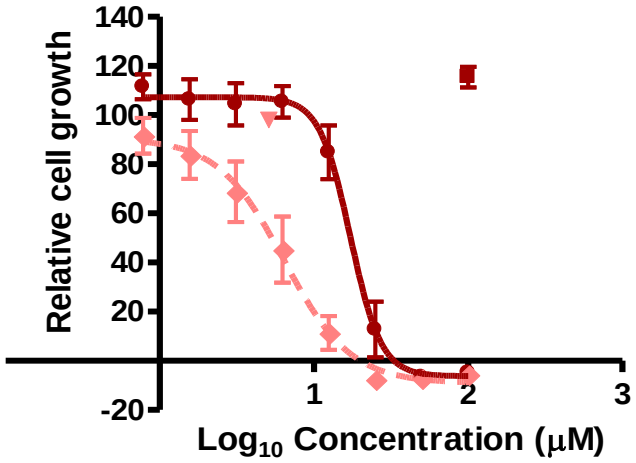


Figure 5.5. Effect of BSO on PEITC-induced growth inhibition in MDA-MB-231 cells. Cells were pretreated with 10 μM of BSO for 24 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated with an equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

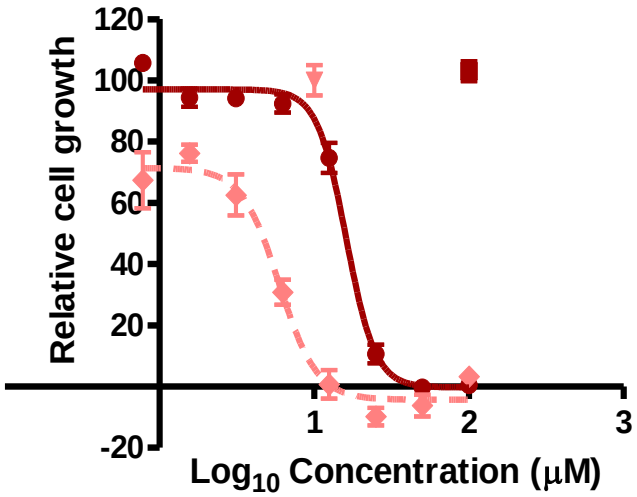


Figure 5.6. Effect of BSO on PEITC-induced growth inhibition in BT549 cells. Cells were pretreated with 10 μM of BSO for 24 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated with an equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

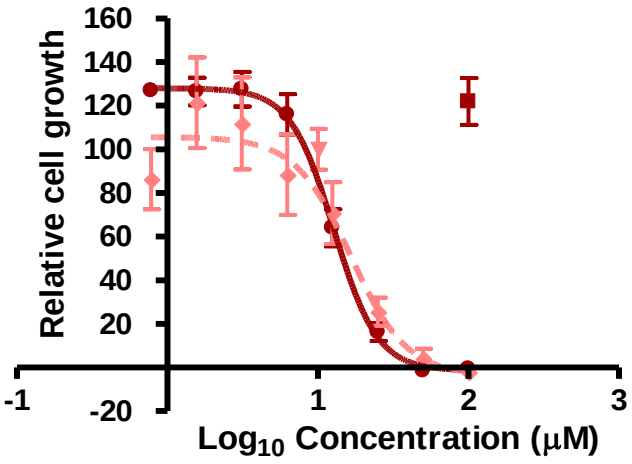


Figure 5.7. Effect of BSO on PEITC-induced growth inhibition in SKBr3 cells.
Cells were pretreated with 10 μM of BSO for 24 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated with an equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

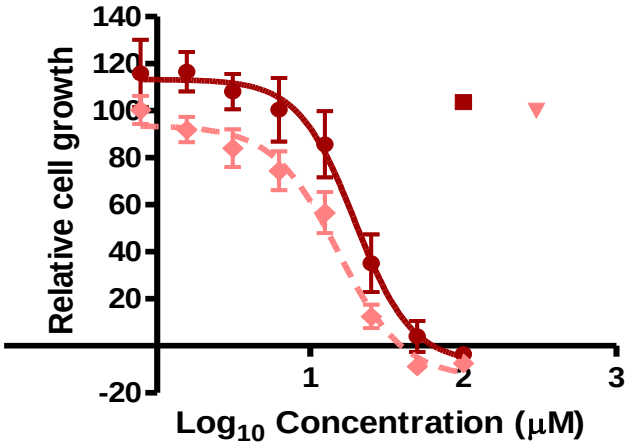


Figure 5.8. Effect of BSO on PEITC-induced growth inhibition in MCF7 cells.
Cells were pretreated with 300 μM of BSO for 24 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated with an equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

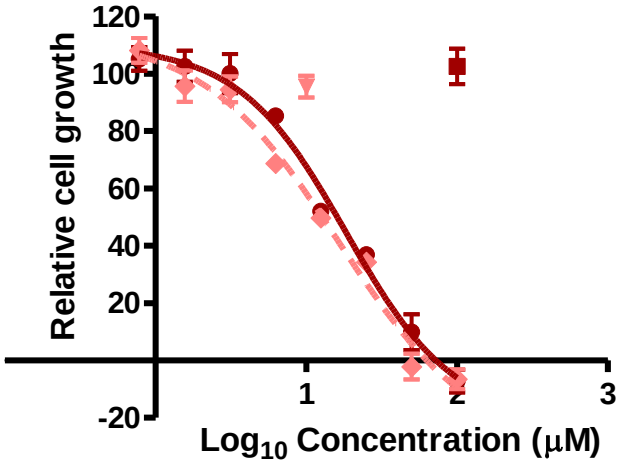


Figure 5.9. Effect of BSO on PEITC-induced growth inhibition in T47D cells.
Cells were pretreated with 10 μM of BSO for 24 hours before being treated with indicated concentrations of PEITC for 6 days (--♦--) or left untreated (▼) or treated with an equivalent amount of DMSO (■). Cells were also treated with indicated concentrations of PEITC alone (●). Proliferation was determined using the MTS assay. Data shown are presented as the means (±SD) cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate.

Table 5.1. Effect of NAC/BSO on the PEITC-induced growth inhibition in breast cancer cell lines. Data shown are the mean (±SD) of cell growth relative to untreated cells (set to 100) from 3 independent experiments each perform in triplicate

CELL LINES	MDA-MB-231	BT549	SKBr3	MCF7	T47D	ZR75.1
MOLECULAR FEATURES						
ESTROGEN RECEPTOR	-ve	-ve	-ve	+ve	+ve	+ve
PROGESTRONE RECEPTOR	-ve	-ve	-ve	+	+	-ve
HER2 STATUS	Normal	Normal	Amplified	Normal	Normal	Normal
RELATIVE POTENCY OF PEITC ± NAC/BSO						
PEITC	7.2 ± 1.6 µM	11.9 ± 7.3 µM	26.4 ± 2.1 µM	13.7 ± 1.0 µM	9.2 ± 3.8 µM	40.4 ± 4.8 µM
NAC	3-fold Increased of IC_{50}	1-fold Increased of IC_{50}	ND	*<1-fold Increased of IC_{50}	No Increased of IC_{50}	No Increased of IC_{50}
BSO	3-fold Reduction of IC_{50} (10 µM BSO)	3-fold Reduction of IC_{50} (10 µM BSO)	<1-fold Reduction of IC_{50} (10 µM BSO)	1-fold Reduction of IC_{50} (300 µM BSO)	No Reduction of IC_{50} (10 µM BSO)	ND

***** - Data obtained from Breeze Cavell and provided for comparison

ND - Not Done

5.2.3 Effect of NAC on the potency of PEITC inducing cell cycle arrest in breast cancer cells

Since effects of NAC and BSO clearly differed between MDA-MB-231 and MCF7 cells, their effects on cell cycle arrest and apoptosis in both breast cancer cells were investigated. This would avoid any potential effects of NAC directly on MTS assays, as potentially observed in MDA-MB-231 cells (Figure 5.1; Table 5.1). MDA-MB-231 and MCF7 cells were pretreated with 10 mM NAC, and the effects on PEITC-induced cell cycle arrest analyzed at 48 hours.

As observed previously, PEITC alone triggered a G2/M arrest in MDA-MB-231 cells, associated with decreased G1 cells and increased cells with G2/M DNA content (Figure 5.10; 5.11). However, NAC pretreatment significantly prevented these changes compared to PEITC treated (Figure 5.11); the cell cycle distribution of PEITC + NAC treated cells was essentially identical to DMSO treated cells (Figure 5.11 [B]). In MCF7 cells, PEITC increased the proportion of cells with G1 DNA content, as expected. Again NAC alone had no effect on the cell cycle distribution of MCF7 cells compared to the control, but significantly reversed the apoptosis effects of PEITC (Figure 5.12, 5.13).

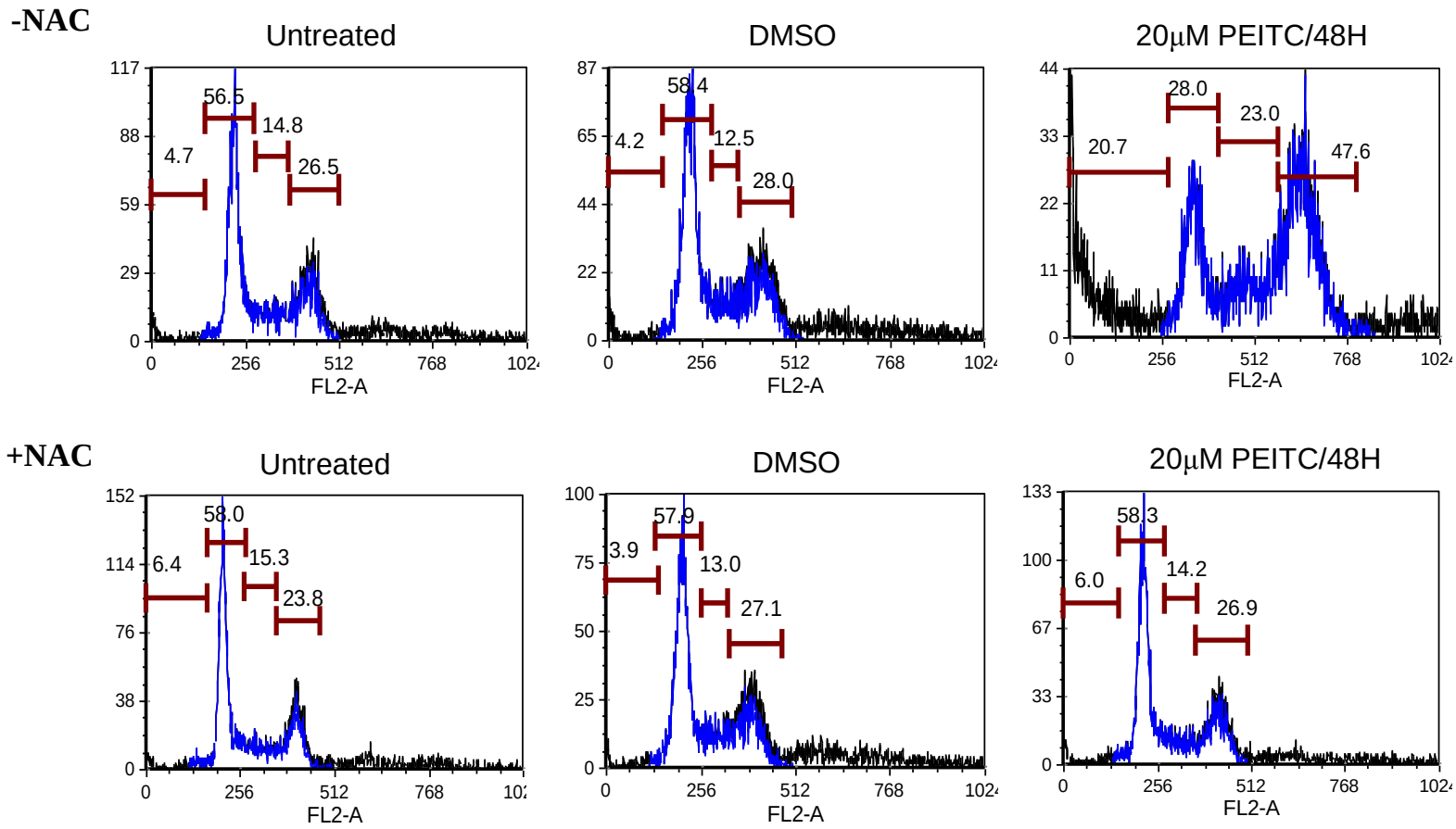
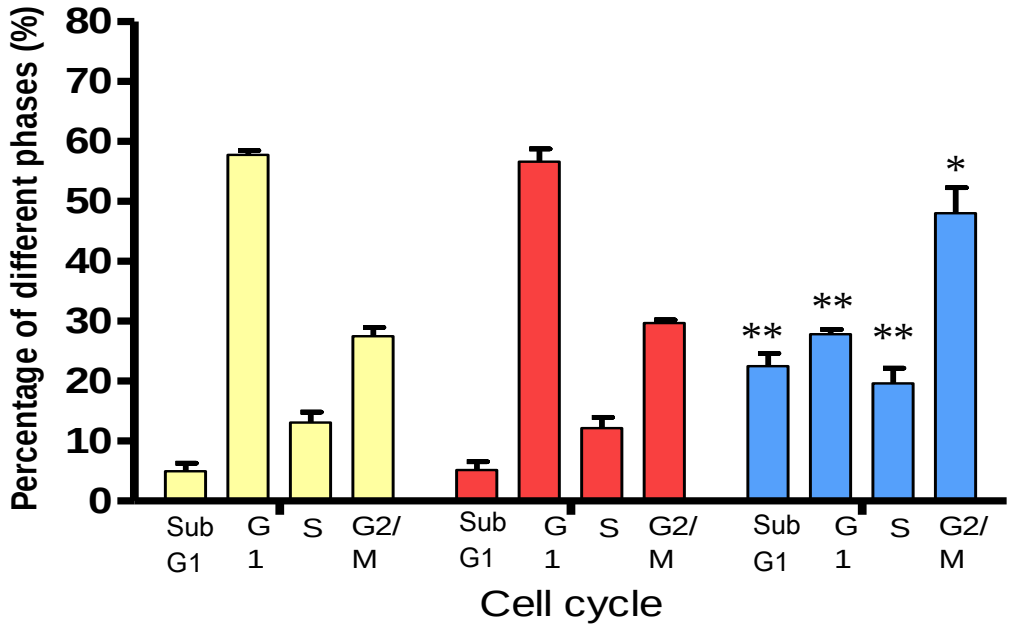
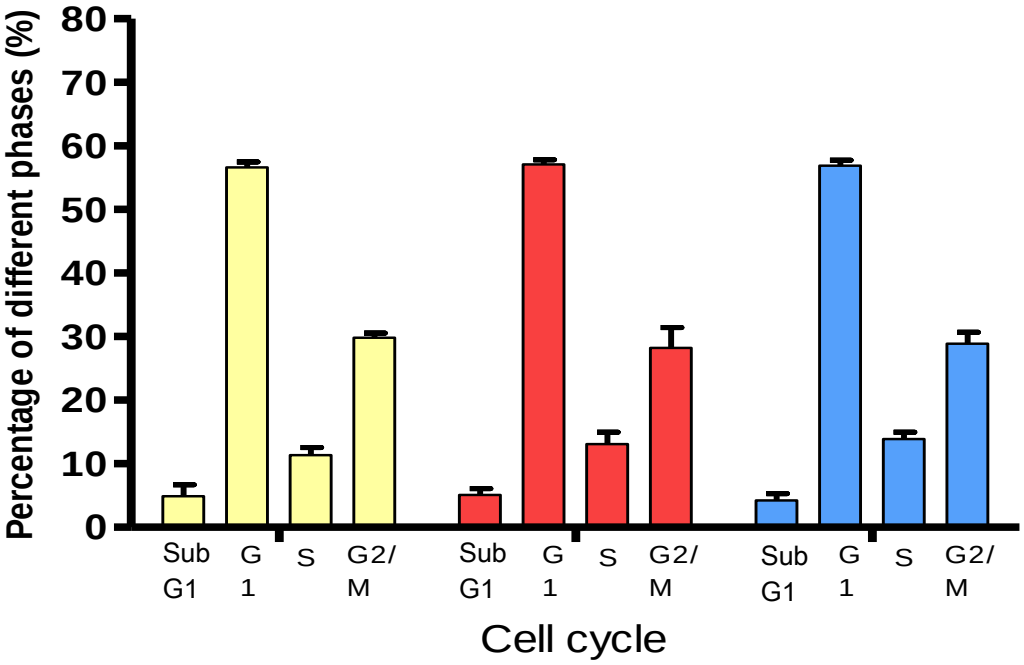


Figure 5.10. NAC pretreatment induced cell cycle arrest in MDA-MB-231 by PEITC. MDA-MB-231 cells pretreated with 10 mM NAC for 6 hours before being treated with PEITC for 48 hours. Propidium iodide staining was determined via flow cytometry. The x axis FL2A shows red fluorochrome for PI labelling. Blue line indicates single cells population which has been set in the dot plot graph (Data not shown). Data shown above are representative of three independent experiments.

A


Percentage of different phases (%)
80
70
60
50
40
30
20
10
0
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
**
**
**
*
Cell cycle

B


Percentage of different phases (%)
80
70
60
50
40
30
20
10
0
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Cell cycle

C

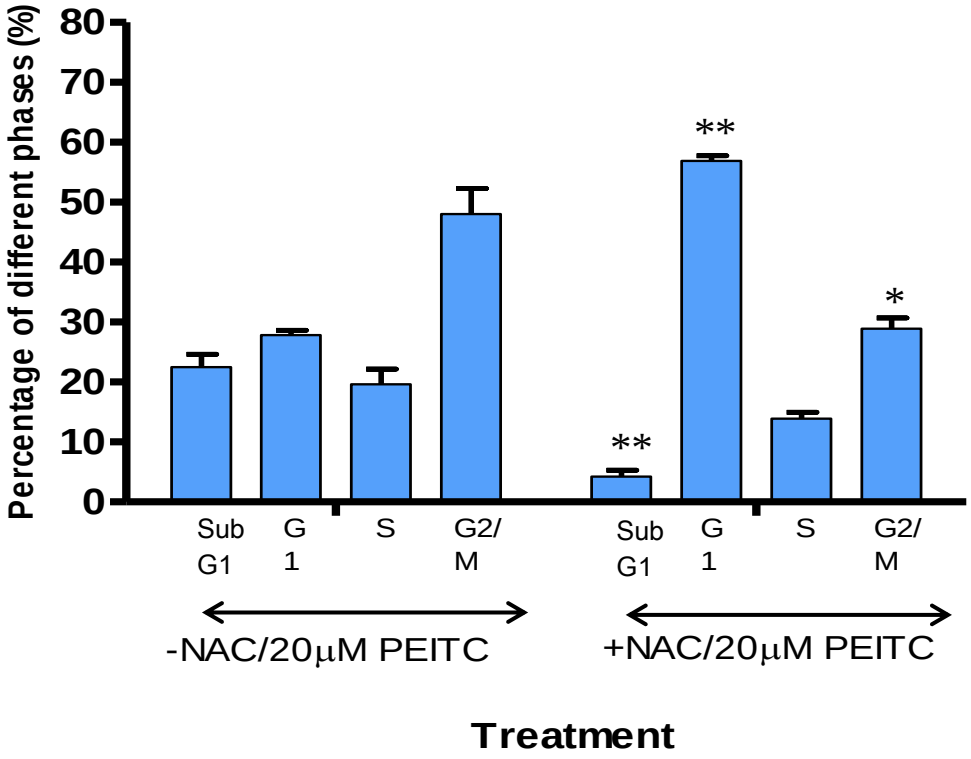


Figure 5.11. Quantitation of cell cycle arrest in MDA-MB-231 cells that were (A) treated with PEITC alone for 48 hours (B) pre-treated with 10 mM NAC followed by PEITC treatment for 48 hours and (C) comparison of PEITC effects with and without NAC pretreatment. Untreated cells ( ), DMSO ( ), 20 μM PEITC ( ). (A) Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$). (C) Statistically significant differences between PEITC alone and PEITC with NAC pretreatment are indicated (*$p < 0.05$; **$p < 0.005$). All other differences were not statistically significant. Data shown represents the average of three independent experiments.

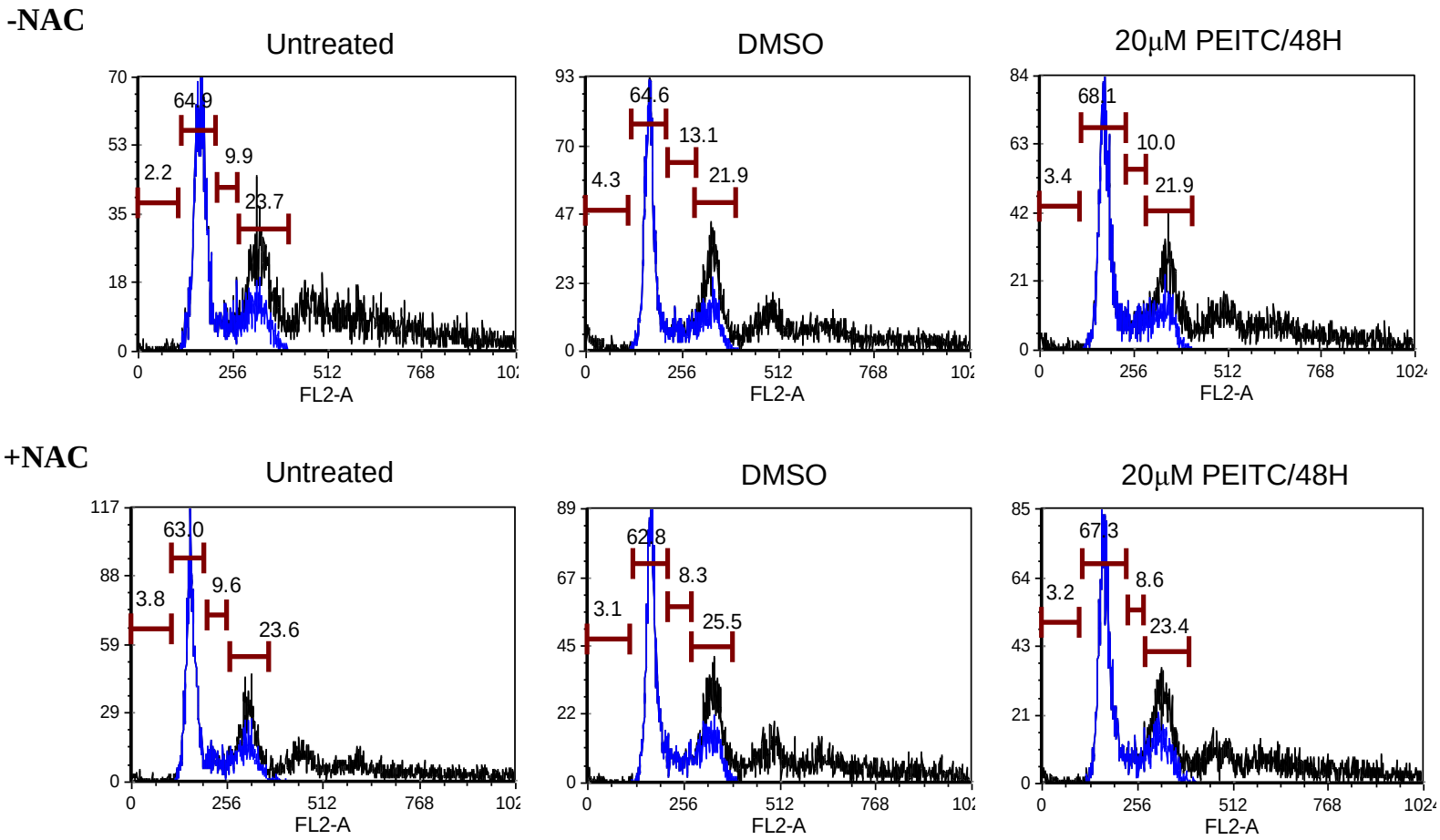
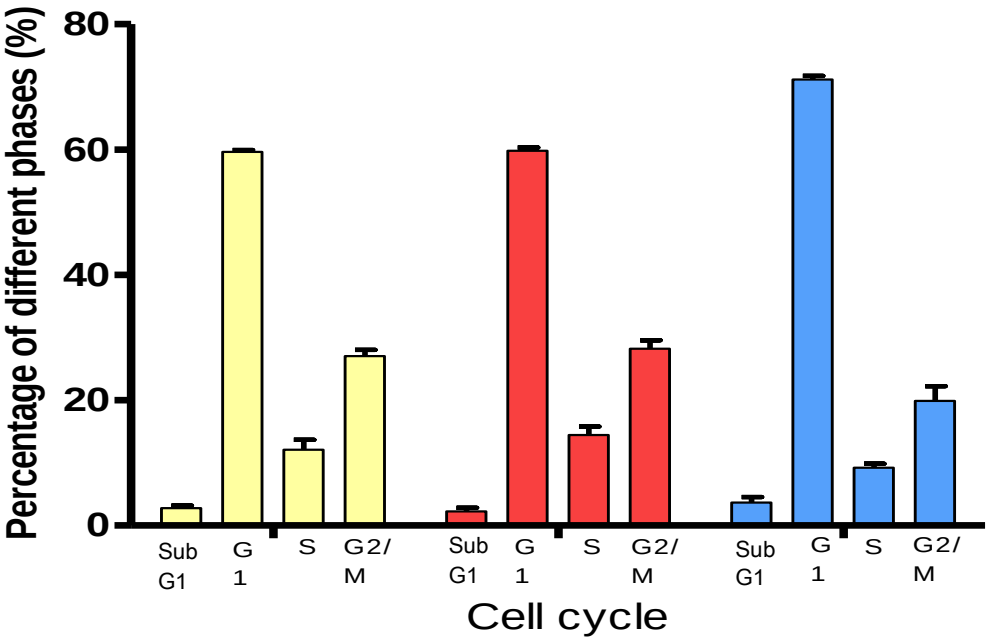
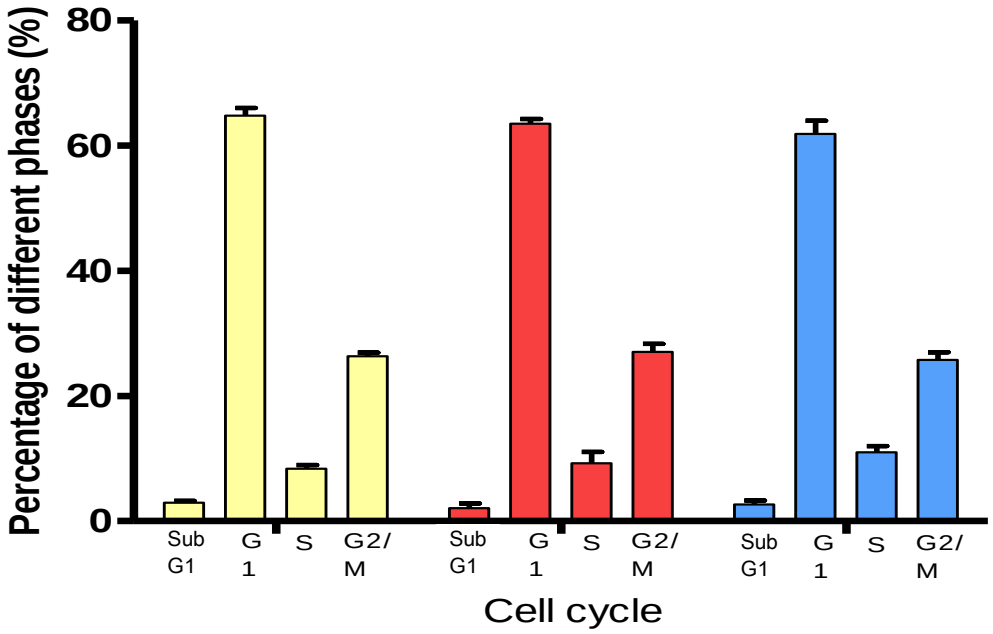


Figure 5.12. NAC pretreatment induced cell cycle arrest in MCF7 by PEITC. MCF7 cells pretreated with 10mM NAC for 6 hours before being treated with PEITC for 48 hours. Propidium iodide staining was determined via flow cytometry. The x axis FL2A shows red fluorochrome for PI labelling. Blue line indicates single cells population which has been set in the dot plot graph (Data not shown). Data shown above are representative of three independent experiments.

A


Percentage of different phases (%)
80
60
40
20
0
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Cell cycle

B


Percentage of different phases (%)
80
60
40
20
0
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Cell cycle

C

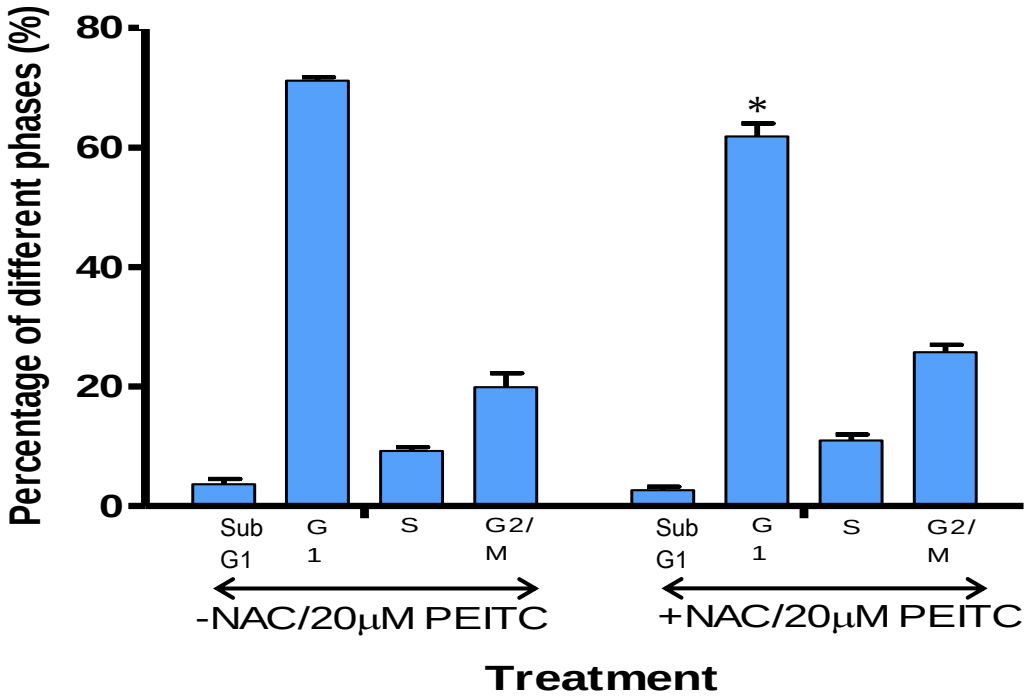


Figure 5.13. Quantitation of cell cycle arrest in MCF7 cells that were (A) treated with PEITC alone for 48 hours (B) pre-treated with 10 mM NAC followed by PEITC treatment for 48 hours and (C) comparison of PEITC effects with and without NAC pretreatment. Untreated cells (□), DMSO (■), 20 μM PEITC (■). (C) Statistically significant differences between PEITC alone and PEITC with NAC pretreatment are indicated (*$p < 0.05$). All other differences were not statistically significant. Data shown represents the average of three independent experiments.

5.2.4 Effect of NAC on the potency of PEITC induced apoptosis in breast cancer cells

Similar experiments were performed to determine the effects of NAC pretreatment on PEITC-induced apoptosis. In MDA-MB-231 cells, PEITC-induced apoptosis was completely prevented by 10 mM NAC pretreatment (Figure 5.14, 5.15). As expected the levels of PEITC-induced apoptosis in MCF7 cells were very low; this was unaffected by NAC pretreatment (Figure 5.16, 5.17).

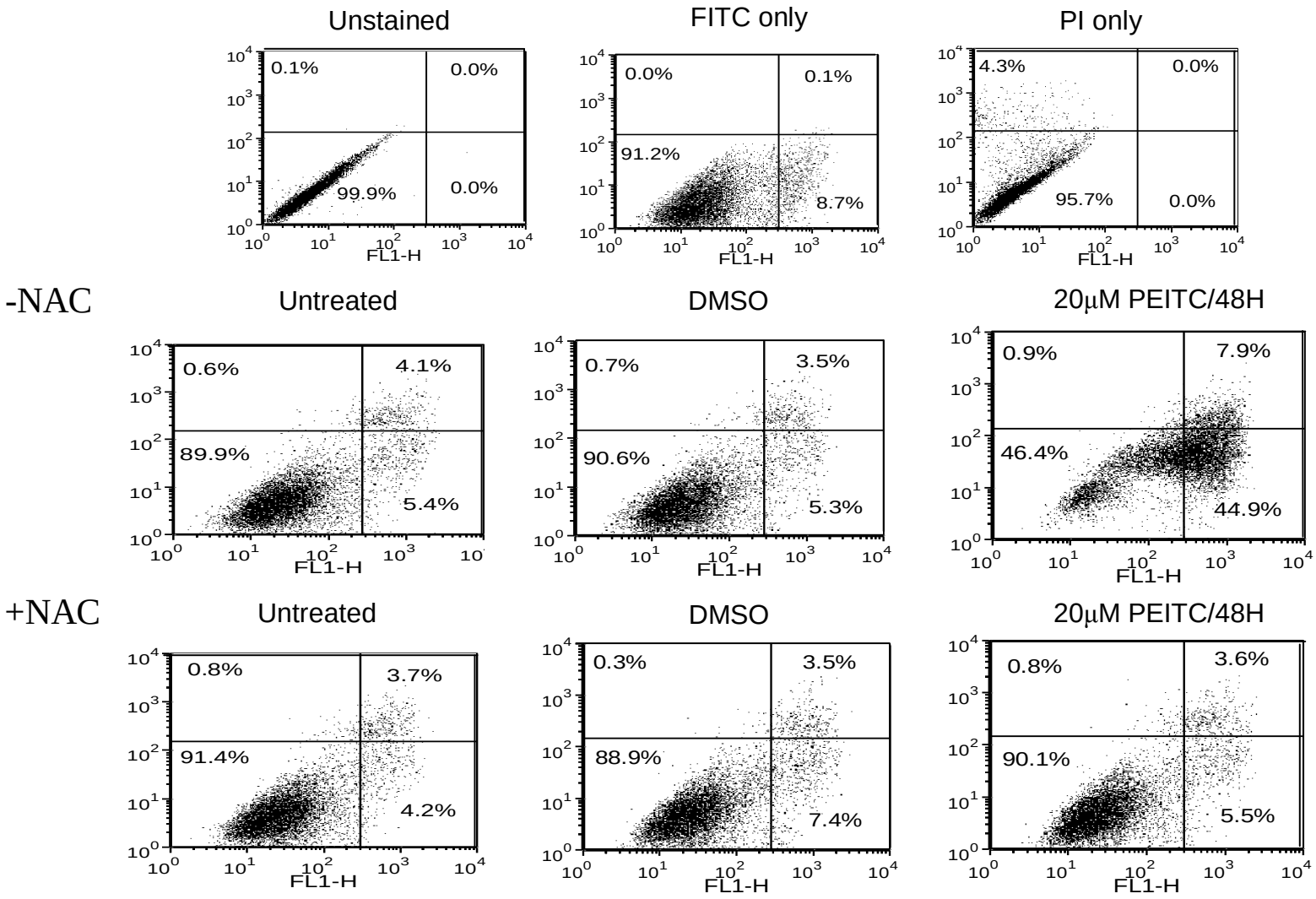
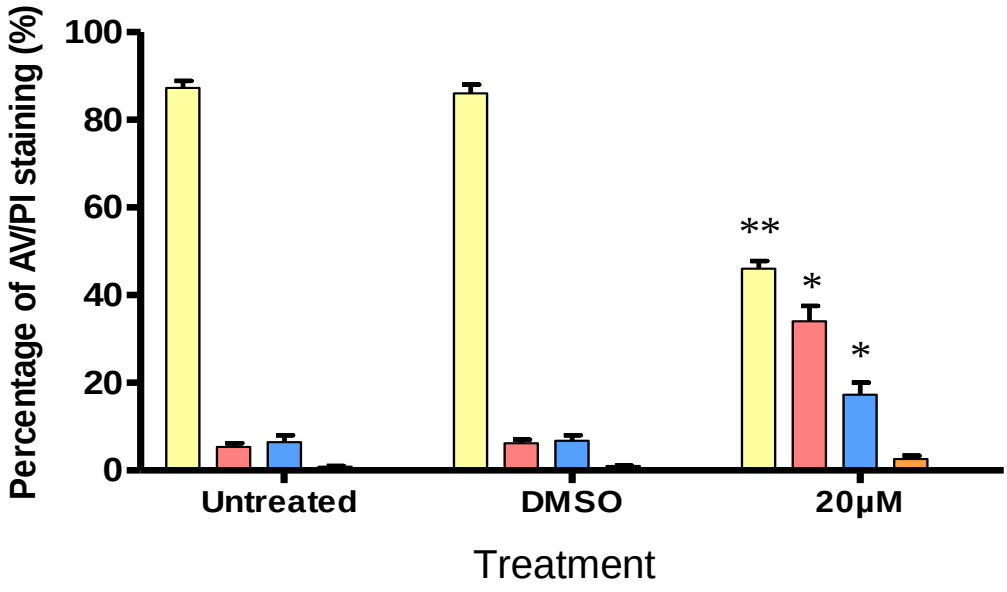
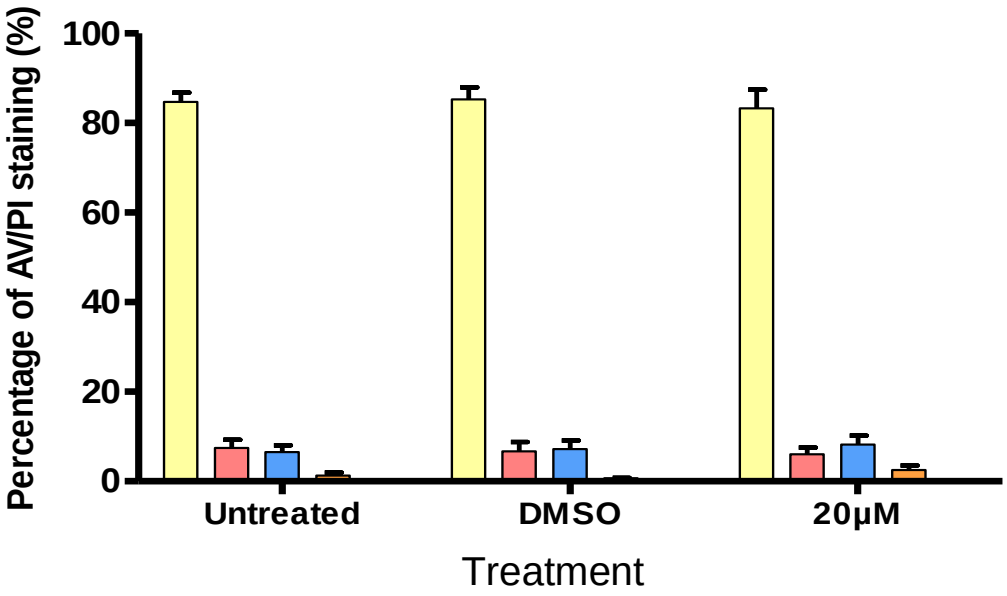


Figure 5.14. Effect of NAC pretreatment on apoptosis in MDA-MB-231 cells. MDA-MB-231 cells pretreated with 10 mM NAC for 6 hours before being treated with PEITC for 48 hours. Annexin V and propidium iodide staining were determined via flow cytometry. The x axis shows FL1-H (log) for annexin V labeling and the y axis shows FL2-H (log) with red fluorochrome for PI labeling. Data shown are representative of three independent experiments.

A


Percentage of AV/PI staining (%)
100
80
60
40
20
0
**
*
*
Untreated
DMSO
20μM
Treatment

B


Percentage of AV/PI staining (%)
100
80
60
40
20
0
Untreated
DMSO
20μM
Treatment

C

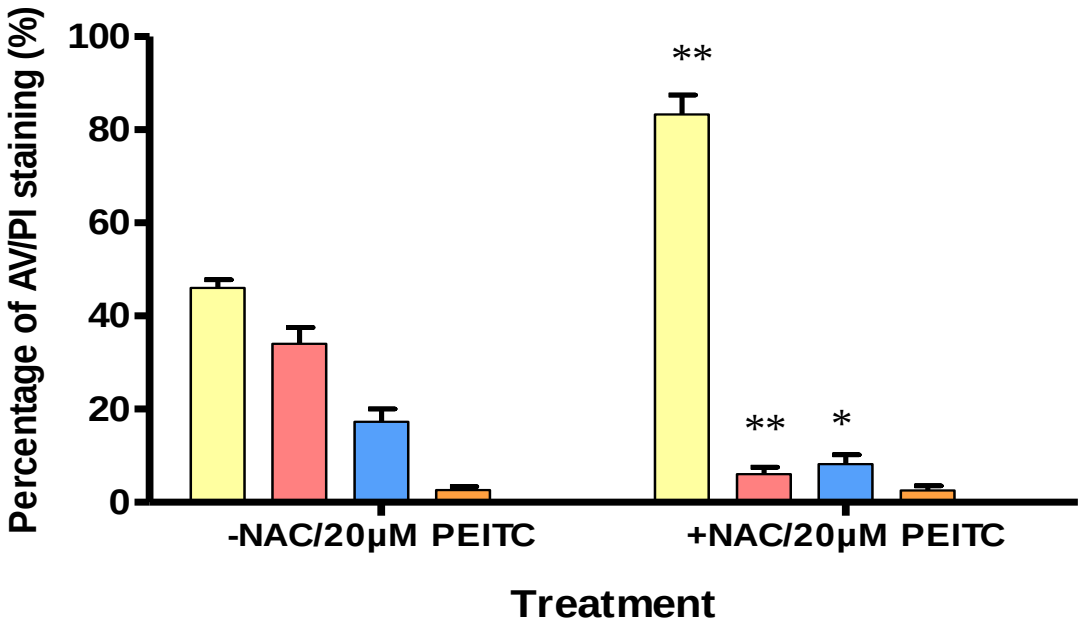


Figure 5.15. Quantitation of apoptosis in MDA-MB-231 cells that were (A) treated with PEITC alone for 48 hours (B) pre-treated with 10 mM NAC followed by PEITC treatment for 48 hours (C) comparison of PEITC effects with and without NAC pretreatment. AV-/PI- ( ), AV+/PI- ( ), AV+/PI+ ( ) and AV-/PI+ ( ). (A) Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.005$). (C) Statistically significant differences between PEITC alone and PEITC with NAC pretreatment are indicated (*$p < 0.05$; **$p < 0.005$). All other differences were not statistically significant. Data shown are the average of three independent experiments**.**

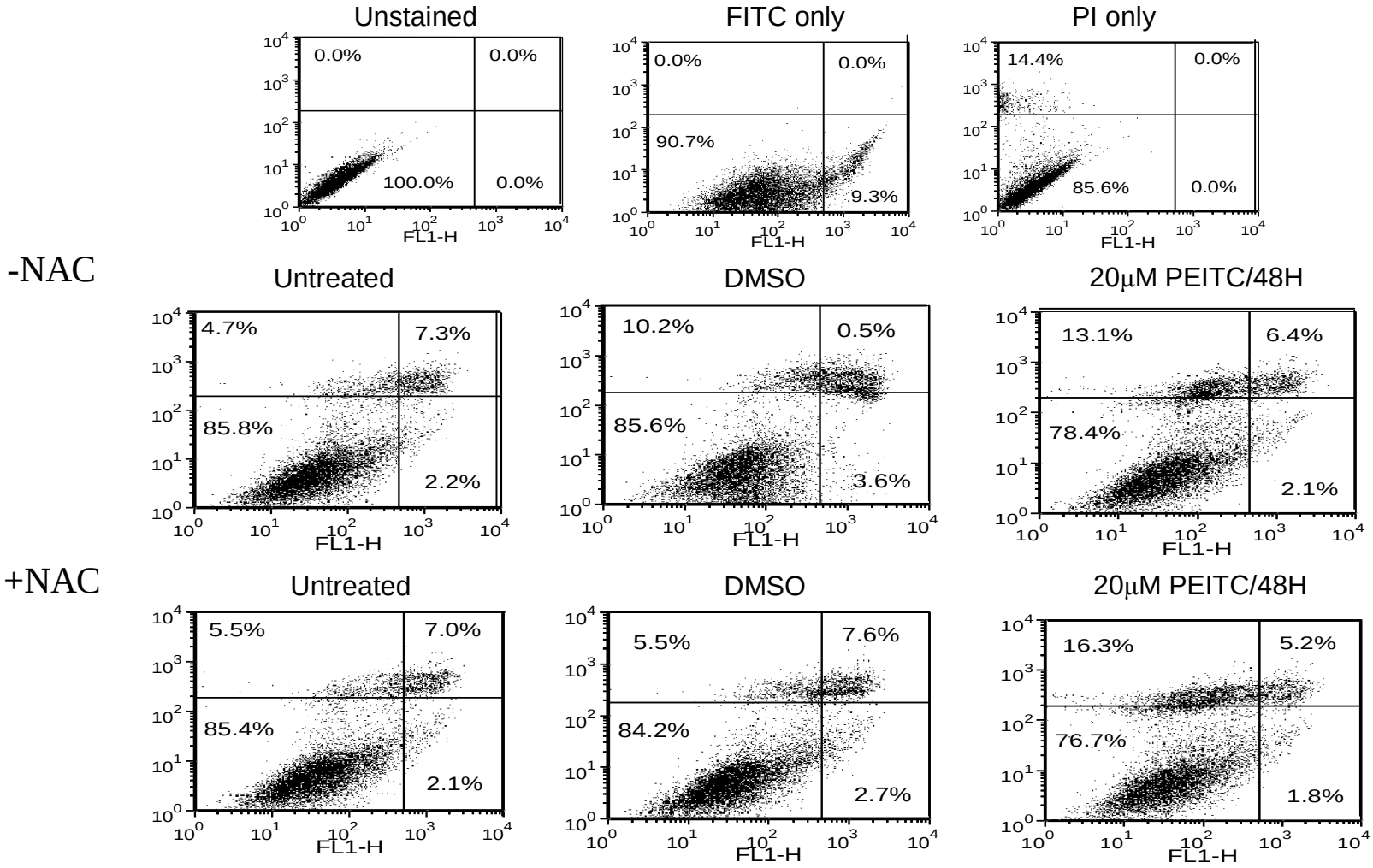
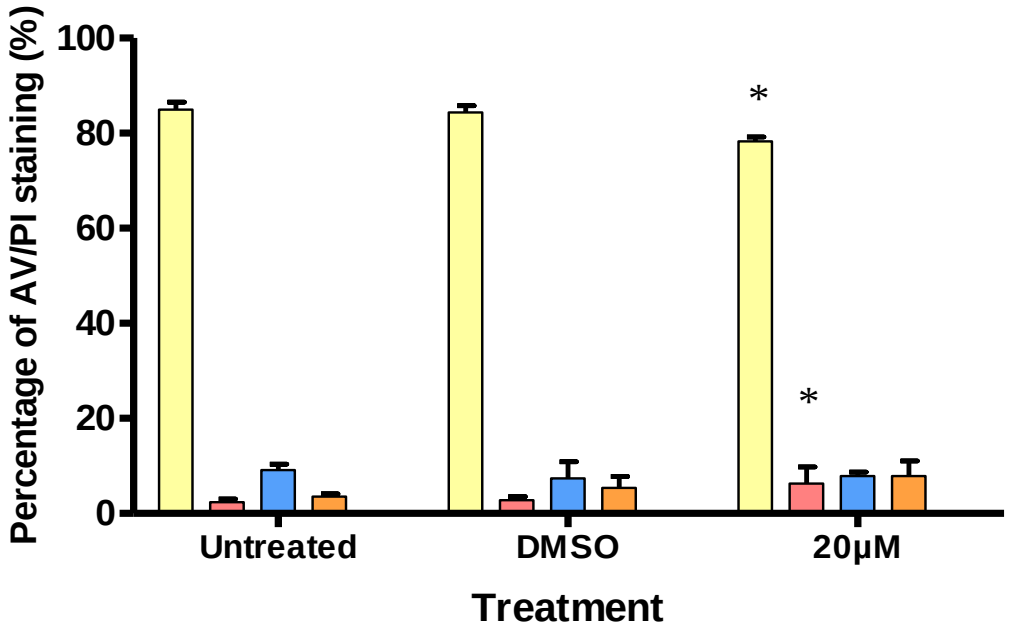
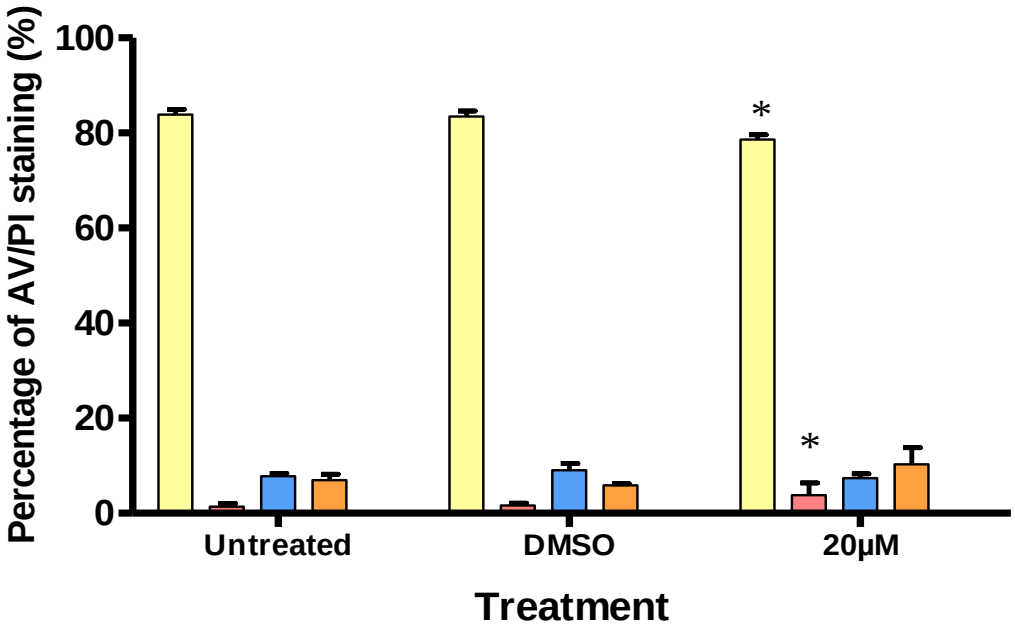


Figure 5.16. Effect of NAC pretreatment on PEITC induced apoptosis in MCF7 cells. MCF7 cells pretreated with 10 mM NAC for 6 hours before being treated with PEITC for 48 hours. Annexin V and propidium iodide staining were determined via flow cytometry. The x axis shows FL1-H (log) for annexin V labeling and the y axis shows FL2-H (log) with red fluorochrome for PI labeling. Data shown are representative of three independent experiments.

A


Percentage of AV/PI staining (%)
100
80
60
40
20
0
*
*
Untreated
DMSO
20µM
Treatment

B


Percentage of AV/PI staining (%)
100
80
60
40
20
0
*
*
Untreated
DMSO
20µM
Treatment

C

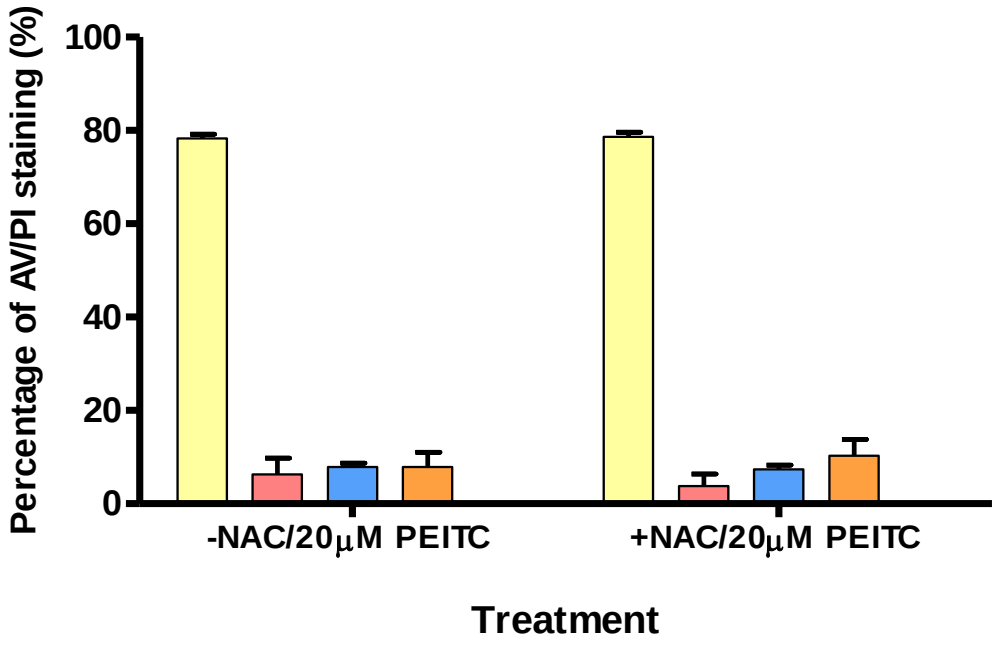


Figure 5.17. Quantitation of apoptosis in MCF7 cells that were (A) treated with PEITC alone for 48 hours (B) pre-treated with 10 mM NAC followed by PEITC treatment for 48 hours (C) comparison of PEITC effects with and without NAC pretreatment. AV-/PI- ( ), AV+/PI- ( ), AV+/PI+ ( ) and AV-/PI+ ( ). (A and B) Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$). All other differences were not statistically significant. Data shown are the average of three independent experiments**.**

5.2.5 Effect of BSO on the potency of PEITC inducing cell cycle arrest in MCF7 cells

Further effects on the modulation of GSH levels in MCF7 cells on the cell cycle arrest were investigated using BSO pretreatment. In this experiment, MCF7 cells were pretreated with similar concentration and time point as in the growth inhibition assay (300 μM for 24 hours), stained with propidium iodide and analyzed by flow cytometry (Figure 5.18). Similar experiment was conducted in MDA-MB-231, however, BSO pretreatment causing major cell death in MDA-MB-231 cells due to high toxicity even at lower concentration.

Similar as in previous chapter, data obtained indicated, treatment with PEITC alone resulted in a significant increased of sub-G1 and G1 arrest compared to the control after 48 hours treatment as observed in the previous chapter (Figure 5.19 [A]). However, there was

a modest reduction in G1 arrest as well as a small increased in S phase arrest in MCF7 cells upon BSO pretreatment compared to MCF7 cells treated with PEITC alone (Figure 5.19 [B]). Both changes of G1 and S-phase arrest in MCF7 cells pretreated BSO did not reach statistically significant compared to the control.

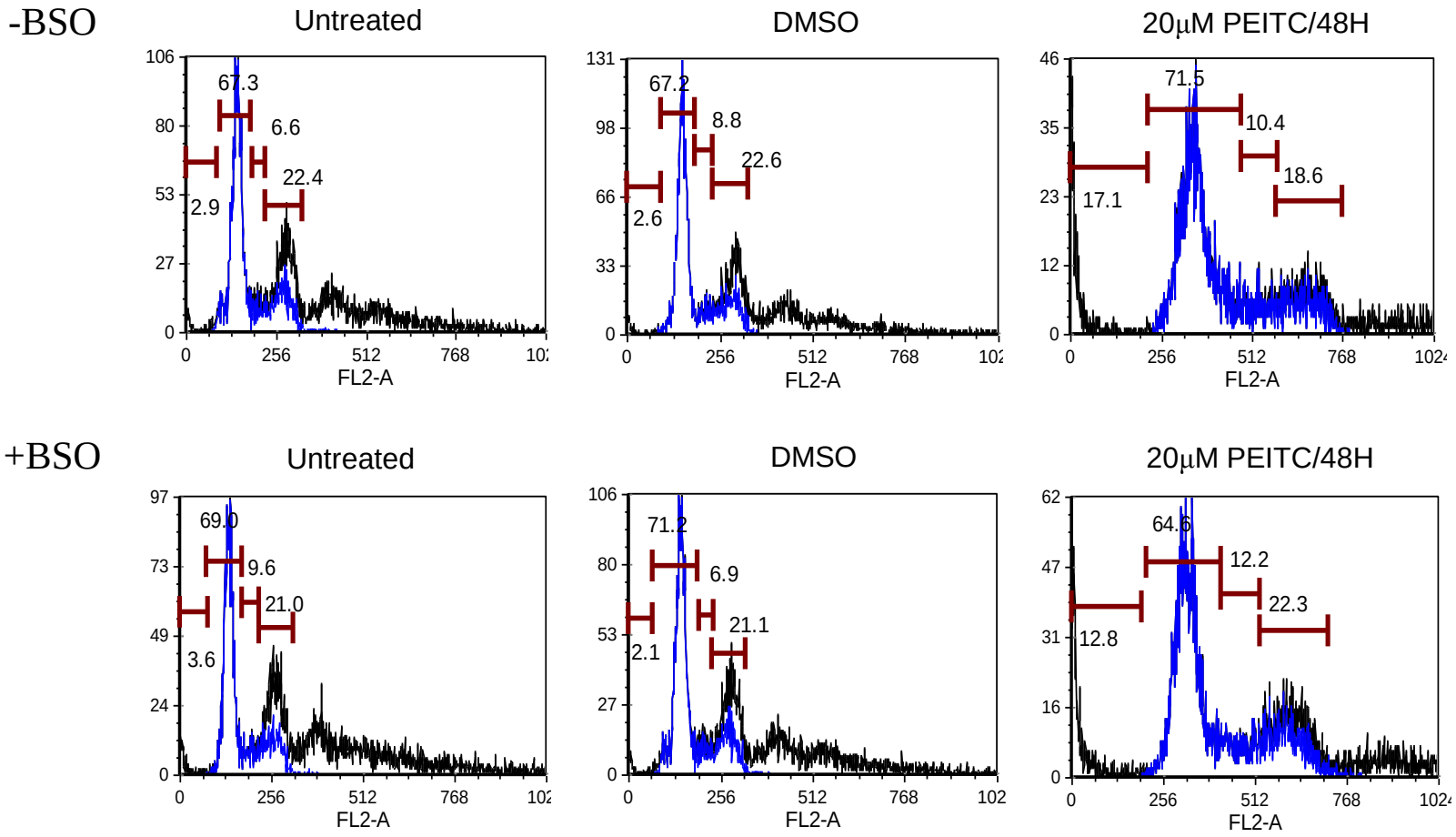
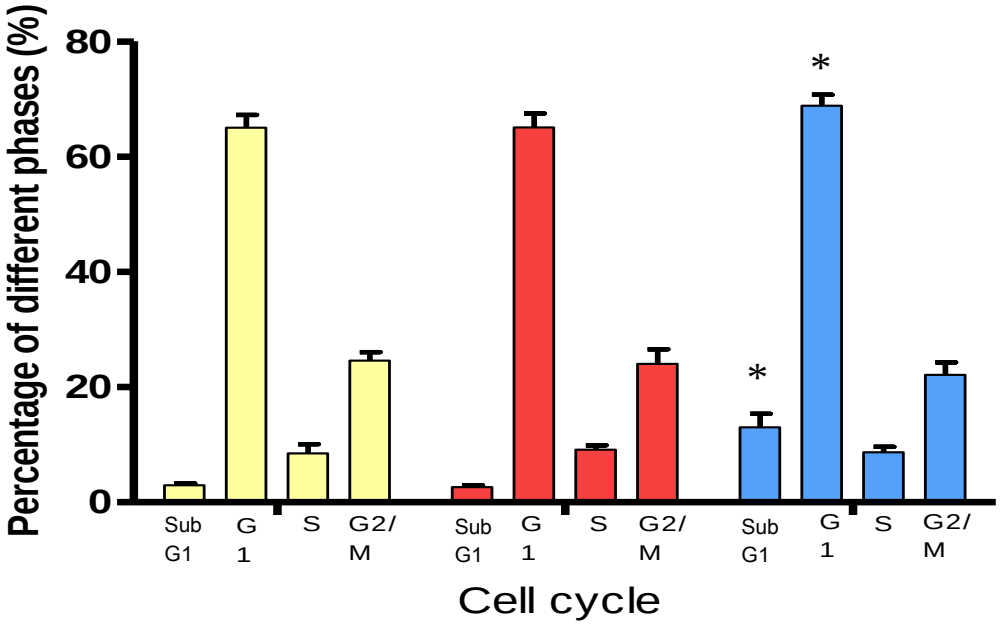
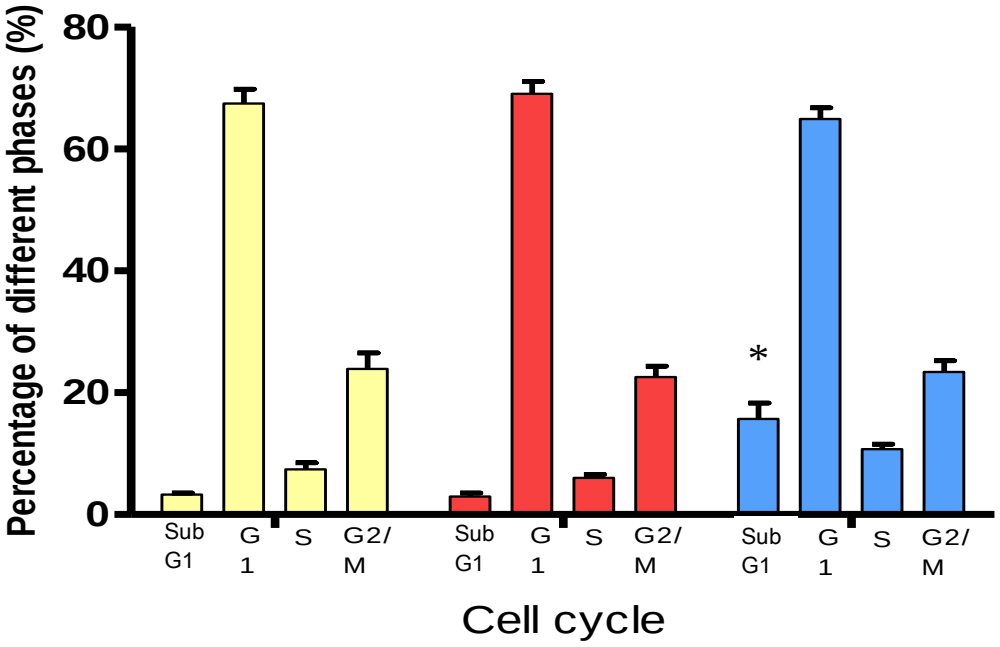


Figure 5.18. BSO pretreatment induced cell cycle arrest in MCF7 by PEITC. MCF7 cells pretreated with 300 μM BSO for 24 hours before being treated with PEITC for 48 hours. Propidium iodide staining was determined via flow cytometry. The x axis FL2A shows red fluorochrome for PI labelling. Blue line indicates single cells population which has been set in the dot plot graph (Data not shown). Data shown above are representative of three independent experiments.

A


Percentage of different phases (%)
80
60
40
20
0
*
*
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Cell cycle

B


Percentage of different phases (%)
80
60
40
20
0
*
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Sub G1
G 1
S
G2/ M
Cell cycle

C

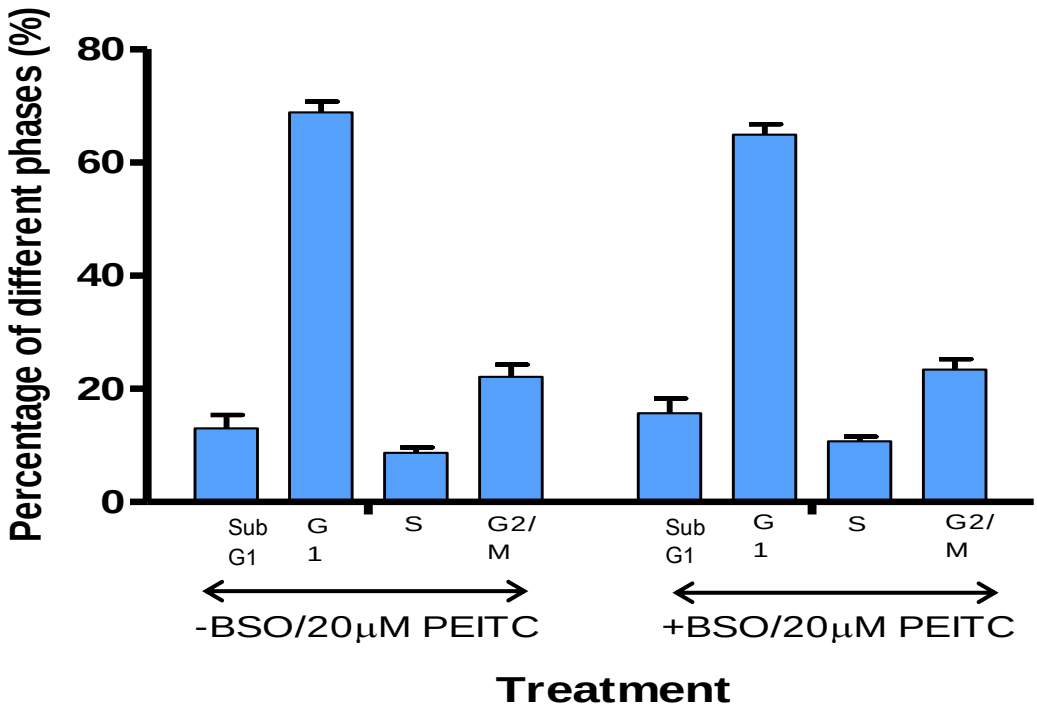


Figure 5.19. Quantitation of cell cycle arrest in MCF7 cells that were (A) treated with PEITC alone for 48 hours (B) pre-treated with 300 μM BSO followed by PEITC treatment for 48 hours and (C) comparison of PEITC effects with and without BSO pretreatment. Untreated cells (□), DMSO (■), 20 μM PEITC (■). (A and B) Statistically significant differences between DMSO and PEITC treated cells are indicated (**p* < 0.05). All other differences were not statistically significant. Data shown represents the average of three independent experiments.

5.2.6 Effect of BSO on the potency of PEITC inducing apoptosis in MCF7 cells

Similar experiments were performed to determine the effects of BSO pretreatment on PEITC-induced apoptosis. In MCF7 cells, PEITC-induced apoptosis was unaffected by 300 μM BSO pretreatment since there was no significant differences between PEITC treated and BSO pretreated in MCF7 cells (Figure 5.20; 5.21).

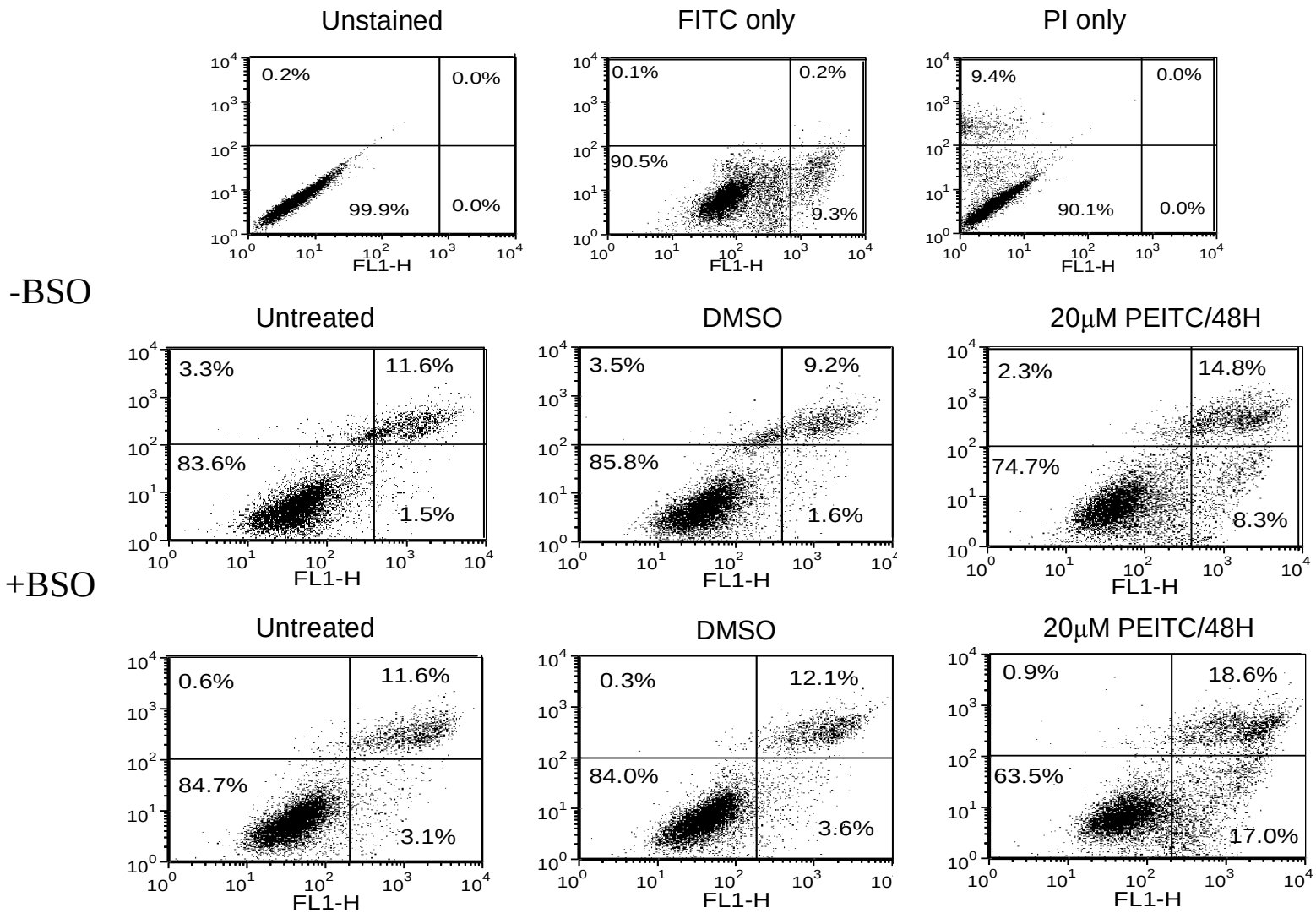
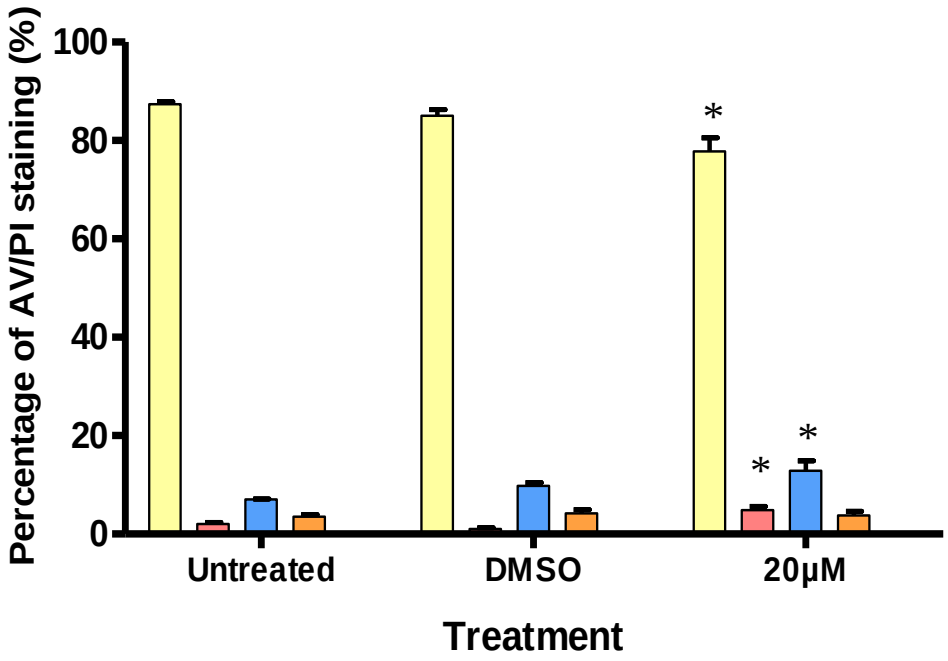
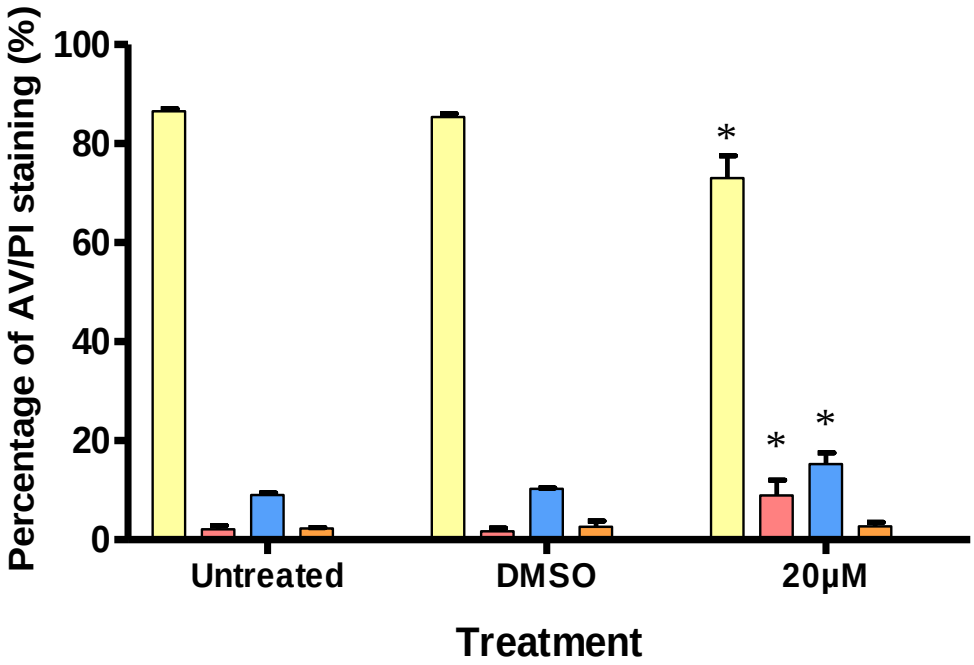


Figure 5.20. BSO pretreatment induced apoptosis in MCF7 cells. MCF7 cells pretreated with 300 μM BSO for 24 hours before being treated with PEITC for 48 hours. Annexin V and propidium iodide staining were determined via flow cytometry. The x axis shows FL1-H (log) for annexin V labeling and the y axis shows FL2-H (log) with red fluorochrome for PI labeling. Data shown are representative of three independent experiments.

A


Percentage of AV/PI staining (%)
100
80
60
40
20
0
*
*
*
Untreated
DMSO
20μM
Treatment

B


Percentage of AV/PI staining (%)
100
80
60
40
20
0
*
*
*
Untreated
DMSO
20μM
Treatment

C

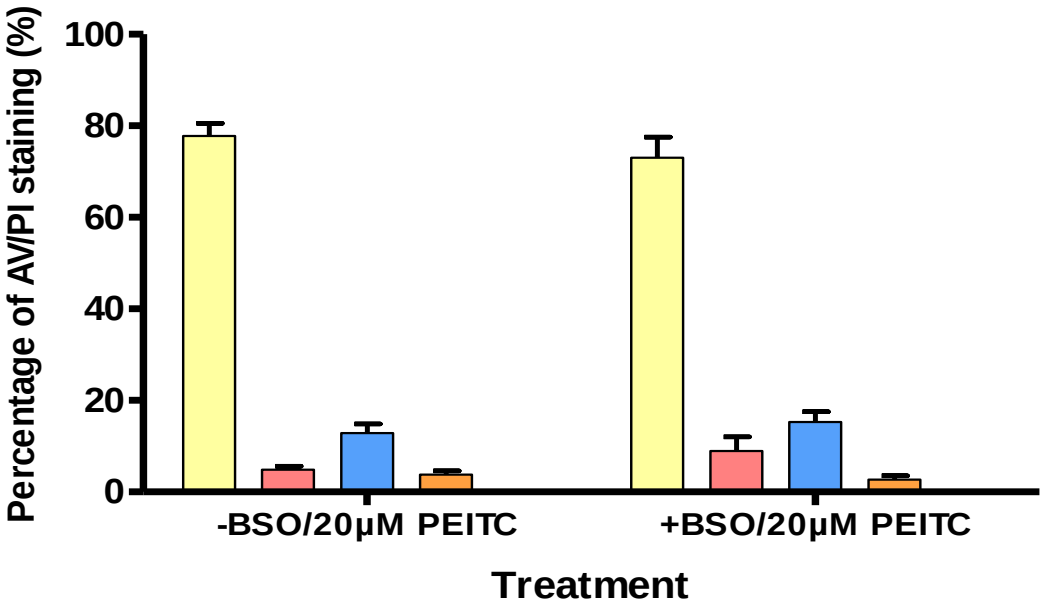


Figure 5.21. Quantitation of apoptosis in MCF7 cells that were (A) treated with PEITC alone for 48 hours (B) pre-treated with 300 µM of BSO followed by PEITC treatment for 48 hours (C) comparison of PEITC effects with and without BSO pretreatment. AV-/PI- ( ), AV+/PI- ( ), AV+/PI+ ( ) and AV-/PI+ ( ). (A and B) Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$). All other differences were not statistically significant. Data shown are the average of three independent experiments**.**

5.2.7 Effect of NAC pretreatment on the expression level of Nrf2 in breast cancer cells

Further effects of NAC pretreatment on Nrf2 expression, as a marker of oxidative stress were investigated. As before, basal levels of Nrf2 were higher in MCF7 cells compared to MDA-MB-231 cells. Nrf2 was induced following PEITC treatment in MDA-MB-231 cells, but not MCF7 cells. Pretreatment with NAC decreased basal Nrf2 levels in MDA-MB-231 cells below the level of detection and completely blocked the PEITC-induced induction (Figure 5.22 [A]). Pretreatment with NAC also reduced Nrf2 expression in MCF7 cells. In NAC pretreated cells, PEITC cause a strong induction in Nrf2 levels, but even at its maximum, this level was lower than that observed in untreated MCF7 cells (Figure 5.22 [B]).

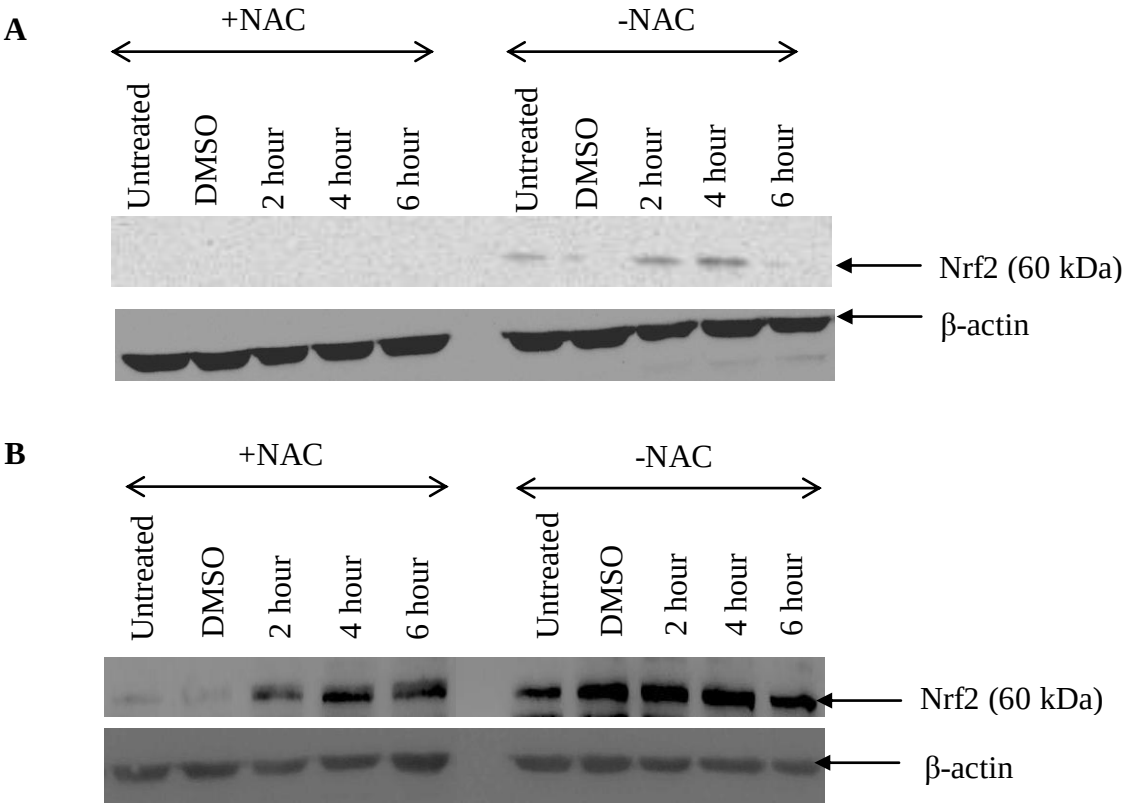
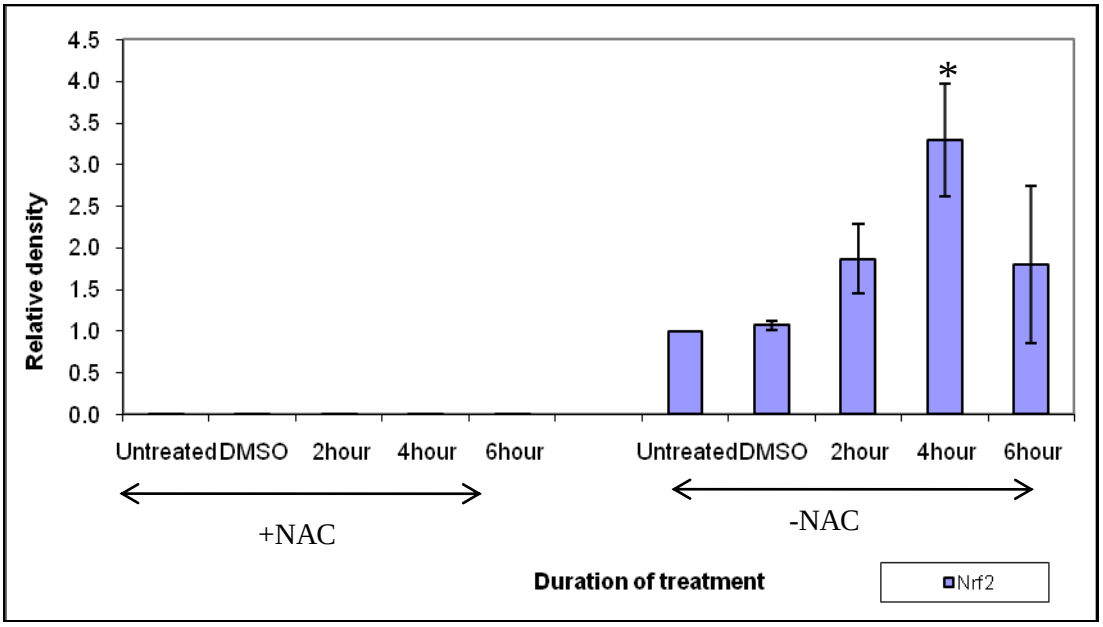


Figure 5.22. Nrf2 expression level in MDA-MB-231 [A] and MCF7 [B] when pretreated with 10mM NAC followed by PEITC treatment as well as in cells treated with PEITC only. Cell lysates were then assayed for Nrf2 regulation via immunoblotting. β-actin acts as a loading control. Experiments are representative of two independent experiments.

A



B

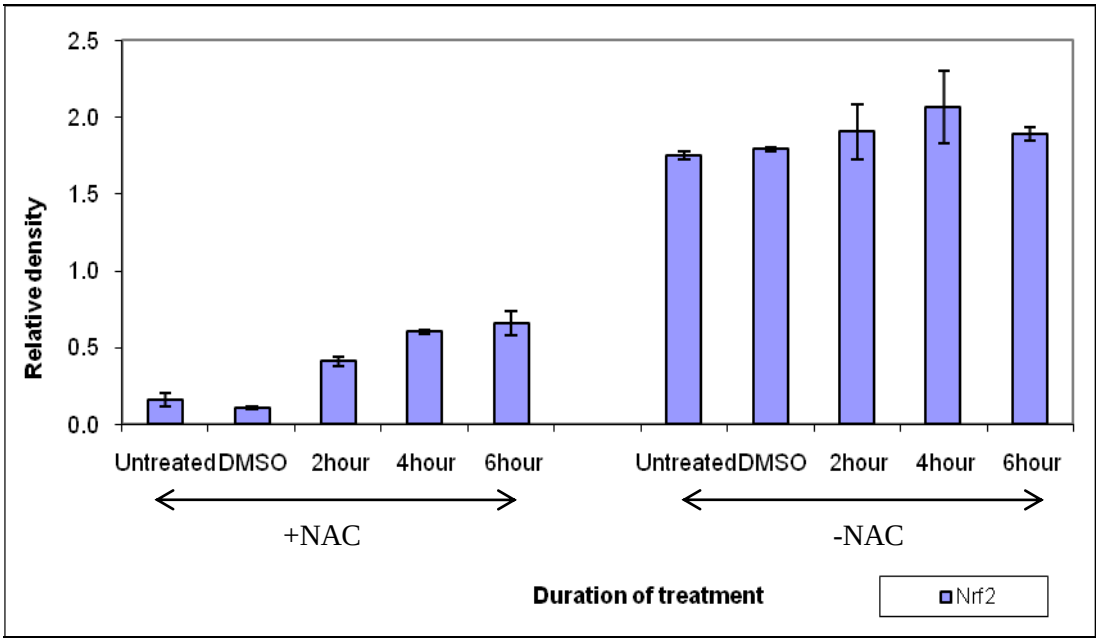


Figure 5.23. Quantitation of Nrf2 expression level in MDA-MB-231 [A] and MCF7 cells [B] upon NAC pretreament. Data shown are means of two independent determination (±SD), normalized to expression of β-actin. Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$).

5.2.8 Glutathione measurement by NAC pretreatment in breast cancer cells treated with PEITC

Results obtained in the previous chapters are consistent with the idea that differences in GSH metabolism contribute to differential responses to PEITC. Thus, to investigate this directly, the levels of GSH in PEITC-treated cells were analyzed.

I first compared "basal" GSH levels in MCF7 and MDA-MB-231 cells (Figure 5.24). Overall, total GSH or GSSG levels in MDA-MB-231 cells were lower compared to MCF7 cells. This is consistent with the idea that MCF7 cells have higher levels of antioxidant defenses, possibly linked to raise basal Nrf2 levels.

Following treatment of MDA-MB-231 cells with PEITC (Figure 5.25), the levels of GSH declined, whereas there was no significant change in the levels of GSSG. Thus, there is an overall decrease in total intracellular GSH, and a decrease in the GSH/GSSG ratio, indicative of oxidative stress. Meanwhile, in MCF7 cells, PEITC treatment also reduced the total levels of GSH, although not to the same extent as in MDA-MB-231 cells. There was also no change in the total GSH/GSSG ratio (Figure 5.26).

As expected, NAC pretreatment of MDA-MB-231 cells increased the basal concentrations of GSH and GSSG (Figure 5.27) and no significant changes observed in GSH/GSSG levels following exposure to PEITC (Figure 5.27). By contrast, NAC pretreatment had little effect on GSH/GSSG levels in MCF7 cells, but did reverse the decrease in GSH concentration observed in cells treated with PEITC alone (Figure 5.28).

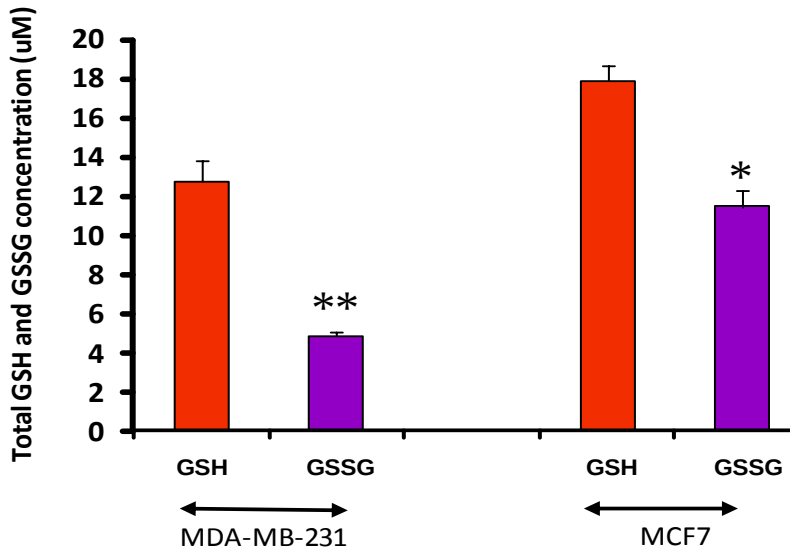


Figure 5.24. Glutathione measurement of the untreated in MDA-MB-231 and MCF7 cells. Total GSH (■) and GSSG (■). Statistically significant differences between total GSH and GSSG for each cell lines are indicated (*$p < 0.05$; **$p < 0.005$). Data shown are the average of three independent experiments.

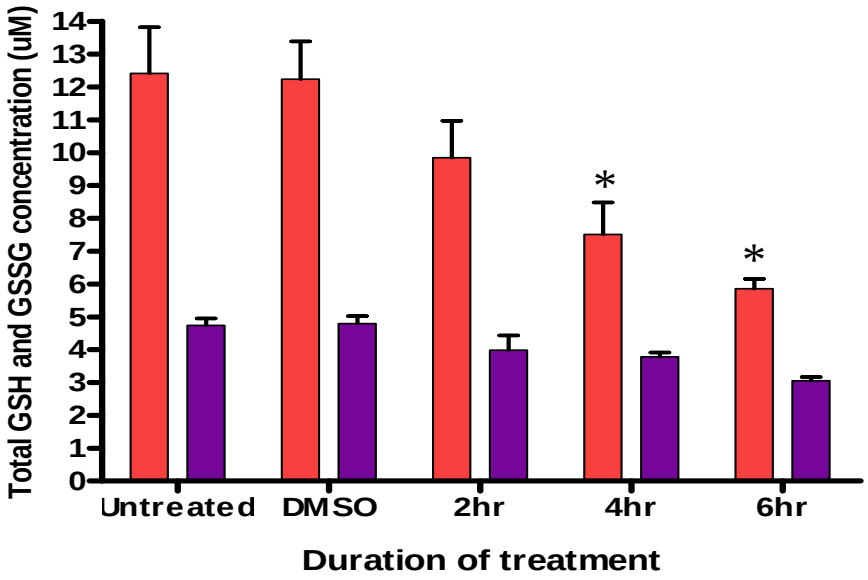


Figure 5.25. Glutathione measurement in MDA-MB-231 cells that were treated with PEITC alone in time dependent manner. Total GSH (■) and GSSG (■). Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$). All other differences were not statistically significant. Data shown are the average of three independent experiments

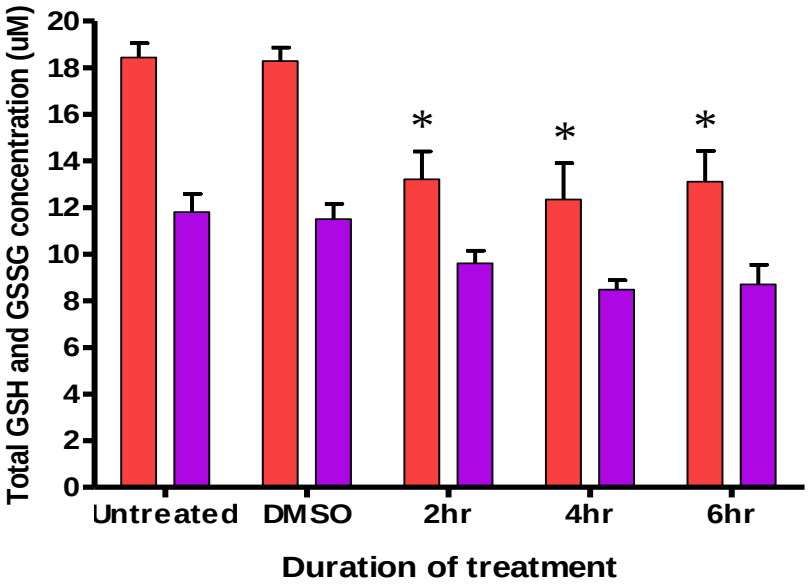


Figure 5.26. Glutathione measurement in MCF7 cells that were treated with PEITC alone in time dependent manner. Total GSH (■) and GSSG (■). Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$). All other differences were not statistically significant. Data shown are the average of three independent experiments.

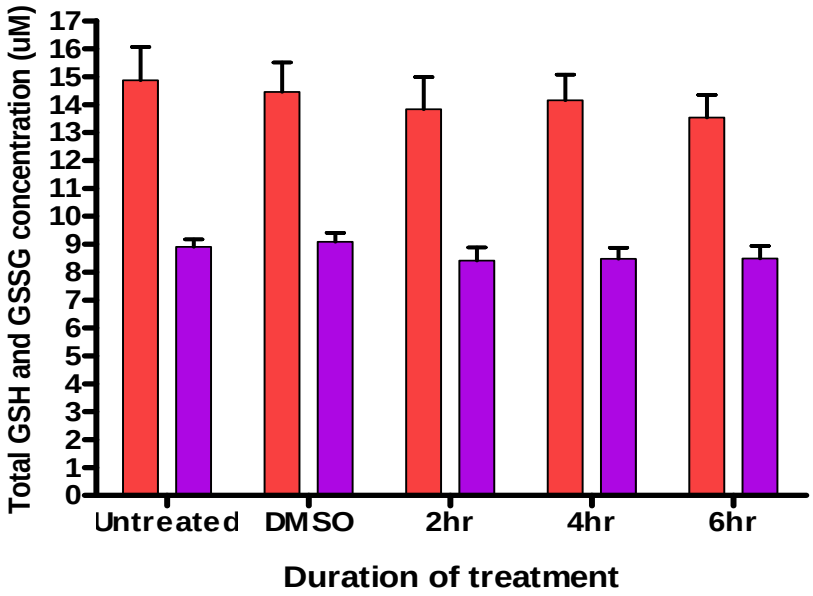


Figure 5.27. Glutathione measurement in MDA-MB-231 cells that were pretreated with 10 mM NAC followed with PEITC treatment in time dependent manner. Total GSH (■) and GSSG (■). No statistically significant differences between DMSO and PEITC treated cells are indicated. Data shown are the average of three independent experiments.

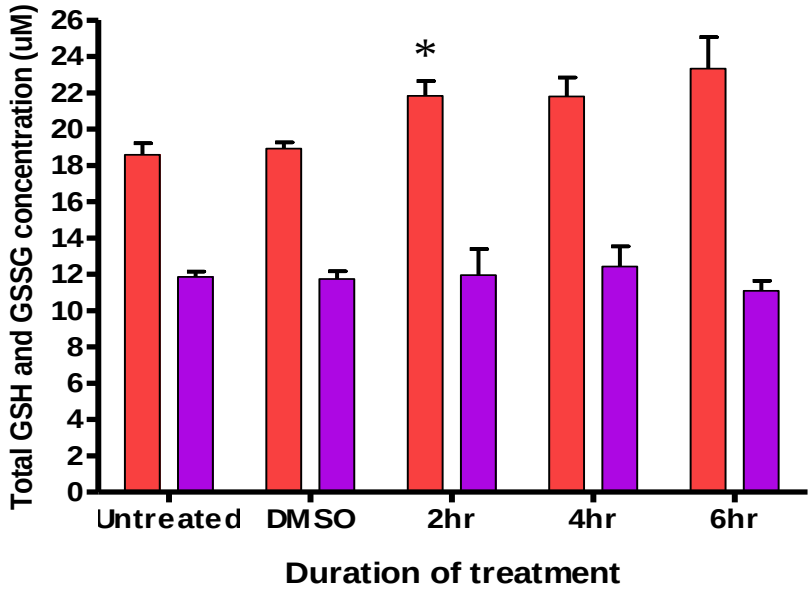


Figure 5.28. Glutathione measurement in MCF7 cells that were pretreated with 10 mM NAC followed with PEITC treatment in time dependent manner. Total GSH (■) and GSSG (■). Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$). All other differences were not statistically significant. Data shown are the average of three independent experiments

5.3 DISCUSSION

The aim of the work described in this chapter was to investigate the role of GSH in determining cell responses to PEITC. Overall GSH levels appeared to be a major determinant of response to PEITC in breast cancer cells.

5.3.1 Effect of GSH manipulation on PEITC responses in breast cancer cells

In this experiment, NAC was used as a natural precursor to elevate intracellular GSH levels. It was observed that NAC strongly reversed the growth inhibitory effects of PEITC (MTS assay) in MDA-MB-231 cells (by ~3 fold), whereas more modest effects (~1-fold) were observed in MCF7 cells. Consistent with this, NAC pretreatment prevented PEITC-induced G2M arrest and apoptosis in MDA-MB-231 cells. By contrast, NAC did not affect cell cycle arrest in MCF7 cells. Thus, elevating intracellular levels of GSH (confirmed in subsequent analysis of GSH/GSSG) appears to sufficient to counter growth inhibitory effects of PEITC. Although NAC can act as a precursor for GSH, it can also act as a direct antioxidant. Therefore one drawback of this approach was that NAC could have acted extracellular to covalent bind PEITC and limits its uptake. However, treatment with a mixed of NAC and PEITC did not substantially affect the growth inhibition by PEITC. NAC also did not exert similar effects in all cell lines and it is likely that its differential effects do reflect its conversion into GSH. Therefore, an alternate approach to elevate GSH levels could be over-express components of the GSH biosynthetic pathway.

Studies with BSO were more difficult to interpret, although also supported the idea that GSH levels were an important determinant of responses to PEITC. BSO is a specific γ-glutamylcysteine synthetase inhibitor and irreversibly blocks the rate-limiting step in the synthesis of glutathione. Most notably, MDA-MB-231 cells were highly sensitive to treatment with BSO alone, and readily underwent apoptosis at concentrations of 50 μM and above. By contrast, MCF7 cells could be pretreated with BSO at 0.3 mM for 24 hours with no significant inhibitory effects since BSO at 0.01 mM did not affect the growth inhibition by PEITC. This is consistent with the idea that MDA-MB-231 cells are more susceptible to GSH depletion, whether by treatment with BSO or PEITC. Both MDA-MB-231 and MCF7 cells were sensitized to growth inhibitory (MTS assay), and the cell cycle arrest and apoptosis inducing effects of PEITC in the presence of BSO. However, the

interpretation of these results is complicated by the fact that different concentrations of BSO were used in MDA-MB-231 (10 µM of BSO) and MCF7 cells (300 µM of BSO).

5.3.2 Effect of NAC pretreatment on the Nrf2 expression level in breast cancer cells treated with PEITC

Previous studies described in chapter 4 had shown that Nrf2 was differentially expressed between MDA-MB-231 and MCF7 cells. Since Nrf2 is both a sensor of oxidative stress and master regulator of antioxidant gene expression, the effects of NAC on its expression was investigated.

As shown before, basal Nrf2 expression was higher in MCF7 cells versus MDA-MB-231 cells. Pretreatment with NAC decreased Nrf2 expression in MCF7 cells, suggesting that on-going production of, perhaps low levels, of ROS mediate the basal expression of Nrf2 in MCF7 cells. When MCF7 cells that had been pretreated with NAC were subsequently treated with PEITC, there was a modest increase in Nrf2 expression, although the levels did not reach those of "basal", untreated MCF7 cells without NAC pretreatment. By contrast, in MDA-MB-231 cells, Nrf2 was induced following PEITC treatment, but this induction was completely blocked upon NAC pretreatment.

5.3.3 Glutathione measurement in breast cancer cells treated with PEITC

To directly measure effects of PEITC, GSH and GSSG levels were quantified using biochemical assays. PEITC has been reported to conjugate with GSH; ITC-GSH conjugates are removed from the cells by transport pumps causing a reduction in total cellular GSH. PEITC has also been shown to increase the relative proportion of GSSG, a marker of intracellular oxidative stress.

Basal levels of glutathione were higher in MCF7 cells, compared to MDA-MB-231 cells. This is consistent with the elevated expression of Nrf2 in MCF7 cells, since many Nrf2 target genes are involved in GSH synthesis. The GSH/GSSG ratio was lower in MCF7 cells indicative of on-going oxidative stress. In MDA-MB-231 cells, PEITC treatment leads to decreased total intracellular GSH levels and a decreased GSH/GSSG ratio. All of these changes were prevented by pretreatment by NAC. In MCF7 cells, there were only modest decreases in total GSH, and no evidence for altered GSH/GSSG ratios. Again, the decline in total GSH was reversed by NAC.

5.3.4 Summary

Results described here suggest that differences in ROS play a major role in determining response to PEITC. The following is a tentative model which attempts to integrate these findings.

In MCF7 cells, there is an on-going, low level production of ROS that leads to activation of Nrf2. This in turn induces expression of antioxidant target genes establishing a raised "basal" level of antioxidant defence. This is supported by the observation that NAC decreases basal Nrf2 expression in these cells, and that total GSH is relatively high. Thus, at rest MCF7 cells exist at an equilibrium whereby basal ROS production leads to elevated antioxidant defences. Thus when these cells are further challenged with PEITC, they are well adapted to counter this insult; PEITC has relatively modest growth inhibitory effects in these cells, and does not induce significant levels of increased ROS or apoptosis. Raised GSH levels perhaps also allow these cells to better tolerate GSH depletion via BSO treatment. Although not sufficient to push these cells to extensive apoptosis, PEITC is not without effects in these cells. PEITC does induce a cell cycle arrest, later changes in molecular markers (Bax, AKT, 4EBP1 phosphorylation), some GSH depletion and does enhance Nrf2; however, overall, MCF7 cells appear to be relatively protected by basal Nrf2 mediated defences to tolerate the effects of PEITC.

By contrast MDA-MB-231 cells appear to be relatively "unprotected" from the effects of PEITC-induced ROS protection. These cells lack substantial basal Nrf2 expression, and therefore have reduced antioxidant defences, consistent with the lower levels of total GSH. When these cells are challenged with PEITC, there is a strong depletion of GSH and shift to increased GSSG, a transient production of ROS and Nrf2 is induced from a very low basal level. However, these responses are presumably either inadequate or too slow to protect MDA-MB-231 cells from growth inhibitory effects/apoptosis induced by PEITC. The critical role of ROS is revealed in experiments with NAC pretreatment. Increased GSH levels (to a level approaching basal levels in MCF7 cells), prevents Nrf2 induction and PEITC induced growth inhibition and apoptosis. The lower levels of basal GSH leaves MDA-MB-231 cells very susceptible to further decreases, as indicating by their extreme sensitivity to BSO.

One key issue is to what extent these differences reflect the specific genetic make-up of the two lines. MDA-MB-231 cells are representative of triple-negative breast cancer, for which treatment options are limited, whereas MCF7 cells are representative of ER

positive tumours, typically treated by surgery and anti-hormone therapy. A major drawback of this study is that these two cell lines will not represent the diverse range of breast cancer subtypes so it is impossible to conclude whether differences in behavior between MCF7 and MDA-MB-231 cells reflects more widely relevance responses that could be linked to clinically important disease subtypes.

To begin to extend my results to other cell lines, the effects of BSO or NAC on growth inhibition (MTS) in BT549, SKBr3, T47D and ZR75.1 cells were investigated. Like MDA-MB-231 cells, growth inhibitory effects of PEITC in ER negative BT549 was strongly increased by BSO compared to ER positive, T47D and MCF7 cells. Meanwhile in SKBr3 cells which are ER negative, BSO failed to increase the growth inhibitory effect by PEITC. Although the exact mechanism is unknown, it is hypothesized might be due to the presence of amplified Her2/Neu. Thus, overall sensitivity of the cell lines to PEITC was MDA-MB-231 > BT549 > MCF7 > SKBr3 > T47D cells. This suggests that although ER status may somehow be linked to GSH levels, other molecular features of the breast cancer cells might influence the sensitivity of the cells towards PEITC treatment.

Meanwhile, the growth inhibitory effects of PEITC in ER negative MDA-MB-231 and BT549 were strongly reversed by NAC, whereas NAC had a modest reversal effects on ER positive MCF7 cells (Data provided by Breeze Cavell). However NAC had no reversal effects on other ER positive cells, T47D and ZR75.1 cells. Therefore, the overall pattern of sensitivity of the various cells to PEITC was MDA-MB-231 > BT549 > MCF7 > T47D > ZR75.1 > NHDFs, and the reversal effect of NAC was most obvious in the relatively sensitive MDA-MB-231 cells.

This does suggest there may be some relationship between ER status and decreased sensitivity to PEITC. This would clearly require much more work to confirm, but it is interested that a recent paper has linked oestrogen receptor signaling to increased basal ROS production via affects on mitochondria proteins (Thomas et al. 2005). Thus siRNA knockdown of ERs or anti-estrogens treatment could be used to directly investigate the role of ER signaling in the modulation of basal ROS levels and responses to PEITC.

Chapter 6

In Vivo Study: Effects of PEITC and watercress on selective biomarker

6.1 Introduction

In previous chapters, PEITC has been shown to have the capacity to activate a number of potential "anti-cancer" responses in cancer cells in vitro. Watercress is a very rich source of PEITC, but it is not clear that normal dietary ingestion of watercress would provide sufficient exposure to exert any of these effects in vivo. In this work, I aim to (i) identify a biomarker that can be used to monitor PEITC responses in normal cells *in vivo* and (ii) investigate the effects of watercress ingestion on this biomarker in circulating blood cells.

6.2 RESULTS

6.2.1 The effect of watercress ingestion on ROS accumulation in Peripheral Blood Mononuclear cells (PBMCs)

Initial biomarker studies were performed using peripheral blood mononuclear cells (PBMCs) isolated from normal individuals following ingestions of 80g of watercress. This amount of watercress was selected since it equates to a standard portion, according to the WHO. PBMCs were studied because they are an easily obtainable population of normal cells that are presumably readily exposed to circulating concentrations of PEITC following absorption in the gut.

PBMCs were isolated from donors at time points up to 12 hours following watercress consumption. The ROS accumulation was measured using flow cytometry and the oxidation sensitive dye DHR123. Increased ROS levels were observed in a portion of PBMCs at 5 hours following watercress ingestion, and levels declined to pre-watercress levels at 12 hours (Figure 6.1).

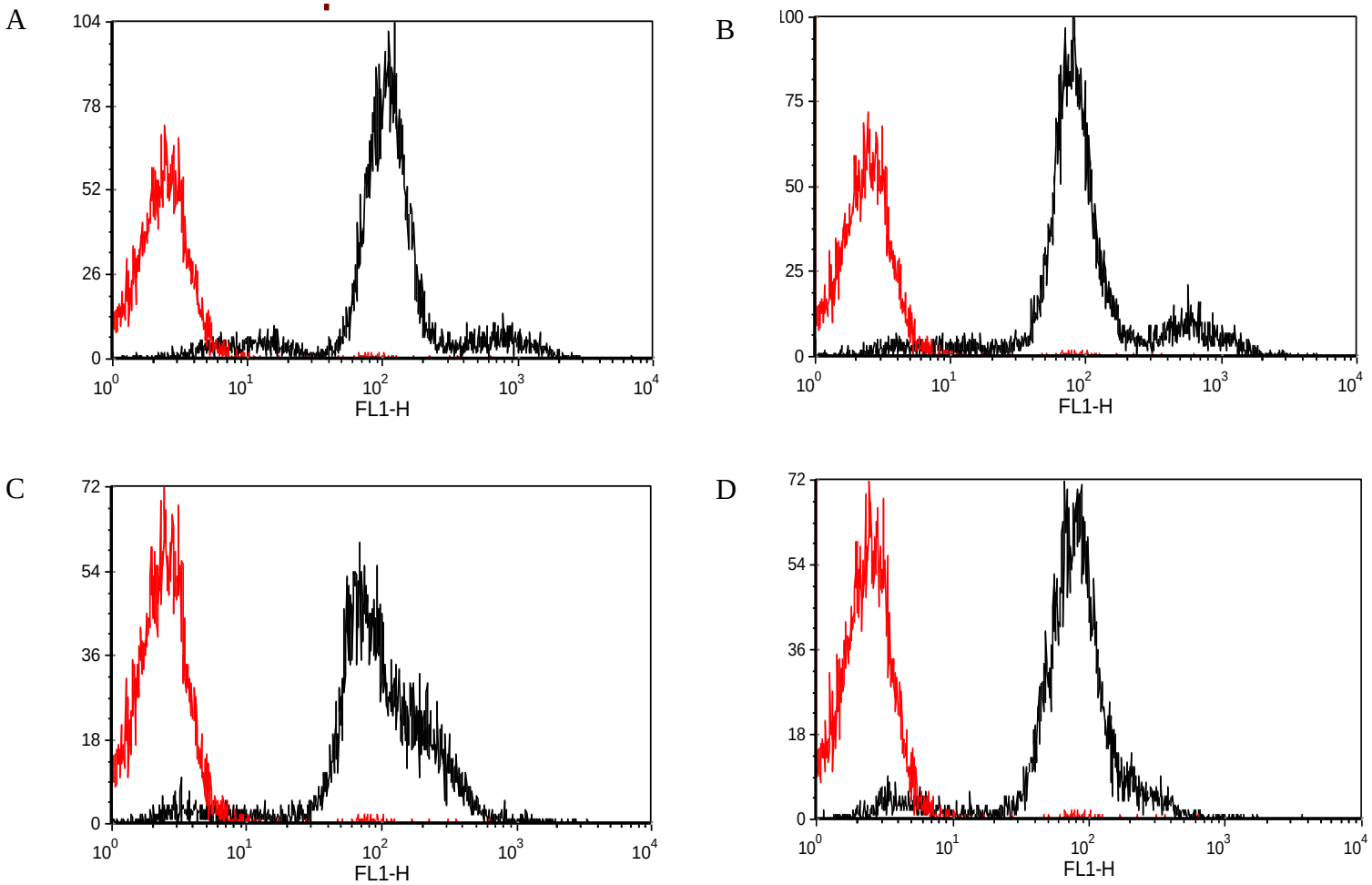


Figure 6.1. Effect of watercress consumption on the accumulation of ROS in PBMCs. PBMCs were isolated from healthy individuals after 0 (A), 2 (B), 5 (C) and 12 (D) hours following watercress consumption. DHR123 staining was determined by flow cytometry. Unstained PBMCs (▬); stained PBMCs (▬). Data shown are representative of two independent experiments.

6.2.2 The effect of watercress on the expression of Keap1 in PBMCs

To further investigate ROS accumulation in PBMCs, the effects of watercress ingestion on Keap1 levels was investigated using immunoblotting. Previous work has shown the ITCs can trigger the ubiquitination and proteosomal degradation of Keap1 (Li & Kong 2009). PBMC Keap1 levels were significantly reduced at 5 and 12 hours of watercress consumption (Figure 6.2). Nrf2 expression was not detected in PBMCs at any time point (data not shown).

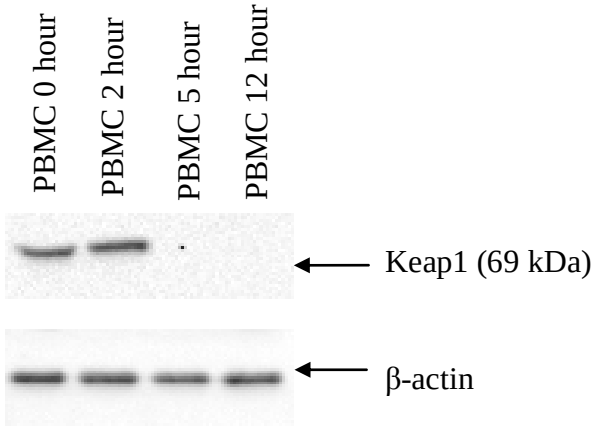


Figure 6.2. Effect of watercress consumption on the expression level of Keap1 in PBMCs. PBMCs at 0, 2, 5 and 12 hours were obtained. Cell lysates were then assayed for Keap1 via immunoblotting. β-actin acts as a loading control. Data shown are representative of two independent experiments

6.2.3 The effect of watercress ingestion on the phosphorylation of 4EBP1 in PBMCs

Finally, the effects of watercress ingestion on 4EBP1 phosphorylation in PBMCs were investigated. Previous studies (http://www.bdbiosciences.com/external_files/pm/doc/tds/cell_bio/live/web_enabled/560285.pdf) have demonstrated that 4EBP1 phosphorylation is detected at high levels within monocytes, but not lymphocytes, within PBMCs. 4EBP1 phosphorylation was therefore measured using single cell flow cytometry to discriminate the these two cell types. In these experiments, the antibody that specifically detected Thr37/46 phosphorylated 4EBP1 was used since it gave stronger signals than a Thr70phospho-specific antibody in pilot experiments (data not shown).

In initial experiments, 4EBP1 phosphorylation was studied in normal PBMCs without watercress ingestion. Side and forward scatter was used to identify separate monocyte and lymphocyte subpopulations. As expected, 4EBP1 phosphorylation was readily detected in monocytes, but not lymphocytes (Figure 6.3). To confirm that 4EBP1 phosphorylation was modulated by PEITC in monocytes, PBMCs were treated *ex vivo* with PEITC. Consistent with studies in breast cancer cells, PEITC significantly reduced the levels of 4EBP1 phosphorylation. To confirm the selectivity of the antibody, PBMCs were treated with the PI3K inhibitor LY294002 (Data not shown). Consistent with the known role of PI3K→mTORC1 signaling in driving 4EBP1 phosphorylation, LY294002 treatment decreased 4EBP1 phosphorylation in monocytes. Further analysis in PBMCs upon watercress consumption showed a small reduction in 4EBP1 phosphorylation after 4 hours ingestion compared to before watercress ingestion (Figure 6.4). Based on these finding, 4EBP1 phosphorylation was selected as a biomarker to monitor *in vivo* exposure to PEITC following ingestion of watercress.

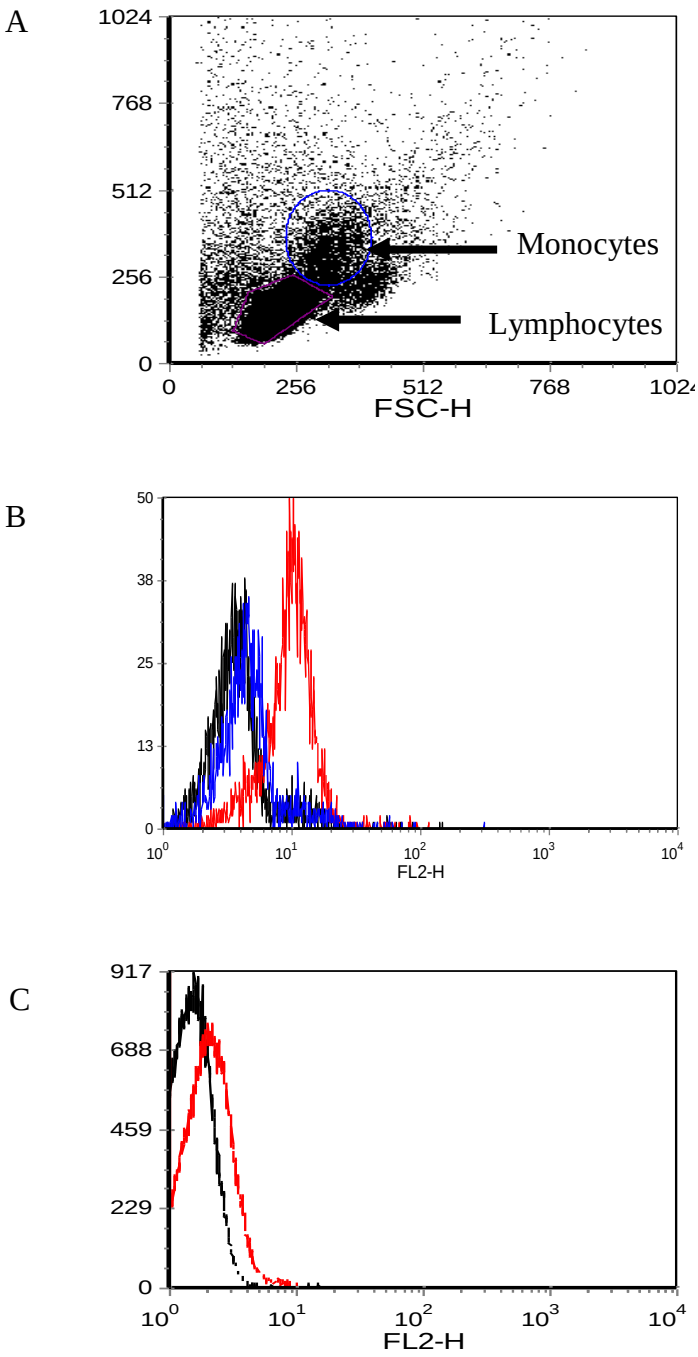


Figure 6.3. Analysis of 4E-BP1 phosphorylation in PBMCs.
PBMCs were isolated from healthy individuals and analysed by flow cytometry using a Thr37/46 4E-BP1 phosphorylation specific antibody. Unstained cells were analysed as a control. (A) Forward/side scatter plot to showing gating of lymphocytes (purple) and monocytes (blue). (B, C) Fluorescence intensity of monocytes (B) and lymphocytes (C). Unstained control (▬); Stained cells (▬). (B) additionally shows the fluorescence intensity of cells treated with PEITC (20 μM) for 2 hours prior to staining with the 4E-BP1 antibody (▬). Data are representative of 4 independent patients.

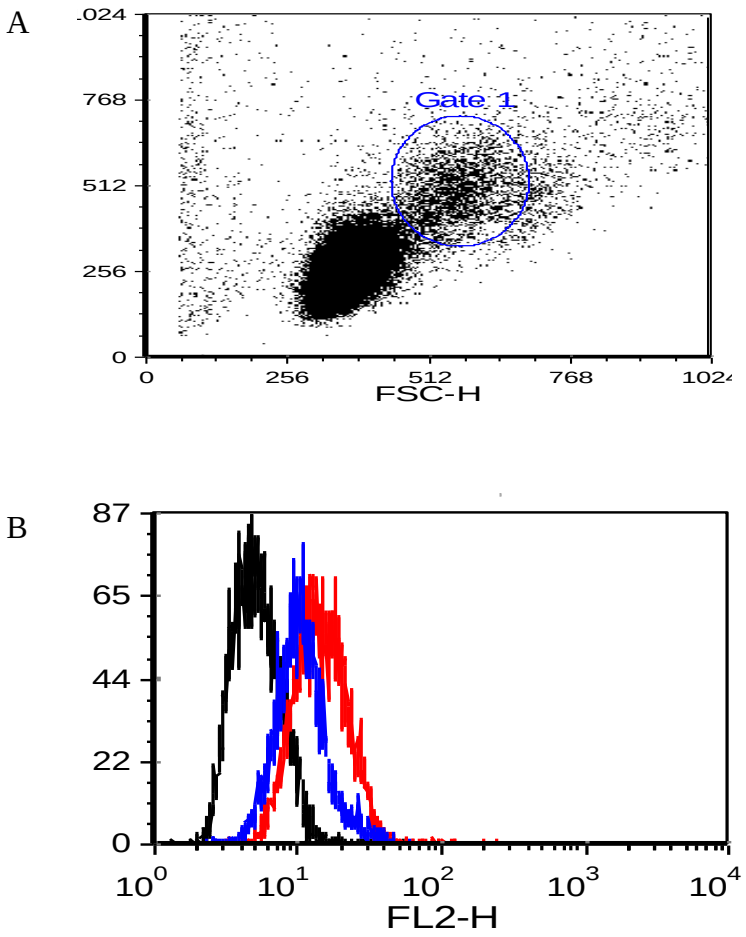


Figure 6.4. Analysis of 4E-BP1 phosphorylation in PBMCs after watercress ingestion. PBMCs were isolated from healthy individuals after watercress consumption and analysed by flow cytometry using a Thr37/46 4E-BP1 phosphorylation specific antibody. Unstained cells were analysed as a control. (A) Forward/side scatter plot to showing gating of monocytes (Gate 1). Unstained control (▬); Stained cells (▬) and fluorescence intensity of cells after watercress consumption for 4 hours (▬) prior to staining with the 4E-BP1 antibody. Data are representative of 3 independent experiments.

6.2.4 Watercress consumption downregulates 4EBP1 phosphorylation *in vivo*.

A pilot feeding study was performed to determine whether ingestion of watercress was sufficient to modulate 4EBP1 phosphorylation *in vivo*. The study design was based on the previous work of Ji & Morris (Ji & Morris et al. 2003). Watercress ingestion and blood sampling was performed at Winchester Hospital and samples were transported to Southampton for analysis.

We enrolled 12 women on the study and analysed plasma PEITC concentrations and/or 4E-BP1 phosphorylation in samples from 9 of these (mean age 58 years, median age 56 years, range 48-82). For 8 participants, we prepared both PBMCs and plasma. For 1 participant we prepared plasma only.

PEITC plasma concentrations were analyzed (Figure 6.5) by Urvi Telang using liquid chromatography-mass spectrometry (LC-MS/MS). Background PEITC concentrations prior to watercress ingestion were very low (generally <1nM). As previously described, plasma PEITC levels rose rapidly before gradually declining over a period of 24 hours. In general, t_{max} was at 2-3 hours, although in one participant (4) tmax was at 8 hours. The mean C_{max} was 297 nM although there was wide inter-individual variation (range 61-656 nM) (Table 8).

Eight participants provided samples for analysis of 4EBP1 phosphorylation and complete data sets were obtained for 4. Two samples were excluded because of high levels of cell death and two because of technical failures. The results showed a marked reduction in 4EBP1 phosphorylation at 6 and 8 hours following ingestion of watercress compared pre-watercress meal values in all 4 participants. 24 hour data was available for 3 participants; 4EBP1 phosphorylation was maintained at a low level until this time in 1 whereas recovery of 4EBP1 phosphorylation was observed in the other 2. The decreases in 4EBP1 phosphorylation at 6 and 8 hours were highly statistically significant ($p=0.001$ and $p=0.002$ from student's t-test, respectively) compared to pre-meal levels. There was considerable variation in levels of 4EBP1phosphorylation prior to 6 hours within individual subjects. We believe this is due to technical variation in transport and sample processing and the average level of 4EBP1 phosphorylation was not significantly altered at these times. Thus, analysis of 4EBP1 Thr37/46 phoshorylation suggests a significant decrease at 6-8 hours post-ingestion of watercress.

Table 6.1. Analysis of plasma PEITC concentrations following watercress consumption

Participant	**Maximal plasma concentration (C_{max}) (nM)**	**Time to reach Cmax (t_{max})(minute)**
1	104	120
2	61	125
3	117	180
4	164	480
5	375	185
6	384	90
7	209	120
8	656	140
9	604	180
Mean	297	180

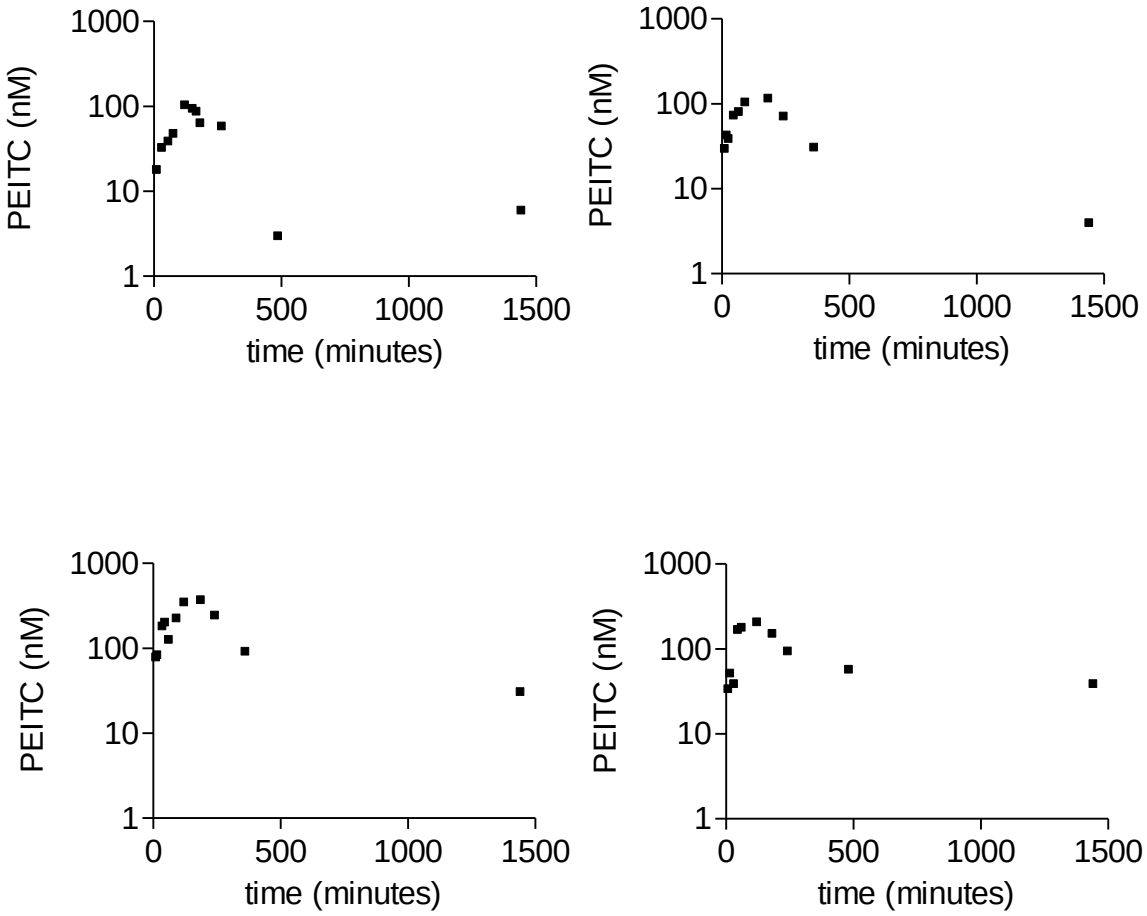


Figure 6.5. Analysis of plasma PEITC concentrations following consumption of watercress.
Plasma concentration of PEITC was determined at various time points following consumption of 80 g watercress. Data from 4 representative subjects is shown. Data provided by Urvi Telang & Marilyn Morris.

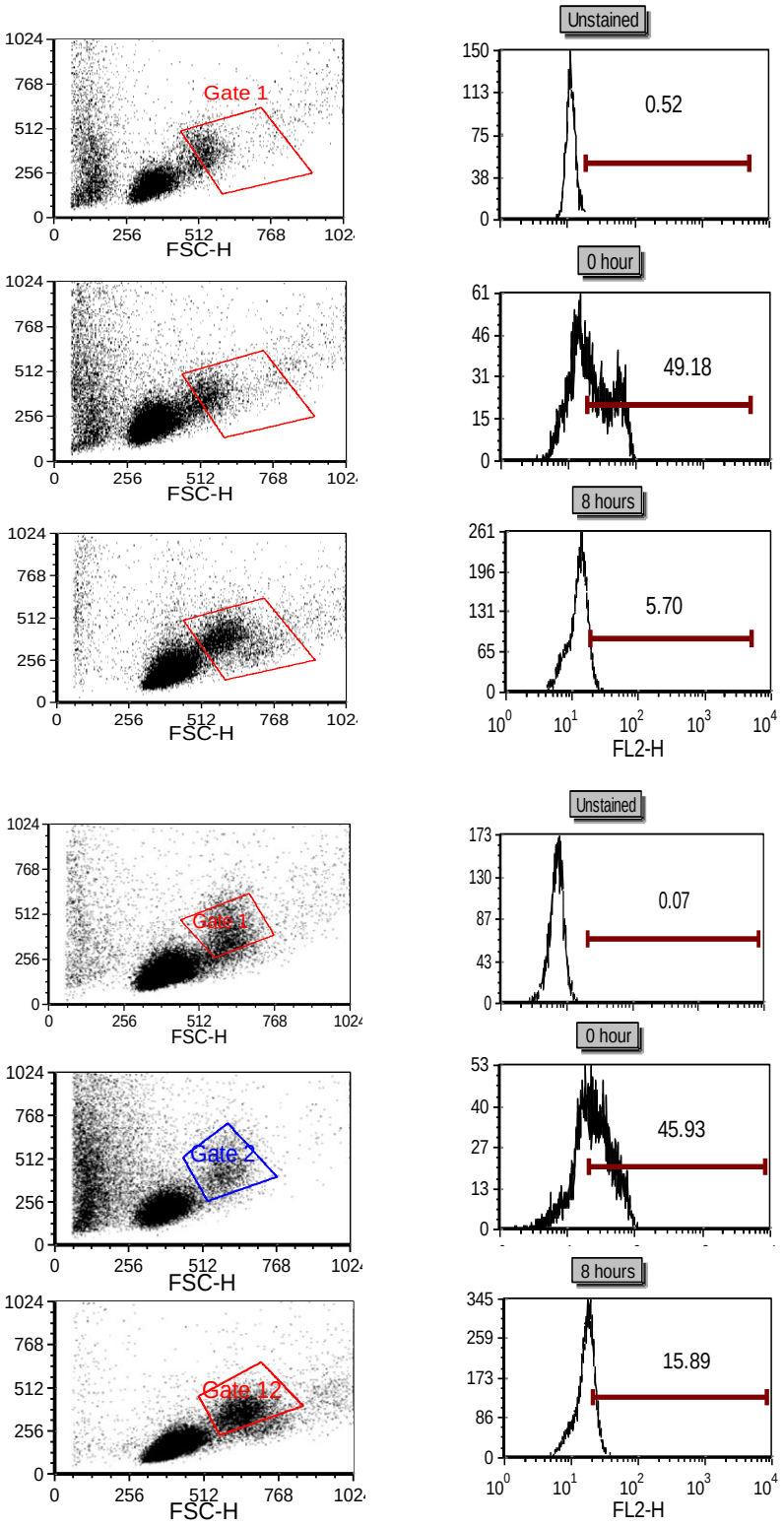
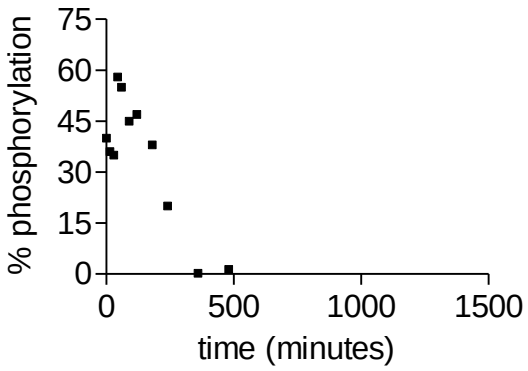
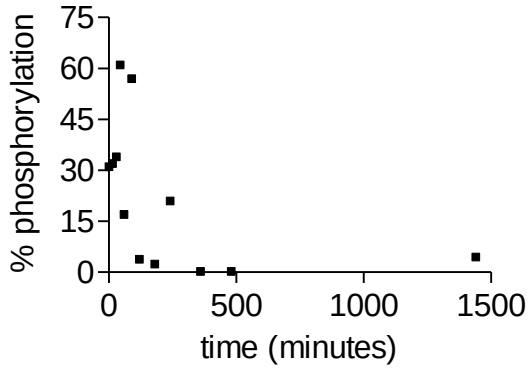


Figure 6.6. Analysis of 4E-BP1 phosphorylation following consumption of watercress. 4E-BP1 phosphorylation was analyzed by flow cytometry in peripheral blood-derived monocytes at various time points following consumption of 80 g watercress. (A) Representative data from two subjects showing FSC/SSC plots (left panels) and fluorescence intensity (right panels) of unstained control cells, and stained cells prior (T_0) and 8 hours (T_{480}) after consumption of watercress.

A



B



C

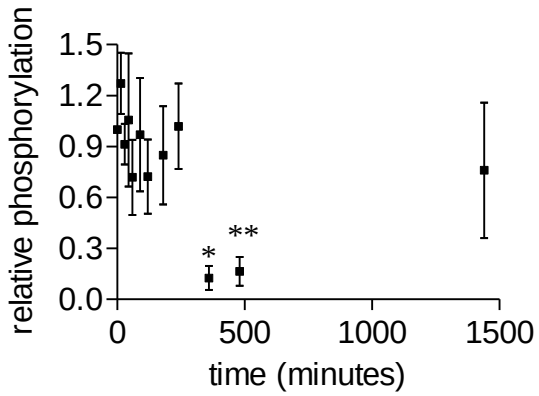


Figure 6.7. Analysis of 4EBP1 phosphorylation following watercress consumption.
Graphs show (A, B) the levels of 4E-BP1 phosphorylation in two representative subjects and (C) the mean (±SEM) levels of 4E-BP1 phosphorylation in all four subjects following consumption of watercress. In (C), the level of 4E-BP1 phosphorylation at T_0 was set to 1.0 to allow comparison between individuals. Statistically significant differences compared to T_0 are indicated (Student's t-test): *P=0.001; **P=0.002.

6.3 DISCUSSION

The aim of the work described in this chapter was to select a biomarker suitable for analysis of potential *in vivo* effects of PEITC and to perform a pilot study to determine the effects of watercress ingestion on this biomarker. PBMCs were selected as target tissue because they are amenable to repeat sampling in healthy individual and are presumably readily exposed to circulating levels of PEITC.

Although watercress ingestion appeared to lead to increased ROS and reduced Keap1 in PBMCs, we did not select these as biomarkers. Firstly, we felt that increases in ROS would be fairly transient and therefore difficult to reliably measure in samples, particular following transport between sites. Although the reduction in Keap1 may be promising, we were unable to detect increased Nrf2 expression so the significance of this change is unclear.

By contrast, decreased 4EBP1 phosphorylation appeared to be an appropriate biomarker of *in vivo* PEITC exposure. First, several *in vitro* studies have reported that reduced 4EBP1 phosphorylation may be an important mechanism contributing to anticancer effects of PEITC and this is therefore likely to be functional relevant (Wang et al. 2009; Hu et al. 2007). Secondly, 4EBP1 phosphorylation was suitable for analysis by flow cytometry which provides quantitative measurements, as well as information on levels within specific cell sub-populations. This was important, since 4EBP1 phosphorylation differed between monocytes and lymphocytes, and the relative proportion of these cells may differ in study participants.

The mechanisms by which PEITC decreases 4EBP1 phosphorylation are unknown. However, modulation of upstream regulators including mTOR and PTEN which contain redox sensitive cysteine residues is suggested to play a role (Dames et al. 2005; Salmeen & Barford 2005). However, it is possible that other watercress derived isothiocyanates, PEITC metabolites or unrelated bioactives may also modulate 4EBP1 phosphorylation. Therefore, the possibility that other compounds are involved in the *in vitro* and *in vivo* effects of watercress is included.

A pilot study was performed to determine the effects of watercress ingestion of plasma PEITC concentrations and 4EBP1 phosphorylation. Flow cytometry analysis demonstrated a statistically significant reduction in 4EBP1 Thr37/46 phosphorylation at 6-8 hours following watercress consumption in 4/4 participants studied. It is required to be cautious since there was significant variability in the levels of 4EBP1 phosphorylation

within individual samples prior to time. However, these differences disappeared when results from the 4 individuals were combined, whereas the consistent downregulation of 4EBP1 phosphorylation at 6-8 hours was highly statistically significant. The considerable variation in levels in the reduction of 4EBP1 phosphorylation might be due to variation in transportation and sample processing.

This suggests that dietary intake of a single 80g portion of watercress is sufficient to modulate this potential anti-cancer pathway. However, further studies are required with larger sample sizes to test this more rigorously. It would be important to further develop the assays. For example, it would be important to minimise time delays between sampling and processing since this appeared to lead to elevated cell death in some samples.

These results are consistent with those previously reported investigating the effects of watercress consumption on carcinogen metabolism or oxidative stress. However, these studies involved repeated ingestion of watercress over a period of 3 days to 8 weeks (Hecht et al. 1999; Hecht et al. 1995; Gill et al. 2007; Hoffmann et al. 2009). Recently, ingestion of a single 50g portion of watercress is reported to associate with reduced plasma immunoreactivity of the proinflammatory cytokine macrophage migration inhibitory factor (MIF), which is a direct target for covalent modification by PEITC (Cross et al. 2009; Brown et al. 2009).

As part of our study, we also analysed the plasma concentrations of PEITC. These results are generally in line with those previously reported by Ji et al. who analysed plasma concentrations of PEITC in 4 participants following consumption of 100g of watercress (Ji & Morris 2003). Both studies shows a rapid increases in plasma PEITC concentrations, peaking at 2 to 3 hours, followed by a decline to near background levels at 24 hours. However, the C_{max} values in this study were generally lower (297 v 928 nM on average) and showed much more inter individual variation in our study. Multiples variables are likely to contribute to these differences.

The lower average C_{max} at least partly reflects the smaller portion size selected for this study (80g versus 100g) and although the differences in intake is small, it is not clear that PEITC accumulation in the plasma is proportionate to dose. It is also possible that differences in glucosinolate content of the crop will have contributed to the variation in mean C_{max} concentrations between the studies since it is known that differences in sunlight exposure, temperature and added fertilizers can all influence gluconasturtin production in watercress (Engelen-Eigles 2006; Kopsell et al. 2007). The average age of the participants in this study was relatively high and age-related changes in absorption may have also

impacted on both the overall C_{max} and contributed to inter-individual variation. Finally, GST variants have previously been demonstrated to modulate ITC metabolism and potential chemopreventive effects and it is also possible that genetic variation may have contributed to some of the differences such as age and genetics.

A key question is why dietary intake of watercress may be sufficient to modulate 4E-BP1 phosphorylation although plasma concentrations may not achieve the concentrations that are required to effect this pathway *in vitro* (typically 1-5 μM) (Wang et al. 2009; Hu et al. 2007). One possible explanation lies in the interaction of PEITC with GSH. Efflux of ITC-conjugates, extracellular hydrolysis and reuptake of ITCs leads to marked accumulation of intracellular ITCs (Zhang 2000; Xu & Thornalley 2001). Thus, the intracellular concentration of PEITC in monocytes may be much higher than is predicted from the plasma concentrations. Similar interactions may account for the time lag between peak plasma concentration and inhibition of 4E-BP1 phosphorylation. It will be important to investigate further how differences in glutathione levels and metabolism alter cellular accumulation of ITCs and their metabolites since GSH levels are altered in many cancer cells (Estrela et al. 2006). As discussed above, it is also important to bear in mind alternate possibilities. For example, *in vivo* modulation of 4E-BP1 phosphorylation may be unrelated to or only partially dependent on PEITC and due to other bioactives derived from watercress. For example, Brown et al. recently reported that the mean plasma concentration of total ITCs and dithiocarbamate metabolites reached ~1.5 μM at 2 hours following ingestion of a 50 g portion of watercress (Brown et al. 2009). At present, it is not known whether dithiocarbamate metabolites may contribute to modulation of 4E-BP1 phosphorylation either directly, of following conversion to ITCs.

Chapter 7

Final Discussion

7.0 FINAL DISCUSSION

The clinical management of breast cancer has been based mainly on reducing the effects of estrogens with anti-estrogens or aromatase inhibitors, combined with chemotherapeutics (Litherland & Jackson 1988; Henderson 1991). However, the disseminated nature of breast cancer and the development of cross-resistant tumors are the primary causes for failure of current therapies. At the time of tumor diagnosis, there is a high probability that metastasis lesions have already occurred and many of these will contain a resistant sub-population of cells (Tong et al. 2002).

Different drugs show different degrees of effectiveness. The tumor mass consists of a heterogenous population of cells that may have different level of sensitivity to a particular drug. Tumor cells often utilize various metabolic pathways to establish progression and chemosensitivity or resistance is determined by which pathway is used. While drug-sensitive cells are killed, resistant cells are left unaffected to divide and replace eliminated cells. After initial chemotherapy, these few cells may acquire the ability to survive and typically return. Thus, the initially sensitive cancers eventually recur as drug-resistant cancers that are no longer sensitive to standard chemotherapy. Thus, the main objective of polychemotherapeutics is to target the different sub-populations of cells with different drugs with the aim of achieving complete elimination via different mechanisms. In light of this, various novel agents are being sought as to complement the existing arsenal of anticancer drugs.

In this study, a compound from watercress has displayed potential as an anticancer agent. PEITC is a member of isothiocyanate family and has been shown to exhibit anti cancer properties in many of human breast cancer cells. PEITC was observed to be more potent towards estrogen negative MDA-MB-231 cells compared to other human breast cancer cells. It is hypothesized this might be due to the invasiveness of MDA-MB-231 cells and the conjugation between PEITC and cellular thiols causing depletion of intracellular thiols and the cells to be more vulnerable to the treatment. Although PEITC also inhibited the growth of normal human dermal fibroblast (NHDFs), PEITC was approximately 4-fold less toxic towards NHDFs compared to MDA-MB-231 cells.

To further elucidate the mechanism of PEITC, the apoptosis induction and cell cycle arrest in MDA-MB-231 and MCF7 cells treated with PEITC were investigated. Cells can be arrested at cell cycle checkpoint to allow repaired cellular damage. In MDA-MB-231 cells, PEITC was observed to induce G2 arrest. As reported previously, PEITC able to

induce G2-M phase arrest in several cancer cell lines such as prostate cancer cells (Chiao et al. 2000; Visanji et al. 2004). Thus, upon GSH depletion, the electrophilic of PEITC continuously binds protein thiols such as the alpha-tubulin. The tubulin-binding that occur in MDA-MB-231 cells potentially act as inhibitors in G2/M phase causing dysregulation of tubulin dynamics leading to cell cycle arrest in tumor cells (Wang et al. 1999). By contrast, PEITC triggered G1 arrest in MCF7 cells. Although the exact mechanism is unknown, it is suggests might due to the expression of p53 or downregulation of CDK4 or cyclin D1. In consistent with the cell cycle data, the percentage of cell death in MDA-MB-231 cells was very significant compared to the control. By contrast, the percentages of cell death in MCF7 cells were very modest.

Following treatment with PEITC, the expression profile of a number of key proteins involved in apoptosis was examined. PEITC was observed to induce classical intrinsic pathway in MDA-MB-231 cells with the activation of caspase-9, -3 and caspase -8 which activated at later hour. However, no clear indication of which pathway triggered by PEITC in MCF7 cells. Further analysis shows the increased in Bax is closely linked to caspases activation in MDA-MB-231. By contrast, although PEITC induced expression of Bax in MCF7 cells, the increased in the level of Bax was not as rapid as in MDA-MB-231 cells. Thus this explained why MCF7 cells being more resistance towards PEITC compared to MDA-MB-231 cells.

Numerous studies reported that PEITC also could increase ROS generation thus lead to apoptotic induction (Yu et al. 1998). In MDA-MB-231 cells, PEITC was observed to induce rapid and initial ROS accumulation compared to MCF7 cells. The ability of PEITC to induce initial ROS accumulation is suggests due to the depletion of antioxidant GSH since PEITC has been generally known with the ability to conjugate with GSH. Additionally, GSH has been reported play an important role in protecting cells against reactive oxygen species (Poot 1991; Gansauge et al. 1998). Thus, the continuous conjugation between PEITC and antioxidant GSH caused GSH depletion that leads to oxidative stress. Moreover, ROS generation has been reported to involve in the release of cytochrome *c* and caspase activation by certain apoptotic stimuli, for example hypoxia (Buccellato et al. 2004). Induction of ROS by PEITC has also been reported to correlate with PEITC-induced apoptosis in PC-3 cells (Xiao et al. 2006).

ROS induction which caused oxidative stress later gives respond to the stimulation of Nrf2/Keap1 signalling pathway. Normally Nrf2 sequestered in cytoplasms by Keap1. However, upon exposure to oxidative stress, cysteine residues in Keap1 will sense the

intracellular redox changes thus caused conformational changes on Keap1 and no longer able to bind to Nrf2. As a consequence, Nrf2 will be translocated to the nucleus and induces the expression of proteins that protects the cells against toxins (Itoh et al. 1999). However, it is also important not to exclude the possibility of PEITC to interact directly to the –SH group of cysteine in Keap1 and therefore triggered the activation of Nrf2. By contrast, the levels of Nrf2 in MCF7 cells were activated even in the control although no ROS accumulation was observed upon PEITC treatment. Therefore, it is suggests due to the continuous activation of Nrf2 even at rest thus preventing or lowered the accumulation of ROS in MCF7 cells.

The initial accumulation of ROS in MDA-MB-231 cells by PEITC is hypothesized might be due to the depletion of GSH. Thus, the effect of the modulation of intracellular GSH on PEITC responses was confirmed in the presence of antioxidant NAC which is also a precursor to the synthesis of GSH. The results obtained demonstrated NAC had a strong reversal effect on the growth inhibition by PEITC in MDA-MB-231 cells. By contrast, NAC had a modest reversal effect on the growth inhibition by PEITC in MCF7 cells. Further analysis on different breast cancer cells shows NAC reversed the growth inhibitory effect of PEITC in ER negative, BT549 cells while no effect at all in ER positive T47D and ZR75.1 cells. Thus it is suggests that the presence of NAC in MDA-MB-231 cells restore and protect the GSH level from being depleted by PEITC.

Further experiment to investigate the effect of NAC on PEITC-induced cell cycle arrest as well as apoptotic induction show, NAC able to protect MDA-MB-231 cells against G2 cell cycle arrest and induction of apoptosis by PEITC. This is due to NAC acts as a precursor to the synthesis of glutathione in MDA-MB-231 cells thus reduced the possibility of PEITC to interact with protein cysteinyl thiols for example alpha tubulin which is important for the formation of microtubule. Interestingly, NAC also prevented Nrf2 activation in MDA-MB-231 cells suggesting the presence of NAC prevent ROS accumulation in MDA-MB-231 cells therefore no activation of Nrf2 observed. Meanwhile in MCF7 cells, although there was not much different in the levels of Nrf2 in MCF7 cells treated with PEITC with and without NAC pretreatment, the level of Nrf2 in the untreated and DMSO treated upon NAC pretreatment appears to be lower compared to the levels of Nrf2 in untreated and DMSO-treated without NAC pretreatment. This suggests the relatively on-going ROS production that leads to the continuous expression of Nrf2 occur in MCF7 cells at rest.

To further confirm if the sensitivity of MDA-MB-231 cells is closely related to GSH modulation, the levels of GSH in MDA-MB-231 cells treated PEITC with and without NAC pretreatment were determined. The decreased in the level of intracellular GSH in MDA-MB-231 cells shows the importance of GSH in MDA-MB-231 cells survival and the initial increased of ROS is due to the conjugation between cystenyl group of GSH and PEITC that leads to depletion of GSH which later compensated by the increased in Nrf2 expression level. However, NAC restores the levels of GSH in MDA-MB-231 cells thus preventing further conjugation between GSH and PEITC as well as protein cysteinyl such as Keap1 and α-tubulin.

Interestingly, although NAC pretreatment did not affect the cell cycle arrest as well as apoptosis, there was a small increased in the levels of GSH observed in MCF7 cells compared to when MCF7 cells treated with PEITC alone suggesting a small conjugation might occur between PEITC and GSH in MCF7 cells upon PEITC treatment and that NAC acts in compensated the levels of intracellular GSH upon PEITC treatment.

Antagonistic, BSO pretreatment caused MDA-MB-231 cells to be more sensitive towards PEITC treatment compared to when treated with PEITC alone. Depletion of intracellular GSH by BSO causing the MDA-MB-231 cells to be more sensitive to cell death. However, MCF7 cells were more resistant towards BSO pretreatment with an unaffected percentage of cell death compared to PEITC treatment alone. This shows GSH depletion in MCF7 cells did not affect the cells viability towards PEITC treatment.

The AKT pathway has been reported to associate with many cells event such as cell proliferation, survival and motility (Datta et al. 1997). Activation of Akt inactivates several pro-apoptotic molecules such as Bad, caspase-9, IkB kinase and p53 through MDM2-mediated phosphorylation (del Peso et al. 1997; Cardone et al. 1998; Romashkova & Makaroz 1999; Zhou et al. 2001) thus promotes cell survival and suppresses apoptosis (Shin et al. 2002; Datta et al. 1997). Akt has also been reported able to stimulate oxidative metabolism in mitochondria which indirectly increases intracellular ROS (Dolado & Nebreda 2008). Akt is differentially activated by growth factors and oxidative stress by sequential phosphorylation of Ser473 by mTORC2 and Thr308 by phosphoinositide-dependent kinase1 (PDK1) (Antico Arciuch et al. 2009). In both cell lines, PEITC increased the phosphorylation of Akt at both sites Ser473 and Thr308. Although the exact mechanism is still unclear, the similar responses of Akt phosphorylation by PEITC show no differential responses in the Akt signalling between two cell lines.

4EBP1 is regulated by phosphorylation. The unphosphorylated form of 4EBP1 binds to eIF4E preventing the translational event. Upon phosphorylation of 4EBP1, eIF4E is free and allow the translation of RNA. However, PEITC inhibited the phosphorylation of 4EBP1 in MCF7 cells but not in MDA-MB-21 cells. A pilot study was performed to determine the effects of watercress ingestion of plasma PEITC concentrations and 4EBP1 phosphorylation as well as to choose a suitable biomarker by measuring ROS accumulation in PBMCs after watercress meal and investigating the levels of Keap1.

The bioavailability of ITCs was first analyzed by measuring the intracellular ROS level in circulating blood cells. Although ROS production was observed after watercress meal, due to the sensitivity of the experiment, complex interplay between metabolism and GSH conjugation as well as transportation, the intracellular concentrations of ITCs may vary from different cell type. The expression of Keap1 in PBMCs was also observed upon watercress ingestion. Although there was a reduction in the levels of Keap1, the significant change in the levels of Nrf2 was not observed and the experiment was continued by looking at the inhibition of 4EBP1 phosphorylation.

The inhibition of 4EBP1 phosphorylation was first determined by treating the PBMCs with PEITC compound since PEITC had been reported in several studies to inhibit the phosphorylation of 4EBP1 (Wang et al. 2009; Hu et al. 2007). Later, a small pilot study to investigate the possibility of measuring 4EBP1 phosphorylation as a biomarker was performed since 4EBP1 inhibition by PEITC has been demonstrated in several cell types such as in human breast cancer MCF7 cells, human renal carcinoma RCC4 cells (Wang et al. 2009), human prostate cancer PC3 cells and human colorectal cancer HCT cells (Hu et al. 2007). Moreover, it was possible to measure 4EBP1 phosphorylation on a single cell basis via flow cytometry analysis. In addition, measuring 4EBP1 phosphorylation in PBMCs which is a relatively accessible tissue source suitable for repeat sampling. Thus, measuring changes in 4EBP1 phosphorylation following watercress consumption via flow cytometry analysis is perhaps a suitable biomarker compared measuring ROS accumulation or Keap1 expression level in PBMCs.

Therefore, based on these studies, it is beneficial to continue with some suggested future experiments:

1. Extend this analysis to additional cell lines with different characteristics
2. Add primary samples into the research to see differential responses of PEITC.

3. Further analysis on the key molecular responses; for examples Nrf2 and ROS in the additional cell lines upon PEITC treatment.
4. Examine the effects of genetic modulation of GSH levels (by siRNA) in the cell lines upon PEITC treatment.

Chapter 8

Appendices

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In vivo modulation of 4E binding protein 1 (4E-BP1) phosphorylation by watercress: a pilot study

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Dietary intake of isothiocyanates (ITC) has been associated with reduced cancer risk. The dietary phenethyl ITC (PEITC) has previously been shown to decrease the phosphorylation of the translation regulator 4E binding protein 1 (4E-BP1). Decreased 4E-BP1 phosphorylation has been linked to the inhibition of cancer cell survival and decreased activity of the transcription factor hypoxia-inducible factor (HIF), a key positive regulator of angiogenesis, and may therefore contribute to potential anti-cancer effects of PEITC. In the present study, we have investigated the in vitro and in vivo effects of watercress, which is a rich source of PEITC. We first demonstrated that, similar to PEITC, crude watercress extracts inhibited cancer cell growth and HIF activity in vitro. To examine the effects of dietary intake of watercress, we obtained plasma and peripheral blood mononuclear cells following the ingestion of an 80 g portion of watercress from healthy participants who had previously been treated for breast cancer. Analysis of PEITC in plasma samples from nine participants demonstrated a mean maximum plasma concentration of 297 nM following the ingestion of watercress. Flow cytometric analysis of 4E-BP1 phosphorylation in peripheral blood cells from four participants demonstrated significantly reduced 4E-BP1 phosphorylation at 6 and 8 h following the ingestion of watercress. Although further investigations with larger numbers of participants are required to confirm these findings, this pilot study suggests that flow cytometry may be a suitable approach to measure changes in 4E-BP1 phosphorylation following the ingestion of watercress, and that dietary intake of watercress may be sufficient to modulate this potential anti-cancer pathway.

Isothiocyanates: Cancer: 4E binding protein 1 (4E-BP1): Phosphorylation

Epidemiological studies have suggested that a diet rich in cruciferous vegetables such as broccoli, cabbage and watercress is associated with reduced risk of multiple cancer types(1). The potential anti-cancer effect of high cruciferous vegetable intake has been linked to the presence of glucosinolates within these foods. Following the release of the plant enzyme myrosinase by chewing or cutting, hydrolysis of glucosinolates gives rise to a number of products including isothiocyanates (ITC), indoles, thiocyanates and nitriles(2,3). Over 100 glucosinolates which give rise to chemically distinct hydrolysis products have been identified. Following absorption, ITC are rapidly conjugated to glutathione (GSH) via the action of glutathione-S-transferase enzymes and metabolised predominantly via the mercapturic acid pathway(2,3). Several studies have indicated that the potential cancer-protective effects of a high-cruciferous vegetable diet are modulated by sequence variations within glutathione-S-transferase enzymes, most notably GSTM1 and GSTT1(4 – 7). These variants may be linked to the enhanced protective effects of a high-cruciferous vegetable diet via potential effects on ITC metabolism(8). The mechanisms of action of the potential anti-cancer activity of ITC are complex, and at present, are incompletely understood. However, alterations of carcinogen metabolism via inhibition of phase I enzymes and induction of phase II enzymes, as well as direct modulation of pathways controlling key cancer hallmarks, such as proliferation, resistance to apoptosis and angiogenesis, are thought to be involved(2,3,9,10).

Following uptake into cells, ITC conjugate rapidly with the free thiol of glutathione(11,12). Cycles of ITC conjugate efflux, regeneration of ITC by extracellular hydrolysis and reuptake of ITC lead to a very marked accumulation of ITC within cells and concomitant depletion of intracellular GSH. Decreased intracellular GSH leads to increased levels of reactive oxygen species, which may play an important role in suppressing growth and survival of transformed cells(13). In the absence of GSH-mediated defence, intracellular ITC are thought to conjugate with various cellular proteins, predominantly via reactive cysteine thiols(2,14). One arm of this response, driven by covalent modification of Keap1, leads to

Abbreviations: C_{max}, maximal plasma concentration; DFO, desferrioxamine; 4E-BP1, 4E binding protein 1; FSC, forward scatter; HIF, hypoxia-inducible factor; ITC, isothiocyanates; PBMC, peripheral blood mononuclear cells; PEITC, phenethyl ITC; SSC, side scatter.

the stabilisation of Nrf2, a master regulator of antioxidant gene expression, and induction of protective antioxidant proteins[15–18]. Similar events are thought to contribute to the direct anti-cancer effects of ITC on proliferation, survival and angiogenesis[2]. Conjugation to a- and b-tubulins may contribute to the mitotic arrest that is frequently observed in ITC-treated cells, although it is likely that ITC exert their biological effects via modulation of multiple downstream effectors[14,19–22]. Metabolites of ITC are also likely to be involved, potentially via effects on chromatin remodelling and gene expression[23].

We have previously investigated the effects of phenethyl ITC (PEITC) on the response of cancer cells to hypoxia[24]. PEITC is derived from the glucosinolate gluconasturtiin, which is found at particularly high levels in watercress (Nasturtium officinale or Rorippa nasturtium-aquaticum). We demonstrated that PEITC interfered with the ability of hypoxia to activate hypoxia-inducible factor (HIF)[24], a key transcription factor that mediates cellular responses to low pO_2[25,26]. Activation of HIF leads to increased transcription of a wide range of genes involved in angiogenesis (e.g. vascular endothelial growth factor), apoptosis (e.g. Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3) and metabolism (e.g. GLUT1). Inhibition of HIF may be important for the anti-cancer effects of PEITC, since PEITC and other ITC have been shown to posses anti-angiogenic activity in vitro and in vivo [27–29], and angiogenesis plays a role in tumourigenesis, enabling growth of nascent tumours beyond a small size limit dictated by the perfusion distance of O_2 away from blood vessels[30].

The mechanism by which PEITC inhibits HIF activity appears to involve inhibition of HIF1a mRNA translation[24]. We[24] and others[31] have demonstrated that PEITC decreases the levels of phosphorylation of 4E binding protein 1 (4E-BP1), and this may play an important role in the modulation of HIF1a mRNA translation. The translation of HIF1a mRNA is highly dependent on the eIF4E translation factor, which is, in turn, regulated by 4E-BP1[32]. Dephosphorylation of 4E-BP1 facilitates its interaction with 4E-BP1, leading to decreased translation of RNA such as HIF1a mRNA. Hu et al.[31] have also demonstrated that PEITC-induced cell death is reversed by overexpression of eIF4E. Thus, 4E-BP1 may be a key target for PEITC-associated anti-cancer effects, leading to the loss of both growth- and angiogenesis-promoting pathways. PEITC decreases the phosphorylation of 4E-BP1 on multiple sites, including Thr_{70}, Ser_{65} and $Thr_{37/46}$[24,31].

A small number of studies have investigated the in vivo effects of watercress consumption, generally, on pathways of carcinogen metabolism and oxidative stress. Pharmacokinetic analysis has demonstrated rapid absorption of PEITC into the blood with a mean maximal plasma concentration (C_{max}) of 928 nM after the ingestion of 100 g watercress[33]. Hecht et al.[34,35] have demonstrated that dietary intake of watercress increased urinary metabolites of the tobacco-specific lung carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and cotinine in smokers. Gill et al.[36] have demonstrated reduced levels of basal and hydrogen peroxide-induced DNA damage in peripheral blood lymphocytes following daily intake of 85 g of watercress for 8 weeks, associated with modest increases in erythrocyte superoxide dismutase and glutathione peroxidase 1 activity in specific cohort individuals carrying the GSTM1*1 allele[37]. A very recent study[21] demonstrated reduced immunoreactivity of the proinflammatory cytokine MIF in plasma following the ingestion of a single 50 g portion of watercress.

Since decreased 4E-BP1 modulation has been functionally linked to in vitro anti-cancer effects of PEITC[24,31], we investigated the effects of watercress extract on cancer cell growth inhibition and HIF activity. We also performed a small pilot study to determine whether dietary intake of watercress was sufficient to modulate 4E-BP1 phosphorylation in vivo.

Experimental methods

Cell culture

Human MCF7 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and were maintained in Dulbecco's Modified Eagle's medium (Lonza Group Limited, Basel, Switzerland) supplemented with 10 % (v/v) fetal calf serum (PAA Laboratories, Yeovil, UK), 1 mM L-glutamine and penicillin/streptomycin (Lonza group Limited). PEITC and desferrioxamine (DFO) were obtained from Sigma Chemicals (Poole, UK). HIF reporter assays were performed as described previously[24]. Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep (Axis-Shield Diagnostics, Dundee, UK) according to the manufacturer's instructions. Growth inhibition assays were performed in duplicate as described previously[24]. LY294002 was obtained from Sigma Chemicals.

Watercress extracts

Watercress samples were snap frozen in liquid N_2 before being ground to a fine powder using a pestle and mortar. Ground watercress (1 g) was decanted into a QIAshredder homogeniser (Qiagen, Crawley, UK), and incubated at room temperature for 1 h. Samples were centrifuged at 16 000 g for 6 min to collect the crude watercress extract.

Analysis of 4E binding protein 1 phosphorylation

The analysis of 4E-BP1 phosphorylation was done by single cell flow cytometry. PBMC were washed in ice-cold Roswell Park Memorial Institute 1640 medium (Invitrogen Limited, Paisley, UK) and resuspended in 1 ml of Roswell Park Memorial Institute 1640 medium. Cytofix buffer (BD Biosciences, Oxford, UK; 1 ml) was added, and the cells were incubated for 10 min at 378C before storage at 2 808C, as per the manufacturer's instructions. On the day of the analysis, samples were thawed, and the cells were washed with flow cytometry buffer (BD Biosciences). Cells were resuspended in 1 ml of Phosflow Permeabilisation buffer III (BD Biosciences), and were incubated on ice for 30 min. Cells were then washed twice with Stain Buffer (BD Biosciences), collected by centrifugation and resuspended in 1 ml of Stain Buffer containing 100 ml of phycoerythrin-conjugated anti-4E-BP1 antibody ($Thr_{37/46}$ phospho-specific) (BD Biosciences). Unstained cells were analysed as controls. Cells were incubated in the dark at room temperature for 30 min, washed with Stain Buffer and resuspended in 500 ml of the same buffer before flow cytometry. Flow cytometry was performed using the FL2 channel

(585 nm) on a BD Biosciences FACSCalibur. An average of 785 000 total events was collected for each sample. For 4E-BP1 fluorescence, we measured the proportion of monocytes with fluorescence values greater than those of unstained controls.

The analysis of 4E-BP1 phosphorylation was performed on cells gated on the basis of their forward scatter (FSC)/side scatter (SSC) properties. We found considerable variation in the FSC/SSC profiles of isolated PBMC following fixation and immunostaining. The impact of fixatives used for intracellular staining with phospho-specific antibodies on scatter profiles has been described previously[38]. We separately gated on lymphocytes (abundant population with low FSC and SSC; Fig. 2(a)) and a population with increased FSC/SSC (see oval gate in Figs. 2(a) and 4) that we tentatively described as 'monocytes' (see 'Discussion'). In some analyses, we detected a third population of cells (to the right of the lymphocytes) that we believe is due to variation in fixation. When present, these were excluded from the analysis. Some samples also contained relatively high amounts of dead cells with very low FSC (see Fig. 4). Again, these were excluded from the analysis.

In vivo study

The watercress feeding study was based on the previous work done by Ji et al.[33], who studied the plasma pharmacokinetics of PEITC following the ingestion of 100 g of watercress in four normal participants. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human participants were approved by the Southampton and South West Research Ethics Committee (Ref 08/H0504/86). Written informed consent was obtained from all the participants. Potential participants were identified using the Winchester and Andover Breast Unit database of patients, where clinical and diagnostic data are routinely compiled for audit purposes using criteria defined by the British Association for Surgical Oncology. Although the participants had previously been treated for breast cancer, they were considered free of clinically detectable disease for a minimum of 2 years and were otherwise healthy. None of the women was taking any pharmacological medications or herbal supplements.

Participants were requested to avoid the following foods for 3 d before the study day to exclude known sources of glucosinolates from the diet: cabbage, brussels sprouts, broccoli, calabrese, cauliflower, turnip, swede, rutabaga, kohlrabi, kale, chinese kale, sea kale, curly kale, collard, pak choi, radish, horseradish, mustard, mustard greens, mustard leaf, wasabi, salad rocket, cress, watercress, capers, papaya (pawpaw) and nasturtium (Indian cress). The work of McNaughton & Marks[39] informed the compilation of the list of food items to be excluded from the diet. Food and drink diaries were completed to confirm avoidance of glucosinolate-containing food items; dietary histories were also taken to describe participants' usual glucosinolate intakes, although these data are not presented here. The participants fasted from midnight on the day of the study. Approximately, 8 h later the volunteers were cannulated, and a baseline blood sample (5 ml) was obtained. The participants then ate 80 g of watercress, obtained from a local grower and packager (Vitacress Salads Limited, Andover, UK). Eighty grams is the weight of a portion of fruit and vegetables defined by the WHO, and is equivalent to one of the five portions of fruit and vegetables that describes one of UK's nutrition public health messages[40,41]. Blood samples were then obtained at approximately 7·5 min, 15 min, 30 min, 45 min, 1 h, 1·5 h, 2 h, 3 h, 4 h, 6 h and 8 h following the ingestion of the watercress meal. A 24 h blood sample was obtained by venipuncture. Blood samples were collected in heparinised glass tubes, and stored on ice before processing. Participants were free to take water throughout the study, and were allowed to eat and drink freely from 3 h after the ingestion of watercress meal, although glucosinolate-containing foods were eliminated until the end of the study.

We enrolled twelve women into the study, and analysed plasma PEITC concentrations and/or 4E-BP1 phosphorylation in samples from nine of these women (mean age 58 years, median age 56 years and range 48 –82). For eight participants, we prepared both PBMC and plasma; samples were overlaid onto Lymphoprep and centrifuged at 800 g for 20 min. The plasma was recovered and stored at 2808C. For one participant (participant 5 in Table 1), we prepared only plasma; samples were centrifuged at 486 g for 15 min, and the plasma was recovered and stored at 2808C. Plasma PEITC concentrations were determined as described previously[33], with the exception that analyses were performed using 0·5 ml of plasma. PBMC were recovered from the interface, washed twice in ice-cold Roswell Park Memorial Institute 1640 medium and fixed using Cytofix (BD Biosciences) as described earlier. Cells were frozen before the analysis, and all the samples from an individual participant were analysed in parallel. The analysis of 4E-BP1 phosphorylation was done as described earlier. The analysis was performed on samples from five participants. However, two were excluded due to technical failures, and another two were excluded because of poor viability of cells.

Results

Effect of watercress extract on cancer cell growth and hypoxia-inducible factor activity

PEITC has been shown to decrease cancer cell growth and to inhibit HIF activity, actions which have been linked to decreased 4E-BP1 phosphorylation[24,31]. Watercress is a particularly rich source of the PEITC precursor glucosinolate,

Table 1. Analysis of plasma phenethyl isothiocyanates concentrations following the consumption of watercress

Participant	C_{max} (nM)	t_{max} (min)
1	104	120
2	61	125
3	117	180
4	164	480
5	375	185
6	384	90
7	209	120
8	656	140
9	604	180
Mean	297	180

C_{max}, maximal plasma concentration; t_{max}, time to reach C_{max}.

gluconasturtiin, and we therefore investigated whether watercress extract could exert similar effects. Extracts were prepared by grinding watercress leaf, which releases myrosinase, the enzyme which catalyses the conversion of gluconasturtiin to PEITC. Watercress extract inhibited the growth of MCF7 breast cancer cells with an half maximal inhibitory concentration (IC_{50}) of 24·6 (SD 12·3) ml/ml (Fig. 1(a)).

To analyse the transcriptional activity of HIF, MCF7 cells were transfected with a HIF reporter construct and treated with the hypoxia mimetic DFO (Fig. 1(b)). DFO displaces Fe from prolyl hydroxylases leading to the stabilisation of HIFa proteins. DFO caused a strong induction of the HIF reporter construct, and this was inhibited in a dose-dependent manner by watercress extract, although this was statistically significant only at the highest concentration. Watercress extract also decreased the basal activity of the HIF reporter in the absence of DFO. As a control, we also analysed the effects of DFO and watercress extract on the activity of the SV40-promoter-based reporter plasmid pGL3-promoter. The activity of the control promoter was reduced following treatment with DFO, and was not affected by watercress extract under any condition. Therefore, similar to PEITC[24], watercress extract inhibits HIF activity in MCF7 breast cancer cells.

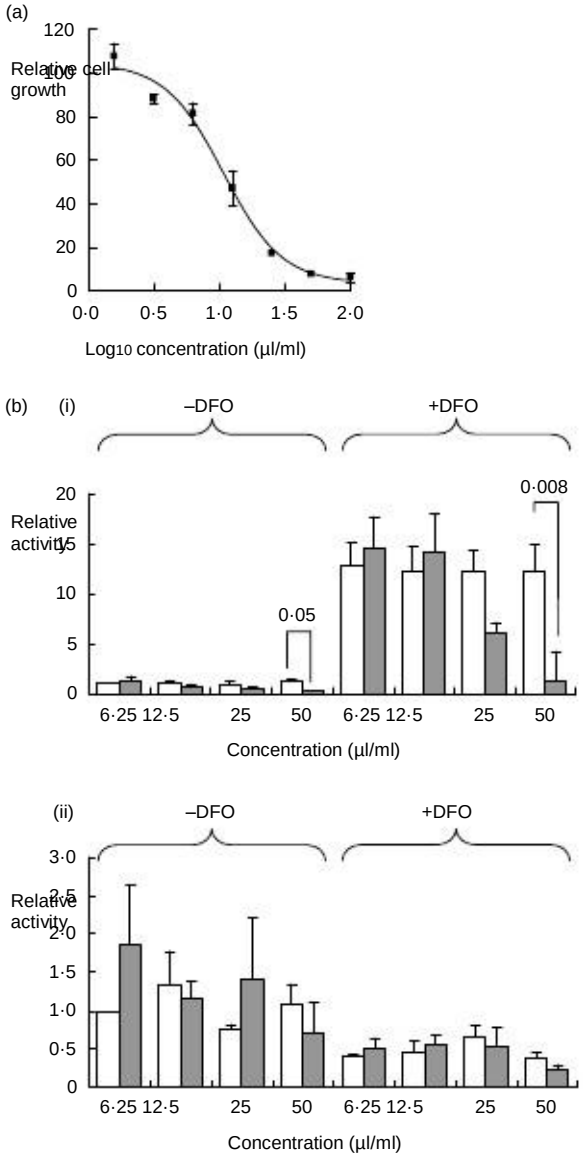

Fig. 1. Inhibition of cancer cell growth and hypoxia-inducible factor (HIF)-dependent transcription by watercress extract. (a) Representative growth inhibition experiment. MCF7 cells were incubated with the indicated concentrations of watercress extract. After 6 d, relative cell numbers were determined using the CellTiter 96w AQueous One Solution reagent. Results are derived from means of duplicate wells. (b) MCF7 cells were transfected with (i) pGL2-9TK-HRE or (ii) control pGL3-promoter reporter constructs, and were treated with the indicated concentrations of watercress extract ( ) or with equivalent amounts of water (A). HIF activity was induced by treating the cells with desferrioxamine (DFO) (100 mM), and luciferase activity was analysed after 24 h. Data indicate mean with their standard errors for luciferase activity relative to control cells (i.e. cell in the absence of DFO and with water equivalent to the lowest concentration of watercress extract tested was set to 1·0) derived from three independent experiments, each performed in triplicate. Statistically significant differences (Student's t test) between dimethyl sulphoxide- and watercress-treated cells are shown. All other differences were not statistically significant.

Phenethyl isothiocyanates decreases 4E binding protein 1 phosphorylation in peripheral blood mononuclear cells

Since PEITC decreases 4E-BP1 phosphorylation and this has been linked to HIF inhibition[24] and growth inhibition[31], we selected 4E-BP1 phosphorylation as a potential biomarker to monitor in vivo exposure to PEITC. To explore the potential utility of 4E-BP1 phosphorylation as a biomarker, we first examined the levels of 4E-BP1 phosphorylation in PBMC and its modulation by PEITC.

PBMC were obtained from healthy donors and analysed by single cell flow cytometry. For these studies, we used an antibody that selectively reacted with 4E-BP1 phosphorylated on $Thr_{37/46}$, since it gave stronger staining compared with a 4E-BP1 Thr_{70} phospho-specific antibody (data not shown). 4E-BP1 phosphorylation varied among different cell populations in peripheral blood based on FSC/SCC properties (see 'Experimental methods' for gating strategy). We readily detected phosphorylated 4E-BP1 in a population of cells that we tentatively described as 'monocytes', whereas the levels of phosphorylation were much lower in lymphocytes (Fig. 2). To confirm that immunostaining was specific for phosphorylated 4E-BP1, cells were treated with LY294002. LY294002 decreases 4E-BP1 phosphorylation via inhibition of Phosphoinositide 3 kinase, a key upstream positive regulator of 4E-BP1 phosphorylation via the phosphoinositide 3 kinase-AKT-mammalian target of rapamycin pathway[42]. Treatment of cells with LY294002 reduced 4E-BP1 phosphorylation by 63 ^ 13 % (mean ^ range of two experiments; Fig. 2). Consistent with previous data[24,31], there was also a significant reduction in 4E-BP1 phosphorylation following in vitro treatment with PEITC (Fig. 2). Thus, 4E-BP1 phosphorylation appears to be a suitable biomarker to monitor in vivo responses to PEITC in PBMC.

Watercress consumption down-regulates 4E binding protein 1 phosphorylation in vivo

We performed a small feeding study to determine whether the ingestion of watercress was sufficient to modulate 4E-BP1 phosphorylation in vivo. We first analysed plasma

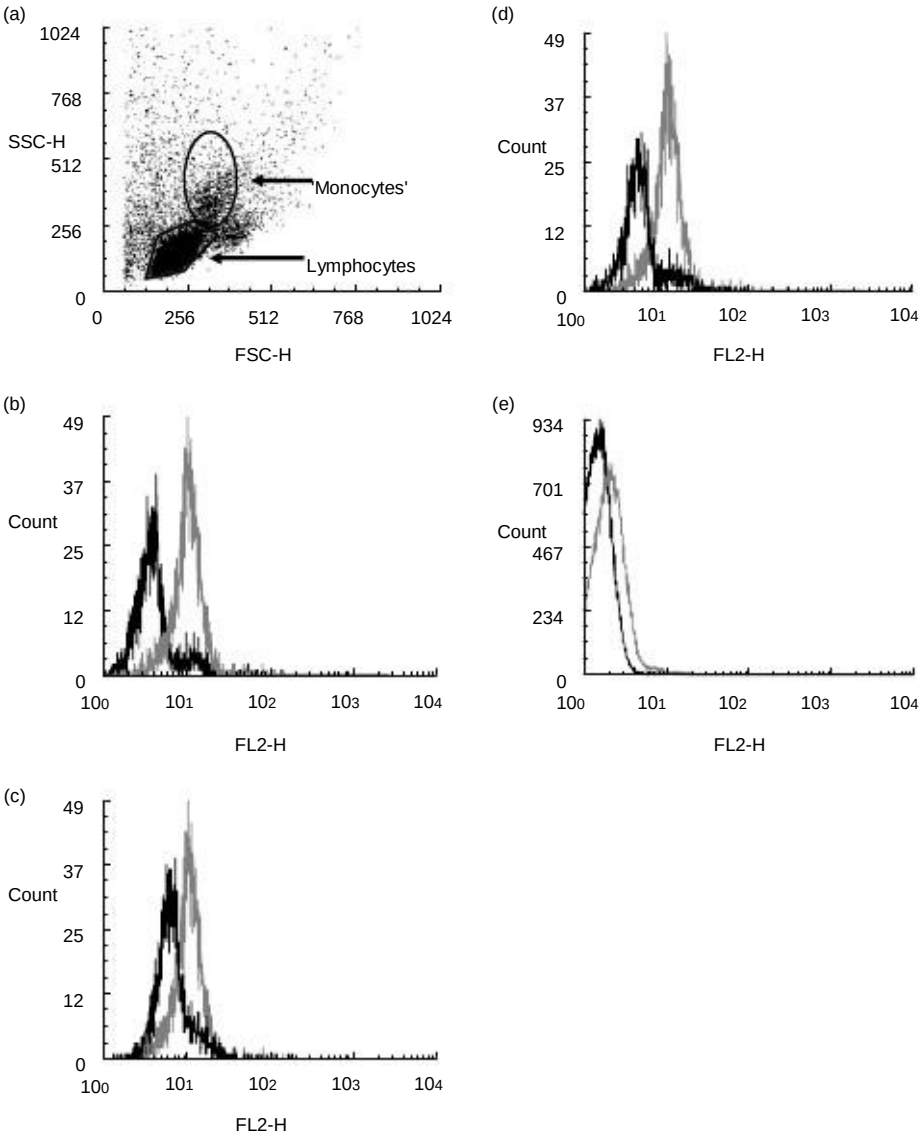

Fig. 2. Analysis of 4E binding protein 1 (4E-BP1) phosphorylation in peripheral blood mononuclear cells (PBMC). PBMC were isolated from healthy individuals and analysed by flow cytometry using a $Thr_{37/46}$ 4E-BP1 phosphorylation-specific antibody. (a) Forward scatter (FSC)/side scatter (SSC) plot showing gating of lymphocytes (lower left) and a population of cells with increased FSC and SSC, which we tentatively classed as monocytes (oval gate). (b –e) Fluorescence intensity of 'monocytes' (b) –(d) and lymphocytes (e). In (b) and (e), black line indicates unstained control, and the grey line indicates antibody-stained cells. In (c), the grey line indicates antibody-stained cells, and the black line indicates cells treated with LY294002 (100 mM) for 2 h before staining with the 4E-BP1 antibody. In (d), the grey line indicates antibody-stained cells, and the black line indicates antibody-stained cells treated with phenethyl isothiocyanates (20 mM) for 2 h before staining with the 4E-BP1 antibody. Data are representative of at least two independent experiments. FL2-H, fluorescence pulse height.

concentrations of PEITC (Table 1). Similar to a previous study(33), there was a rapid increase in plasma PEITC, which, on average, reached a maximal concentration at 3 h (Fig. 3). The mean C_{max} was 297 nM , although there was a wide interindividual variation (range 61 –656 nM). Background PEITC concentrations before the ingestion of watercress were very low (generally ,1 nM).

The analysis of 4E-BP1 phosphorylation was done in four participants (Fig. 4). All the participants showed a marked reduction in 4E-BP1 phosphorylation at 6 and 8 h following the ingestion of watercress compared with pre-watercress meal values. Twenty-four hour data were available for three participants; in one participant, 4E-BP1 phosphorylation was maintained at a low level until this time point, whereas recovery of 4E-BP1 phosphorylation was observed in the other two participants. The decreases in 4E-BP1 phosphorylation at 6 and 8 h were highly statistically significant (P¼0·001 and 0·002, respectively; Student's t test compared to pre-watercress samples). There was a considerable variation in the levels of 4E-BP1 phosphorylation before 6 h within individual subjects. It is possible that this is due to technical variation in transport and sample processing, and the average level of 4E-BP1 phosphorylation was not significantly altered at these time points. Thus, the analysis of 4E-BP1 $Thr_{37/46}$ phosphorylation suggests a significant decrease at 6 and 8 h after the ingestion of watercress.

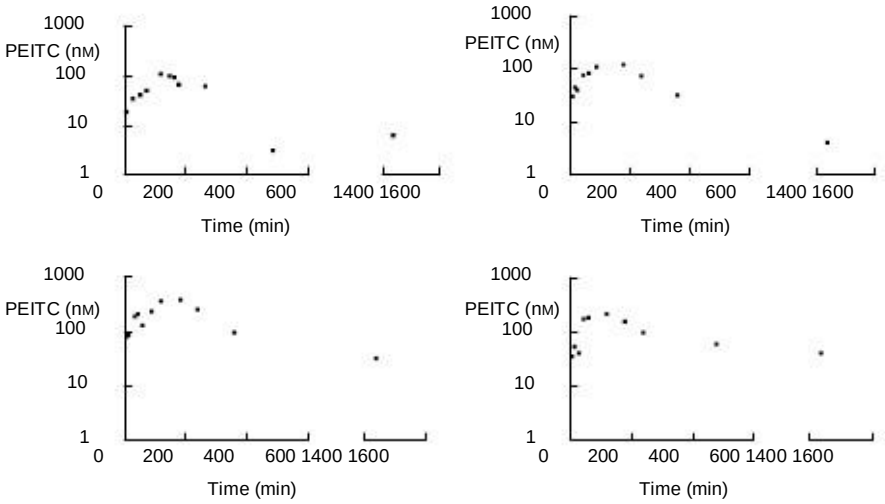

Fig. 3. Analysis of plasma phenethyl isothiocyanates (PEITC) concentrations following the consumption of watercress. Plasma concentration of PEITC was determined at various time points following the consumption of 80 g watercress. Data obtained from four representative subjects are shown.

Discussion

Numerous epidemiological studies have suggested that high dietary intake of cruciferous vegetables is associated with reduced cancer risk, and in vitro studies have indicated that reduced 4E-BP1 phosphorylation may be an important mechanism contributing to anti-cancer effects of PEITC[24,31]. In this work, we have performed a pilot study to determine whether the ingestion of watercress, a rich dietary source of PEITC, is sufficient to modulate 4E-BP1 phosphorylation levels in vivo. The mechanisms by which PEITC decreases 4E-BP1 phosphorylation are not known. Modulation of upstream regulators, including mammalian target of rapamycin and phosphatase and tensin homologue (PTEN), both of which contain redox-sensitive cysteine residues, may play a role[43,44].

We selected 4E-BP1 phosphorylation as a potential molecular biomarker for several reasons. First, modulation of 4E-BP1 phosphorylation by PEITC has been demonstrated in multiple cell types[24,31]. Secondly, modulation of 4E-BP1 phosphorylation has been mechanistically linked to both growth-inhibitory and anti-angiogenic effects of PEITC[24,31]. Thirdly, it was possible to measure 4E-BP1 phosphorylation on a single cell basis using a quantitative flow cytometry assay. Finally, we were able to measure 4E-BP1 phosphorylation in PBMC, a relatively accessible tissue source suitable for repeat sampling. However, it is possible that other watercress-derived ITC, PEITC metabolites or unrelated bioactives may also modulate 4E-BP1 phosphorylation. We therefore cannot exclude the possibility that other compounds are involved in the in vitro and in vivo effects of watercress. Although we consider it unlikely, all the participants ate the watercress at the same time of day, and we therefore also cannot exclude the possibility that decreased 4E-BP1 phosphorylation is unrelated to watercress consumption, and may reflect diurnal modulation of activity.

Although flow cytometric analysis of 4E-BP1 phosphorylation appeared to be a promising approach for the evaluation of in vivo responses to ITC, this pilot study identified a number of key technical challenges that should be addressed in future studies. First, intracellular antibody staining requires cell fixation, and this resulted in a large variation in the scatter properties of the cells. The difficulties caused by variation in scatter properties, specifically in the context of staining with phospho-specific antibodies, have been discussed previously[38]. Our gating strategy excluded lymphocytes and encompassed a population that we tentatively identified as monocytes. However, in future studies, it would be important to combine intracellular staining with specific surface markers to unambiguously identify specific cell subpopulations, and to improve the flow cytometric analyses. Secondly, we observed significant levels of cell death in some samples. The clinical and laboratory sites were geographically separate, and transportation between these sites may have resulted in increased cell death. For technically demanding analyses, such as phospho-specific flow cytometry, we recommend that samples be processed with the minimum of delay.

Flow cytometric analysis demonstrated a statistically significant reduction in 4E-BP1 $Thr_{37/46}$ phosphorylation at 6 and 8 h following the consumption of watercress in 4/4 participants studied. Caution is required in interpreting our data, since there was significant variability in the levels of 4E-BP1 phosphorylation within individual samples before this time point. However, these differences disappeared when results from the four individuals were combined, whereas the consistent down-regulation of 4E-BP1 phosphorylation at 6 and 8 h was highly statistically significant. This suggests that dietary intake of a single 80 g portion of watercress is sufficient to modulate this potential anti-cancer pathway. These results are consistent with those reported previously from studies investigating the effects of watercress consumption on carcinogen metabolism/oxidative stress, although it should be noted that these studies involved repeated ingestion of watercress over a period of 3 d–8 weeks[34–37]. Recently, Brown et al.[20,21] have shown that the ingestion of a single 50 g portion of watercress is associated with reduced plasma immunoreactivity of the proinflammatory cytokine macrophage migration inhibitory factor, which is a direct target for covalent modification by PEITC.

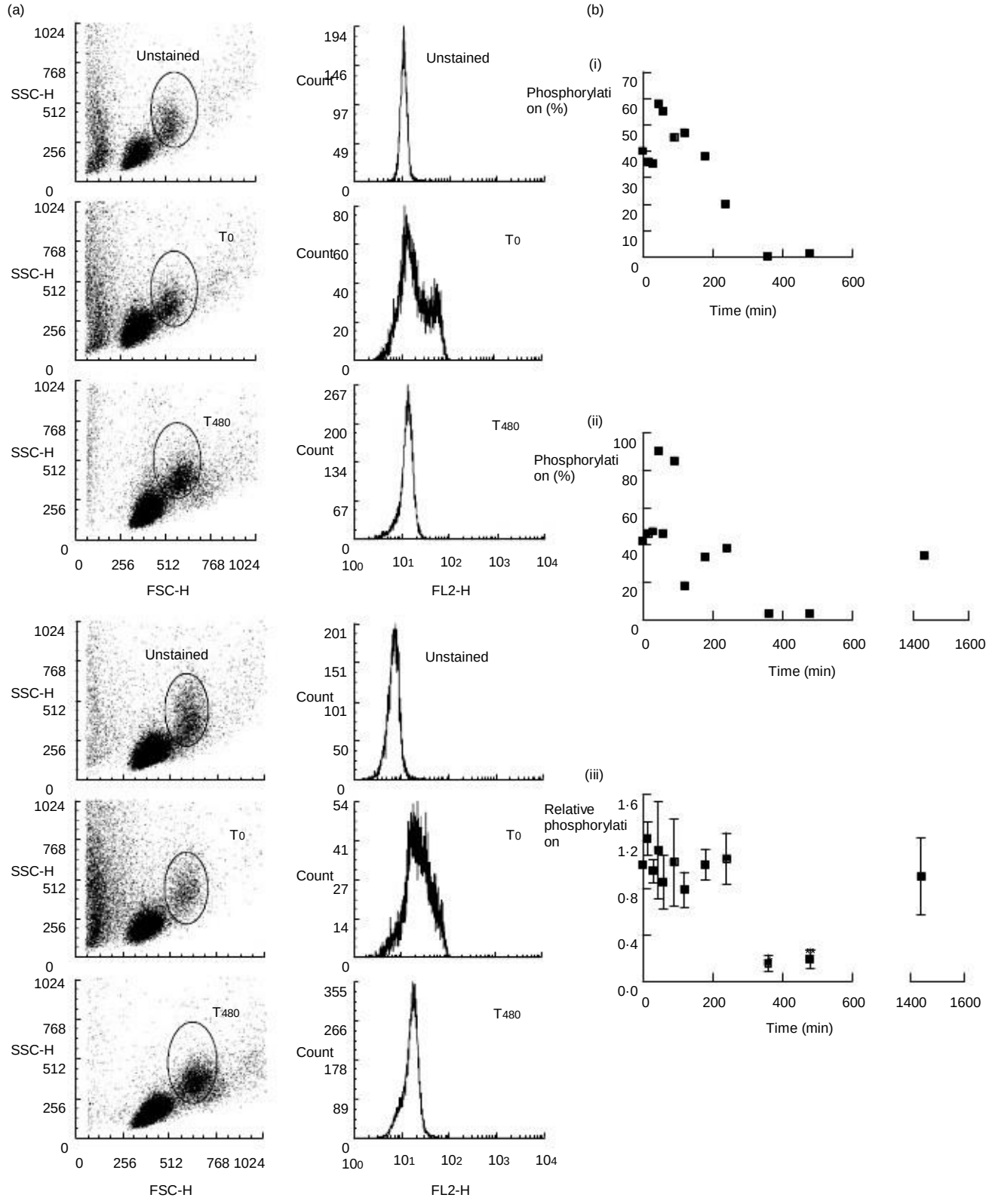

Fig. 4. Analysis of 4E binding protein 1 (4E-BP1) phosphorylation following the consumption of watercress. 4E-BP1 phosphorylation was analysed by flow cytometry in peripheral blood-derived monocytes at various time points following the consumption of 80 g watercress. (a) Representative data obtained from two participants showing forward scatter (FSC)/side scatter (SSC) plots with 'monocyte' gate (a) and fluorescence intensity (b) of unstained control cells and stained cells before (T_0) and 8 h (T_{480}) after the consumption of watercress. (b) Graphs showing (i and ii) the levels of 4E-BP1 phosphorylation in two representative participants (1 and 4) and (iii) the mean with their levels of 4E-BP1 phosphorylation in all four subjects following the consumption of watercress. In (iii), the level of 4E-BP1 phosphorylation at T_0 was set to 1·0 to allow comparison between individuals. FL2-H, fluorescence pulse height. Mean values were significantly different compared to T_0 are indicated (Student's t test): * P¼ 0·001, ** P¼ 0·002.

As part of the present study, we also analysed the plasma concentrations of PEITC. These results are generally in line with those reported previously by Ji et al.(33), who analysed the plasma concentrations of PEITC in four participants following the consumption of 100 g of watercress. Both the studies showed a rapid increase in plasma PEITC concentrations, peaking at 2–3 h, followed by a decline to near background levels at 24 h. However, the C_{max} values in this study were generally lower (297 v. 929 nM on average), and showed much more interindividual variation in the present study. Multiple variables are likely to contribute to these differences.

The lower average C_{max} at least partly reflects the smaller portion size selected for the present study (80 v. 100 g), and although the difference in intake is small, it is not clear whether PEITC accumulation in the plasma is proportionate to dose. It is also possible that differences in glucosinolate content of the crop will have contributed to the variation in mean C_{max} concentrations between the studies, since it is known that differences in sunlight exposure, temperature and added fertilisers can all influence gluconasturtiin production in watercress(45,46). The average age of the participants in the present study was relatively high, and age-related changes in absorption may have also impacted on the overall C_{max} and contributed to interindividual variation. Finally, glutathione-S-transferase variants have previously been demonstrated to modulate ITC metabolism and potential chemopreventive effects, and it is also possible that genetic variation may have contributed to some of the differences. Such age-, genetic- and crop-related differences are all 'real world' variables that are likely to interact to determine exposure, and thus complicate the analysis of biological effects of plant-derived agents.

A key question is why dietary intake of watercress may be sufficient to modulate 4E-BP1 phosphorylation, although plasma concentrations may not achieve the concentrations that are required to effect this pathway in vitro (typically 1– 5 mM (24,31)). One possible explanation lies in the interaction of PEITC with GSH. Efflux of ITC conjugates, extracellular hydrolysis and reuptake of ITC lead to marked accumulation of intracellular ITC(11,12). Thus, the intracellular concentration of PEITC in monocytes may be much higher than what is predicted from the plasma concentrations. Similar interactions may account for the time lag between peak plasma concentration and inhibition of 4E-BP1 phosphorylation. It will be important to investigate further how differences in glutathione levels and metabolism alter cellular accumulation of ITC and their metabolites, since GSH levels are altered in many cancer cells(47). As discussed earlier, it is also important to bear in mind the alternate possibilities. For example, in vivo modulation of 4E-BP1 phosphorylation may be unrelated to or only partially dependent on PEITC, and may be due to other bioactives derived from watercress. For example, Brown et al.(21) recently reported that the mean plasma concentration of total ITC and dithiocarbamate metabolites reached approximately 1·5 mM at 2 h following the ingestion of a 50 g portion of watercress. At present, it is not known whether dithiocarbamate metabolites may contribute to the modulation of 4E-BP1 phosphorylation, either directly or following conversion to ITC.

In summary, we have performed a small pilot study to investigate the feasibility of measuring 4E-BP1 phosphorylation as a biomarker to monitor in vivo effects of PEITC. We tentatively conclude that flow cytometry may be a suitable approach to measure changes in 4E-BP1 phosphorylation following the ingestion of watercress. However, further studies are required with larger sample sizes to test this more rigorously.

Acknowledgements

We thank the medical assistants and ward staff at the Royal Hampshire County Hospital for their assistance and expertise. We particularly thank the women who volunteered for the in vivo study. This work was supported by the Watercress Alliance and BBSRC. The authors declare no conflicts of interest. G. P. and B. M. P. were the principal investigators of the study, and were responsible for the study design and supervision. M. E. M. was responsible for the design and supervision of LC/MS/MS analysis. S. S. A. A. was responsible for the analysis of 4E-BP1 phosphorylation. B. E. C. was responsible for the analysis of HIF activity and cell growth inhibition assays. U. T. was responsible for LC/MS/MS analysis. G. P. wrote the manuscript, which was reviewed by all the authors.

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Cell Stress and Chaperones
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ORIGINAL PAPER

Differential induction of apoptosis in human breast cancer cell lines by phenethyl isothiocyanate, a glutathione depleting agent

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Abstract Phenethyl isothiocyanate (PEITC) is a naturally occurring electrophile which depletes intracellular glutathione (GSH) levels and triggers accumulation of reactive oxygen species (ROS). PEITC is of considerable interest as a potential chemopreventive/chemotherapeutic agent, and in this work, we have investigated the effects of PEITC on human breast cancer cell lines. Whereas PEITC readily induced apoptosis in MDA-MB-231 cells (associated with rapid activation of caspases 9 and 3, and decreased expression of BAX), MCF7 cells were relatively resistant to the apoptosis promoting effects of PEITC. The relative resistance of MCF7 cells was associated with high basal expression of NRF2, a transcription factor that coordinates cellular protective responses to oxidants and electrophiles and raised intracellular levels of GSH. This raised basal expression of NRF2 appeared to be a response to on-going production of ROS, since treatment with the antioxidant and GSH precursor N-acetylcysteine (NAC) reduced NRF2 expression. Moreover, pre-treatment of MDA-MB-231 cells with NAC rendered these cells relatively resistant to PEITC-induced apoptosis. In summary, our data confirm that PEITC may be an effective chemopreventive/therapeutic agents for breast cancer. However, differences in the basal expression of NRF2 and resultant changes in GSH levels may be an important determinant of sensitivity to PEITC-induced apoptosis.

Keywords Phenethyl isothiocyante . Breast cancer . NRF2 . Glutathione . Reactive oxygen species

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Introduction

Phenethyl isothiocyanate (PEITC) is a naturally occurring electrophilic compound that readily undergoes thiocarbamoylation reactions with cellular thiols. Following uptake into cells, the predominant initial reaction of PEITC is with glutathione (GSH) the major intracellular antioxidant (Zhang 2000, 2001). PEITC conjugates are then effluxed from the cell but breakdown of extracellular PEITC conjugates results in liberation of PEITC which is free to re-enter the cell. The net outcome is a rapid depletion of intracellular GSH and accumulation of intracellular PEITC. GSH depletion results in increased accumulation of reactive oxygen species (ROS). The production of intracellular ROS may also increase due to PEITC-mediated inhibition of mitochondrial oxidative phosphorylation (Trachootham et al. 2006; Xiao et al. 2010). Free intracellular PEITC then reacts with cysteinyl thiols of cellular proteins, potentially leading to altered protein function (Mi et al. 2007; Xu and Thornalley 2001).

PEITC has received considerable attention due to its potential chemopreventive and chemotherapeutic activities (Cavell et al. 2011; Cheung and Kong 2010; Hayes et al. 2008). PEITC decreases carcinogen-induced cancer development in vivo and interferes with the activation of carcinogens via inhibition of phase I metabolism and activation of phase II metabolism. PEITC also exerts direct anti-cancer effects against established cancer cells in vitro, including inhibition of cell cycle progression, induction of apoptosis, decreased migration/invasion and/or suppression of angiogenesis. Consistent with this, PEITC exerts therapeutic effects in genetically induced and xenograft tumour models in vivo. PEITC and other related dietary isothiocyanates are thought to play an important role in mediating potential anti-cancer effects associated with diets with a high content of

cruciferous vegetables (Kim and Park 2009; Higdon et al. 2007). The chemopreventive/therapeutic effects of PEITC are currently being explored in clinical studies in low grade B-cell lymphoma and lung cancer (NCT00968461, NCT00691132; http://clinicaltrials.gov/).

It is likely that both increased ROS levels and protein thiocarbamoylation contribute to the biological effects of PEITC in cancer cells. For example, thiocarbamoylation of KEAP1 is thought to be important for the induction of the NRF2 transcription factor, a master regulator of antioxidant/electrophile responsive genes (Cheung and Kong 2010; Hayes and McMahon 2009). In normal cells, KEAP1 associates with NRF2 and targets it for degradation via the proteasome. Treatment of cells with isothiocyantes triggers the release of NRF2 from KEAP1 resulting in stabilisation of NRF2. Once NRF2 accumulates in the nucleus it activates a battery of downstream target genes involved in antioxidant defences, including γ-glutamylcysteine synthetase, the rate-limiting enzyme for the biosynthesis of GSH, GSH reductase and GSH peroxidase. Thus, pro-oxidant/electrophilic stress activates a NRF2-dependent feed-back loop resulting in increased expression oxidant/electrophile protective proteins.

Breast cancer is the most common malignancy amongst women in the Western world, responsible for over 450,000 deaths worldwide in 2008 (Ferlay et al. 2008). Breast cancer can be divided into various molecular subtypes (Perou et al. 2000), and these each have prognostic and therapeutic implications. A major subset of tumours is characterised by the expression of oestrogen receptor (ER), and in these patients, tamoxifen reduces the risk of recurrence of breast cancer by approximately one half, and reduces the risk of death from breast cancer by one quarter. A second subset of tumours express the HER2 receptor, and in these patients, the anti-HER2 antibody trastuzumab reduces risk of death from breast cancer by approximately half and the risk of recurrence by about one third (Smith et al. 2007). In contrast triple negative breast cancer, characterised by lack of expression of ER, progesterone receptor, and HER2 constitute ~15% of women (Foulkes et al. 2010), are associated with adverse prognosis, and currently have no biological therapies available.

Several studies have suggested that PEITC may be an effective preventive/therapeutic agent in breast cancer. PEITC has been shown to promote apoptosis in breast cancer cells (Hahm and Singh 2011; Lee and Cho 2008; Tseng et al. 2004) and to decrease ER expression (Kang and Wang 2010). Induction of apoptosis has been associated with increased expression of the pro-apoptotic BAX and BIM proteins (Hahm and Singh 2011; Lee and Cho 2008; Tseng et al. 2004). PEITC can also interfere with pro-angiogenic pathways via down-modulation of the HIF1α transcription factor (Wang et al. 2009).

In this work, we have investigated the effects of PEITC on human breast cancer cell lines. In particular, we have investigated potential mechanisms that mediate differential sensitivity of these cells to PEITC. Our work suggests that differences in basal levels of NRF2 influence the apoptotic response to PEITC.

Materials and methods

Cell culture and chemicals

MCF7, BT549, MDA-MB-231, ZR-75-1, SKBR3, and T47D human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM; Lonza group Ltd, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (PAA Laboratories, Yeovil, UK), 1 mM L-glutamine and penicillin/streptomycin (Lonza group Ltd). PEITC, indol-3-carbinol, quercetin, buthionine sulfoximine (BSO), staurosporine, and N-acetylcysteine (NAC) were from Sigma Chemicals (Poole, UK). Dimethylsulfoxide (DMSO) was used as a solvent control and was added at a dilution equivalent to the highest concentration of PEITC tested in each assay.

Growth inhibition

Cells were plated at a density of 20,000 cells per well of a 96-well plate in 50 µl complete growth media. The following day cells were treated with PEITC or DMSO as a solvent control or were left untreated. After 6 days, relative cell number was determined using the CellTiter 96® AQ_{ueous} One Solution Reagent (Promega, Southampton, UK) according to the manufacturer's instructions. Relative cell number was calculated as a percentage of untreated cells. IC_{50} values were determined by linear regression using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Apoptosis and cell cycle

To determine the proportion of cells in different phases of the cell cycle, drug-treated cells were collected by centrifugation and fixed in 70% (v/v) ice cold ethanol and stored at 4°C. On the day of analysis, cells were collected by centrifugation and resuspended in 300 µl phosphate buffered saline containing 100 µg ml^{-1} RNAse and 8.3 µg ml^{-1} propidium iodide (Sigma Chemicals) for 15 min. Cell fluorescence was analysed using a FACS Canto (Becton Dickinson, Oxford, UK). The proportion of cells in G1, S, or G2/M phases of the cell cycle was calculated as a proportion of

all cells in cycle, and the proportion of cells with <G1 content was calculated as a proportion of all cells. Apoptosis was analysed by annexin V/propidium iodide staining (Pickering et al. 2007).

Immunoblotting

Immunoblots were performed as previously described (Brimmell et al. 1999) using equal amounts of protein lysates (quantified using the BioRad assay) and antibodies specific for caspase 9, caspase 7, cleaved caspase 9, cleaved caspase 3, (all Cell Signalling Technology, Beverly, MA,USA), BAX, BCL2, NRF2 (all Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit anti-β-actin antibody (Sigma Chemicals). Horseradish peroxidase conjugated secondary antibodies were from GE Healthcare UK (Amersham, UK) and bound immunocomplexes were detected using SuperSignal West Pico Chemiluminescent reagents (Perbio Science UK Ltd, Northumberland, UK). Immunoblot signals were quantified using Quantity One image analysis software (BioRad, Hemel Hempstead, UK).

GSH assays

GSH/GSSG assays were performed using kits from Cambridge Bioscience (Cambridge, UK) Company. The concentration of GSH and GSSG were determined by the end-point method.

Results

PEITC-induced growth inhibition in human breast cancer cell lines

We first investigated the effects of PEITC on the growth of a panel of human breast cancer cell lines using the MTS assay. All cell lines were sensitive to PEITC but there was a ~4-fold variation in their sensitivity (Table 1). The most sensitive cell line was MDA-MB-231, whereas the least sensitive was ZR-75-1. Overall, there was no clear correlation between breast cancer “sub-type” and sensitivity, at least in this small panel of cell lines. MCF7 and MDA-MB-231cells were selected for more detailed studies since these lines are widely used as models in breast cancer research and showed a significant difference in sensitivity to PEITC (mean (±SD) IC_{50} values from up to 9 determinations; 7.2±1.4 μM and 10.6±1.4 μM for MDA-MB-231 and MCF7 cells, respectively; p00.0003 Student's t test).

We also investigated the response of MDA-MB-231 and MCF7 cells to other phytochemicals, indol-3-carbinol and quercetin. Whereas MDA-MB-231 and MCF7 were approximately equally sensitive to indol-3-carbinol (IC_{50}s of 77±5.1 μM and 89±8.1 μM, respectively), MCF7 cells were more sensitive to quercetin-mediated growth inhibition compared to MDA-MB-231 cells (IC_{50}s of 74±8.0 μM and 111±8.2 μM, respectively). Thus, the relative sensitivity of MDA-MB-231 cells to PEITC does not reflect a general increase in sensitivity to growth inhibition.

Table 1 Summary of results for PEITC-induced growth inhibition

Cell line	Status			IC_{50} (μM)[c]
	ER[a]	PR[b]	HER2	
MDA-MB-231	−ve	−ve	Normal	7.2±1.4
T47D	+ve	+ve	Normal	9.2±3.8
BT549	−ve	−ve	Normal	11.9±7.3
MCF7	+ve	+ve	Normal	10.6±1.4
SKBR3	−ve	−ve	Amplified	26.4±2.1
ZR-75-1	+ve	−ve	Normal	40.4±4.8

[a] Estrogen receptor

[b] Progesterone receptor

[c] PEITC IC_{50}, mean±SD derived from a minimum of three independent experiments

Effect of PEITC on cell cycle arrest and apoptosis

To investigate in more detail the differential responses of MDA-MB-231 and MCF7 cells, we first analysed the effects of PEITC (10 or 20 μM) on cell cycle parameters at 24 and 48 h after treatment (Fig. 1). In MDA-MB-231 cells, PEITC predominantly induced a G2/M phase arrest associated with an increased proportion of cells in G2/M and a reduction of cells in G1 phase. There was also an increase in the proportion of cells in S phase, and cells with sub-G1 DNA content, indicative of cell death, especially after 48 h. PEITC also induced a G2/M phase arrest at 24 h in MCF7 cells, although to a lesser extent than in MDA-MB-231 cells, whereas at 48 h, there was a tendency towards a modest increase in the proportion of cells in the G1/S phases. Overall, there was only a very modest increase in the proportion of cell with sub-G1 DNA content in PEITC-treated MCF7 cells.

We also directly investigated the effects of PEITC on apoptosis (Fig. 2). In MDA-MB-231, PEITC induced relatively high levels of apoptosis, especially at 48 h after treatment (Fig. 2a). By contrast, in MCF7 cells, PEITC induced only low levels of apoptosis (Fig. 2b).

Therefore, MCF7 and MDA-MB-231 cells show different biological responses to PEITC. PEITC readily induces apoptosis in MDA-MB-231 cells but not in MCF7 cells. PEITC induces cell cycle arrest in both lines, although the specific phase of arrest may be distinct.

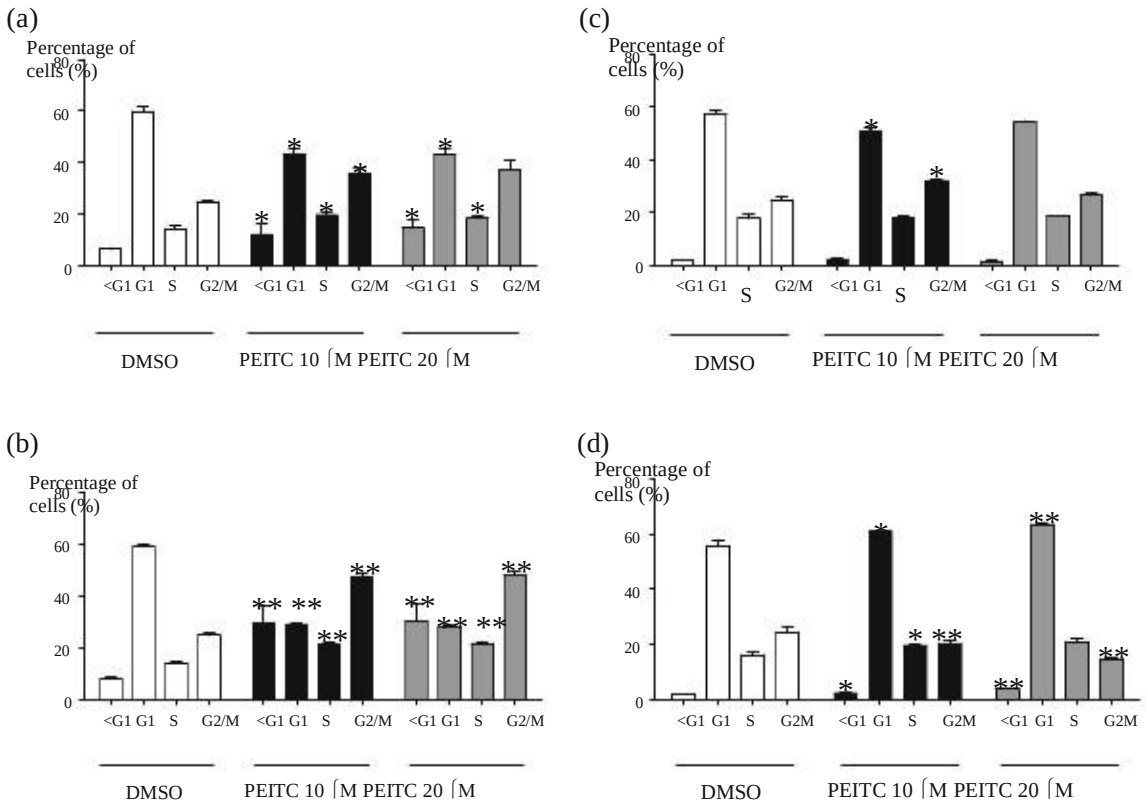

Fig. 1 Cell cycle analysis in PEITC-treated cells. a, b MDA-MB-231 cells or c, d MCF7 cells were treated with PEITC (10 or 20 μM) or DMSO as a control for a, c 24 or b, d 48 h. The proportion of cells in different cell cycle phases was determined using propidium iodide staining and flow cytometry. Data shown are means±SD derived from three independent experiments. Statistically significant differences between DMSO and PEITC treated cells are indicated (*p<0.05; **p<0.005)

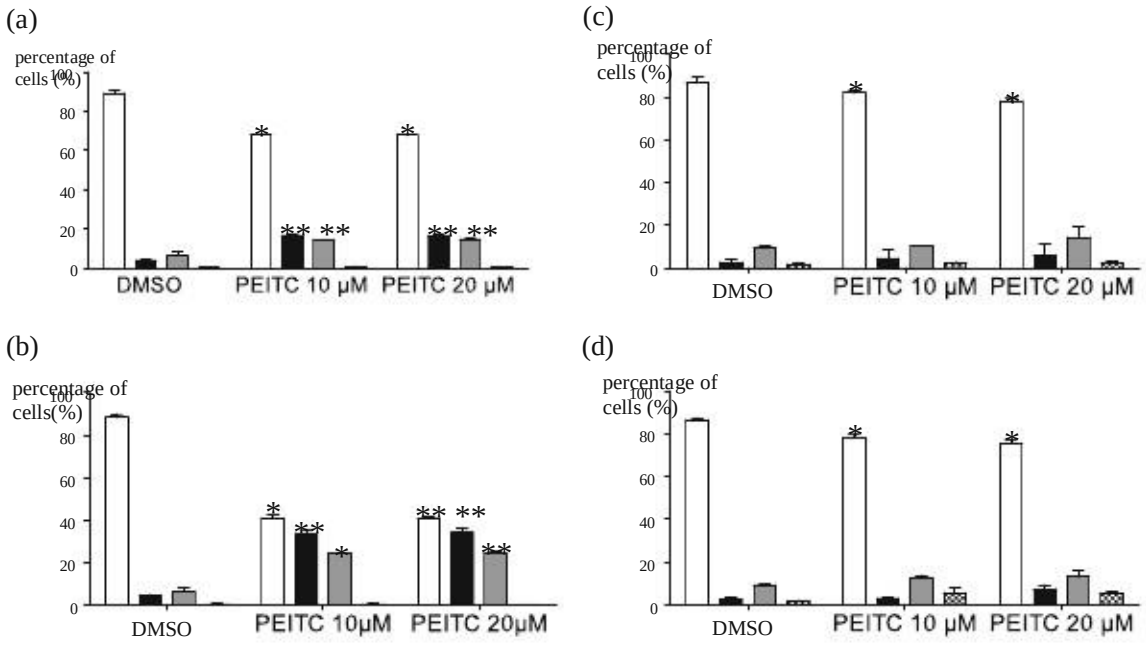

Fig. 2 Apoptosis analysis in PEITC-treated cells. a, b MDA-MB-231 cells or c, d MCF7 cells were treated with PEITC (10 or 20 μM) or DMSO as a control for a, c 24 or b, d 48 h. Induction of apoptosis was analysed using annexin V (An)/propidium iodide (PI) staining (open bars, An−/PI−; closed bars, An+/PI−; grey bars, An+/PI+; hatched bars, An−/PI−). Data shown are means±SD derived from three independent experiments. Statistically significant differences between DMSO and PEITC treated cells are indicated (*p<0.05; **p<0.005)

Effect of PEITC on caspases, BAX, and BCL2

The major difference between MDA-MB-231 and MCF7 in terms of response to PEITC was their differential sensitivity to PEITC-induced apoptosis. To confirm this, we investigated the expression of caspases in PEITC treated cells. In MDA-MB-231 cells, we analysed activation of the initiator caspases for the intrinsic and extrinsic pathways, caspase 9 and caspase 8, respectively, using antibodies that detected both the pro-caspase and activated (cleaved) forms of these caspases. We also analysed activation of the executioner caspase, caspase 3, using an antibody specific for the activated form of caspase 3 (Fig. 3). Similar experiments were performed in MCF7 cells, except we used an antibody that detected pro-caspase and activated (cleaved) forms of the executioner caspase, caspase 7, since these cells do not express caspase 3.

In MDA-MB-231 cells, PEITC treatment resulted in clear processing of caspase 9 and accumulation of active caspase 3. Caspase activation was detected at 2 h and was further increased at 4 and 6 h after addition of PEITC. By contrast, there was no evidence for processing of caspase 8. In MCF7 cells, there was a general decrease in the expression of the pro-caspase forms of caspases 9, 8 and 7, but no evidence for accumulation of active forms (Fig. 3b). These results confirm the differential induction of apoptosis in MDA-MB-231 and MCF7 cells and indicate that PEITC induces apoptosis via the intrinsic pathway.

We analysed the effects of PEITC on expression of BAX, a pro-apoptotic BCL2 family protein which has previously been demonstrated to be increased in PEITC-treated in human ovarian and breast cancer cell lines (Hahm and Singh 2011; Lee and Cho 2008; Satyan et al. 2006). An increase in BAX expression was observed at 2 h after addition of PEITC in MDA-MB-231 cells, and BAX levels further increased at 4 and 6 h (Fig. 4). By contrast, PEITC treatment did not alter BAX expression in MCF7 cells. PEITC also did not alter expression of BCL2 in either cell line.

Effect of PEITC on NRF2

PEITC treatment can elicit increases in intracellular ROS and one possible explanation for the differential response of MCF7 and MDA-MB-231 cells was that these cells differed in their basal levels of ROS or in their ability to tolerate increased ROS (Trachootham et al. 2006). We therefore went on to analyse the expression of NRF2 as a surrogate marker of oxidative stress since NRF2 is induced in cells with high levels of oxidative or electrophilic stress and plays a key role in induction of downstream antioxidant defences. Immunoblot analysis demonstrated that basal (i.e., unin-duced) levels of NRF2 were 60±17% lower in MDA-MB-231 cells compared to MCF7 cells (Fig. 5a; mean of 3 determinations, Student's t test p00.025). PEITC increased NRF2 expression by ~3-fold in MDA-MB-231 cells at 4 h after treatment with PEITC. By contrast, NRF2 expression in MCF7 cells was not effected by PEITC (Fig. 5).

Effect of PEITC on total GSH and GSSG

The association between basal NRF2 expression and sensitivity to PEITC suggested that differences in GSH content might influence responses of MDA-MB-231 and MCF7 cells. We therefore analysed the levels of total GSH and oxidised GSH (GSSG) in MDA-MB-231 and MCF7 cells, before and following addition of PEITC (Fig. 6).

In untreated cells, the levels of both total GSH and GSSG were higher in MCF7 cells, compared to MDA-MB-231 cells. GSH levels were ~50% higher in MCF7 cells. The ratio of GSSG to total GSH was also higher in MCF7 cells compared to MDA-MB-231 cells.

Total GSH and GSSG levels were reduced in MCF7 cells at 2 h after treatment with PEITC, but then remained at this level for the remainder of the time course. Overall, the GSH/GSSG ratio was not substantially altered and even after 6 h treatment the concentration of total GSH was approximately equivalent to that of untreated MDA-MB-231 cells (~12 μM). By contrast, in MDA-MB-231 cells, total GSH levels decreased up to 6 h and were reduced by ~50% at this time. There was also an increase in the GSSG/GSH ratio, indicative of increasing oxidative stress.

Effects of NAC on NRF2 expression and PEITC-induced apoptosis and cell cycle arrest

We next determined whether the relatively high levels of basal NRF2 expression in MCF7 cells was influenced by on-going oxidative stress in these cells. MCF7 cells were pretreated with the antioxidant and GSH precursor NAC, or left untreated as a control, and then treated with PEITC for various times. Treatment with NAC alone reduced basal NRF2 expression in MCF7 cells by ~90% (Fig. 5). Whereas NRF2 was not induced by PEITC in the absence of NAC, treatment with PEITC after NAC pre-treatment resulted in a ~4-fold induction in NRF2 expression from this reduced basal level. In MDA-MB-231 cells, pretreatment with NAC alone further reduced the already low levels of basal NRF2 expression in MDA-MB-231 cells and NRF2 was no longer detectable in these cells. Addition of PEITC to NAC-pretreated cells did not detectably increase NRF2 expression.

To investigate the role of GSH in determining responses to PEITC, we analysed the effect of NAC on PEITC-induced growth inhibition (MTS assay) in MCF7 and MDA-MB-231 cells. Pre-treatment with NAC increased the IC_{50} of MDA-MB-231 cells by ~5-fold (i.e., to 45±

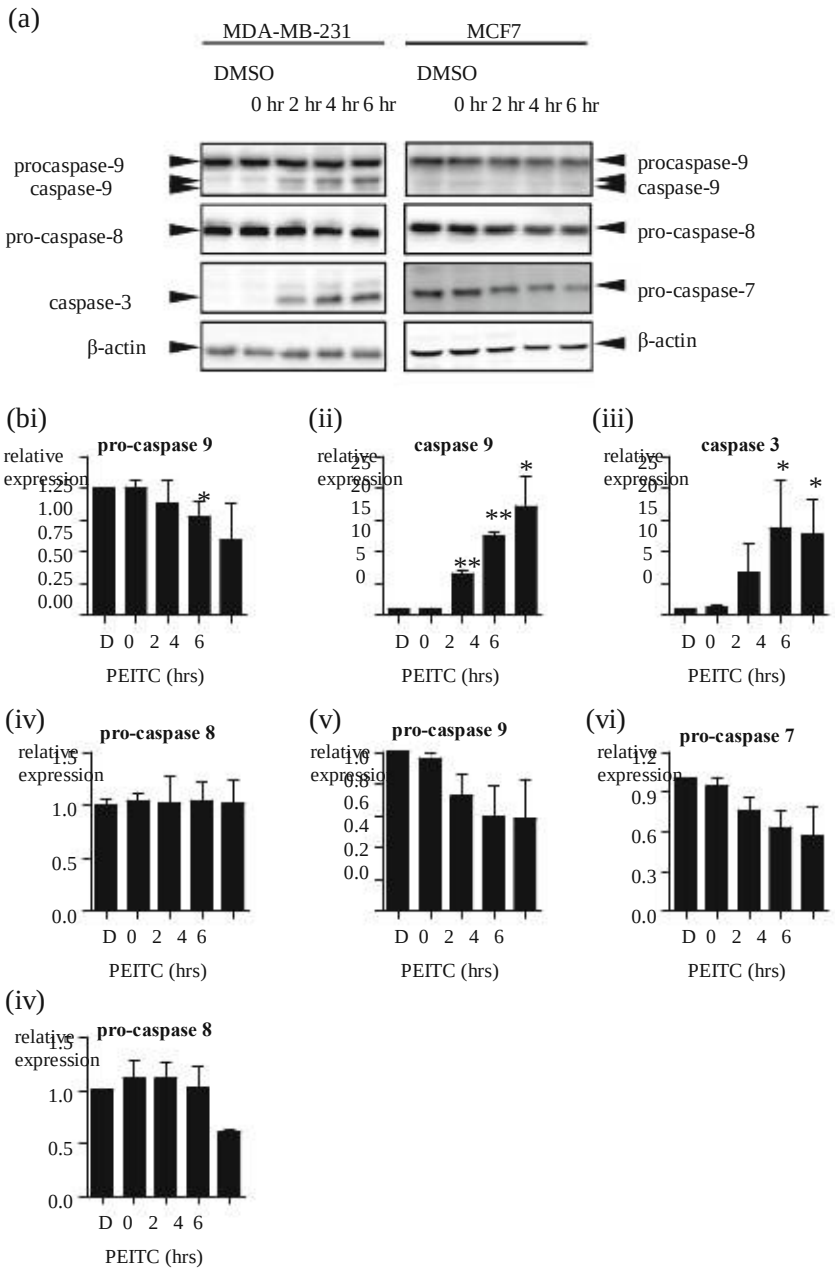

Fig. 3 Caspase expression in PEITC-treated cells. MDA-MB-231 or MCF7 cells were treated with PEITC (20 μM) for the indicated times or DMSO for 6 h as a control. Expression of caspase 9, caspase 8, caspase 3 (MDA-MB-231 cells) and caspase 7 (MCF7 cells) was analysed by immunoblotting. β-actin was analysed as a loading control. a Representative immunoblots. The positions of migration of pro- and cleaved forms of caspases are indicated. b Quantitation. Data shown are means±SD derived from two independent experiments for pro-caspase 9, caspase 9, caspase 3 and pro-caspase 8 in MDA-MB-231 cells (i–iv, respectively) and pro-caspase 9, pro-caspase 7 and pro-caspase 8 in MCF7 cells (v–vii, respectively). Expression values were normalized to that of β-actin. Statistically significant differences between DMSO and PEITC treated cells are indicated (*p<0.05; **p<0.005). Active caspase 9 was not detected in MCF7 cells

4.0 μM, Student's t test versus no NAC pre-treatment, p0 0.004). By contrast, the IC_{50} for PEITC in MCF7 cells was not significantly affected by NAC pre-treatment (11.4± 4.7 μM versus 18.3±1.4 μM, Student's t test, p00.267). Pre-treatment with NAC effectively prevented both the PEITC-induced G2/M arrest and induction of apoptosis in MDA-MB-231 cells (Fig. 7).

Discussion

PEITC is a potential chemopreventive/chemotherapeutic agent, and, with other related phytochemicals, is thought to contribute to the potential protective effects of diets rich in cruciferous vegetables. It is therefore important to understand the mechanisms by which this electrophilic compound exerts its anti-cancer effects and what determines differential responses. Here, we show that individual breast cancer cell lines differ in response to PEITC and that the basal levels of NRF2/GSH may be an important determinant of sensitivity to PEITC-induced apoptosis.

Several previous studies have investigated effects of PEITC in breast cancer cells, demonstrating that PEITC induces apoptosis of human and mouse breast cancer cell lines in vitro (Hahm and Singh 2011; Lee and Cho 2008; Tseng et al. 2004) and decreases breast cancer growth in vivo in a mouse model (McCune et al. 2010). PEITC has also been shown to decrease expression and function of ER

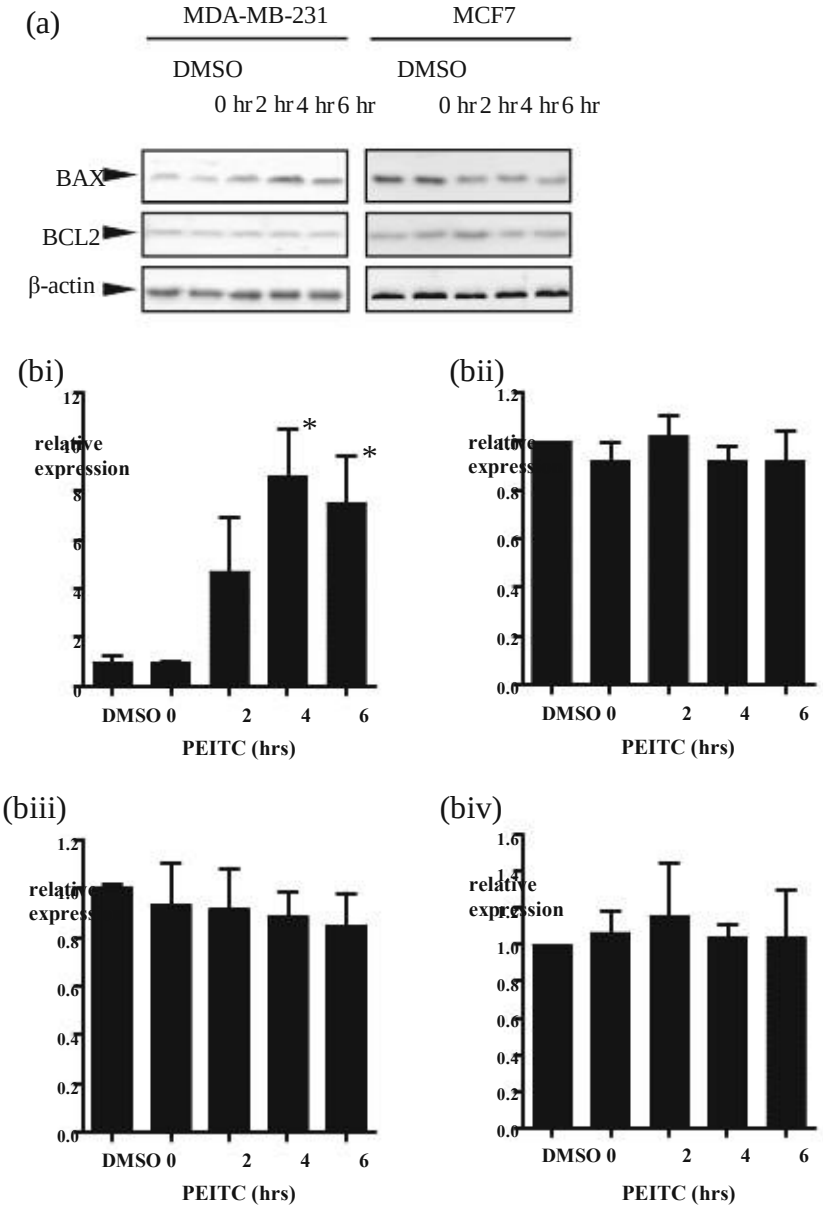

Fig. 4 BCL2 and BAX expression in PEITC-treated cells. MDA-MB-231 or MCF7 cells were treated with PEITC (20 μM) for the indicated times or DMSO for 6 h as a control. Expression of BAX and BCL2 was analysed by immunoblotting. β-actin was analysed as a loading control. a Representative immunoblots. b Quantitation. (i) BAX MDA-MB-231 cells, (ii) BAX MCF7 cells, (iii) BCL2 MDA-MB-231 cells, (iv) BCL2 MCF7 cells. Data shown are means±SD derived from three independent experiments. Expression values were normalized to that of β-actin. Statistically significant differences between DMSO and PEITC treated cells are indicated (*p< 0.05; **p<0.005)

(Kang and Wang 2010). Here, we have extended this work demonstrating that PEITC, dependent on the cell line tested, has the potential to induce both cell cycle arrest and apoptosis, associated with activation of the intrinsic cell death pathway. The key observation from this work is that breast cancer cells demonstrate a variable response to PEITC, with overall sensitivity assessed using the MTS assay varying up to 4-fold between individual cell lines. There was no clear correlation between PEITC responsiveness and the breast cancer subtype represented by these lines. However, the number of cell lines studied was relatively small and further studies are required to determine whether distinct breast cancer subtypes are differentially sensitive to PEITC.

Our mechanistic studies focused on MCF7 and MDA-MB-231 cells as two very well characterised cell line models. The relative sensitivity of MDA-MB-231 cells did not reflect a general increased sensitivity of these cells to phytochemicals, since these lines showed very similar response to indol-3-carbinol, and MCF7 cells were more sensitive to quercetin compared to MDA-MB-231 cells. The major determinant of differential response appeared to be sensitivity to PEITC-induced apoptosis. MDA-MB-231 cells were relatively sensitive to PEITC-induced apoptosis, whereas PEITC did not induce significant levels of apoptosis in MCF7 cells. It is important to recognise that the differential induction of apoptosis in MDA-MB-231 and MCF7 cells is relative and not absolute. For example, PEITC has been shown to induce apoptosis in MCF7 cells in previous studies (Lee and Cho 2008; Hahm and Singh 2011). However, our study has focused on relatively early

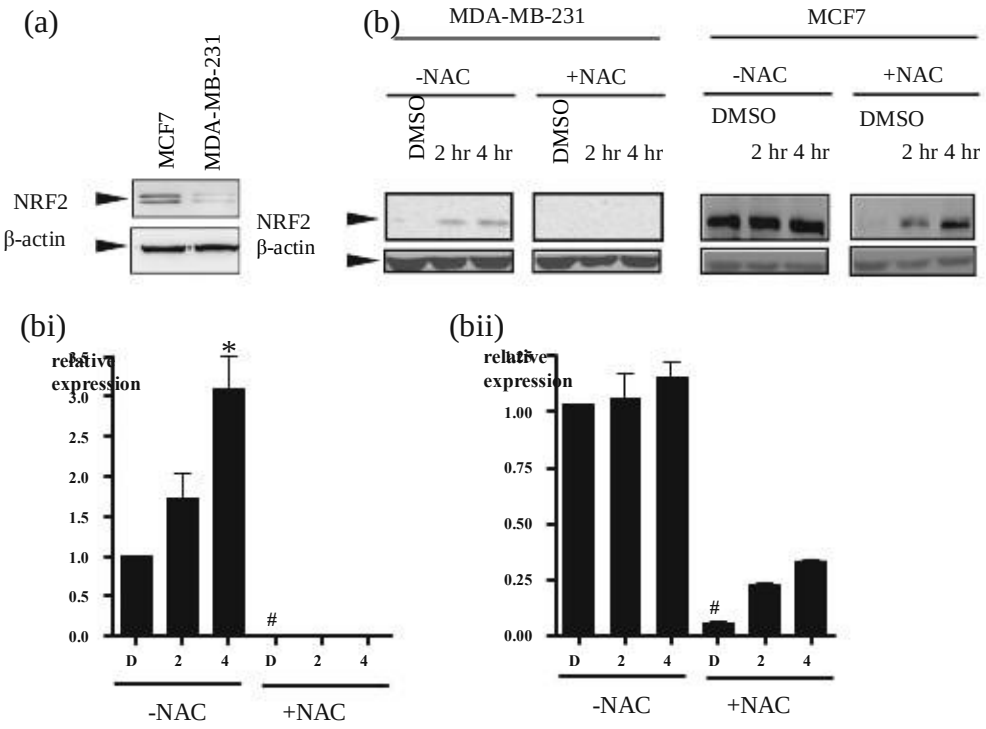

Fig. 5 NRF2 expression in PEITC-treated cells. a NRF2 expression in untreated MDA-MB-231 or MCF7 cells was analysed by immunoblotting. b, c MDA-MB-231 or MCF7 cells were treated with PEITC (20 μM) for the indicated times or DMSO for 6 h as a control. Expression of NRF2 and β-actin (loading control) was analysed by immunoblotting. b Representative immunoblots. c Quantitation. (i) MDA-MB-231 cells, (ii) MCF7 cells. Data shown are means±SD derived from three independent experiments. Expression values were normalized to that of β-actin. Statistically significant differences between DMSO and PEITC treated cells in the absence of NAC are indicated (*p<0.05). Statistically significant differences between NAC treated and untreated cells are indicated (#p<0.05)

time points to probe some of the more immediate changes in PEITC-treated cells. For example, we studied caspase activation at up to 6 h, whereas other studies which have demonstrated PEITC-induced caspase activation in MCF7 cells have studied caspase activation and BCL2 family protein expression at 24 h (Lee and Cho 2008). PEITC-induced apoptosis in MDA-MB-231 cells appeared to be mediated via the intrinsic pathway since it was associated with increased expression of BAX, and activation of caspase 9, but not caspase 8. In contrast to apoptosis, PEITC induced cell cycle arrest in both cell lines, although there were some differences in the specific phase of arrest between the two lines.

Our work suggests that NRF2 status may influence response to PEITC. NRF2 is a transcription factor that is induced by oxidative/electrophilic stress and regulates a set of 100–200 genes encoding proteins involved in cytoprotection, including many components of the GSH system (Hayes and McMahon 2009). NRF2 was constitutively expressed at ~2.5-fold higher levels in MCF7 cells compared to MDA-MB-231 cells in the absence of PEITC. Raised NRF2 expression appears to be functionally relevant since MCF7 cells also contained higher GSH levels than MDA-MB-231 cells. NRF2 expression is regulated at multiple levels, including transcription, degradation, and phosphorylation (Cheung and Kong 2010; Hayes and McMahon 2009), and the mechanisms that mediate increased NRF2 expression in MCF7 cells may be complex. Basal NRF2 expression appeared to be dependent on on-going ROS production in both cell lines since treatment with NAC effectively reduced NRF2 expression. Elevated ROS may release NRF2 from KEAP1 mediated negative control, or may cause activation via enhanced phosphorylation, perhaps associated with ROS-mediated inhibition of phosphatase activity. We speculate that MCF7 cells may be protected from the pro-apoptotic effects of PEITC relative to MDA-MB-231 cells by enhanced expression of NRF2 and resultant increases in levels of GSH. Notably, NRF2 expression was induced in MDA-MB-231 cells following treatment with PEITC. However, maximal induction of NRF2 was relatively delayed compared to activation of capases in these cells. Thus, it seems likely that although MDA-MB-231 can mount a NRF2-mediated antioxidant response to PEITC, the kinetics are too effectively counter the rapid induction of apoptosis in these cells, at least at the concentrations of PEITC tested here.

The mechanisms leading to increased ROS in MCF7 cells are unclear. However, similar differences between MDA-MB-231 and MCF7 cells in response to pro-oxidants have been observed in previous studies. For example, the pro-oxidant (tert-butyl-2(4,5-dihydrogen-4,4,5,5-tetramethyl-3-O-1H-imidazole-3-cationic-1-oxyl-2-pyrrolidine-1-carboxylic ester) L-NNP induced higher levels of ROS and apoptosis in MDA-MB-231 cells compared to MCF7 cells (Zhang et al. 2011) and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces greater levels of GSH depletion and increased ROS in MDA-MB-231 cells compared to MCF7 cells (Lin et al. 2007). Oncogenic transformation has been shown to elevate intracellular ROS (Trachootham et al. 2006) or to promote NRF2-dependent ROS detoxification (DeNicola et al. 2011), possibly dependent on the level of oncogene expression and or cell type.

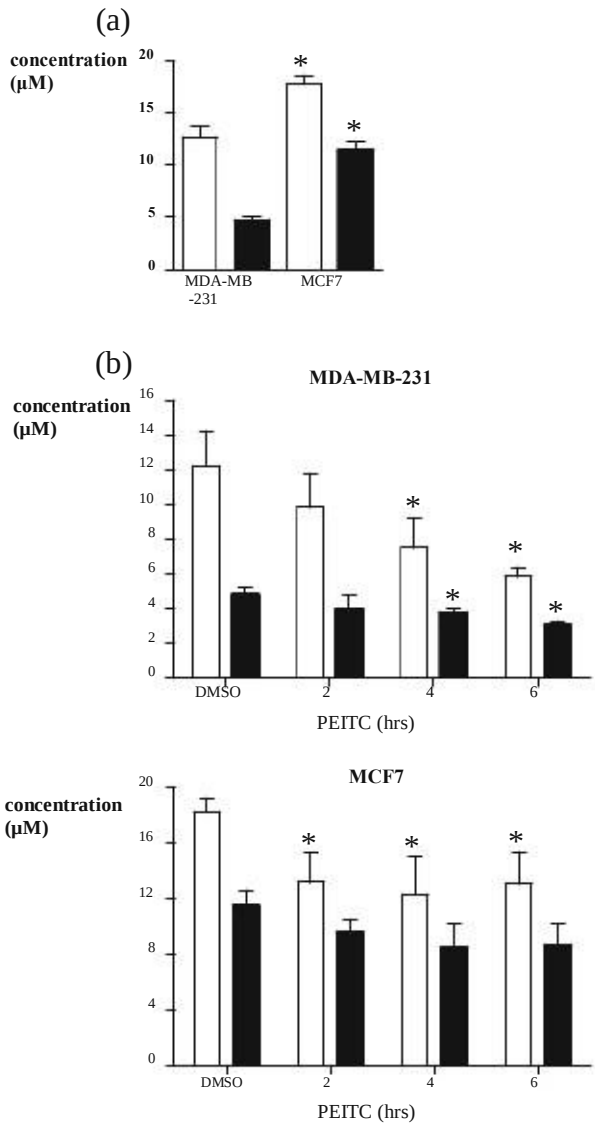

Fig. 6 GSH levels in MCF7 and MDA-MB-231 cells. a Total GSH and GSSG concentration in untreated MDA-MB-231 and MCF7 cells. Data are means±SD derived from three independent experiments. Statistically significant differences between MDA-MB-231 and MCF7 cells are indicated (*p<0.05). b Total GSH (open bars) and GSSG (closed bars) concentrations in (i) MDA-MB-231 and (ii) MCF7 cells treated with PEITC (20 μM) for the indicated times or DMSO (6 h) as a control. Data are means±SD derived from three independent experiments. Statistically significant differences between DMSO and PEITC treated cells are indicated (*p<0.05)

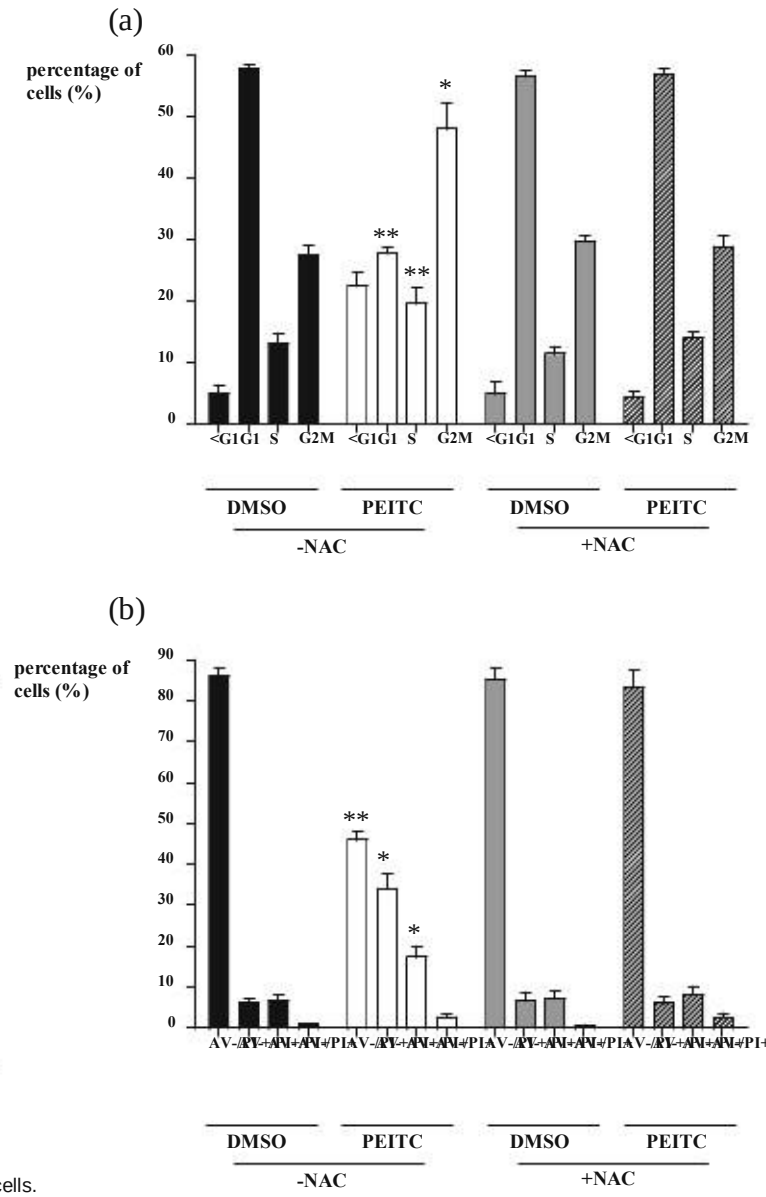

Fig. 7 Effect of NAC on PEITC-induced cell cycle arrest and apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were treated with PEITC (20 μM) or DMSO as a control, in the presence or absence of NAC. In a, the proportion of cells in different cell cycle phases was determined using propidium iodide staining and flow cytometry. In b, induction of apoptosis was analysed using annexin V (An)/propidium iodide (PI) staining (open bars, An−/PI−; closed bars, An+/PI−; grey bars, An+/PI+; hatched bars, An−/PI−). Data shown are means±SD derived from three independent experiments. Statistically significant differences between DMSO and PEITC treated cells are indicated (*p<0.05; **p<0.005)

Regardless, differences in the underlying oncogenic drives in MCF7 and MDA-MB-231 cells may account for differences in ROS production and sensitivity to PEITC.

In summary, our data confirm that PEITC may be an effective chemopreventive/therapeutic agents for breast cancer. However, it will be important to investigate in more detail the molecules that influence PEITC responses in individual tumour types. Cells that have adapted to increased transformation-associated ROS production via upregulation of antioxidant defence may be less sensitive to PEITC-induced apoptosis.

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